Regulation of innate and adaptive immune responses by Toll-like receptor ligands

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Für meine Familie

1 Summary

Toll-like receptors (TLR) recognize conserved molecular patterns of microorganisms and are essential for the activation of host immunity. Whereas the immune-stimulatory effects of TLR ligands are well described, the circumstances of TLR-mediated inhibition of immune responses are still largely unknown. In this thesis, it was investigated how ligands for TLR9 and TLR7 modulated cytotoxic T cell responses in mice infected with recombinant adenovirus or *Plasmodium ssp. in vivo*. The results of this thesis point out, that the anatomical site, dose, and time-point of TLR ligand application determined whether an immune response to an infection was stimulated or suppressed. In contrast to subcutaneous application of TLRs that stimulate adaptive immune responses, systemic application of TLR ligands in high doses prior to infection suppressed CD8 T cell cytotoxicity. TLR-ligand induced suppression of adaptive immune responses was characterized by reduced numbers of antigen-specific T cells and a (subsequent) lack of cytotoxicity and was crucially dependent on TLR expression. Importantly, by adoptive transfer of activated antigen-specific $CD4^+$ T helper cells into TLR ligand-treated mice it was possible to overcome TLR-ligand mediated suppression of CTL responses. This indicated that a block in $CD4^+$ T cell activation is likely to account for insufficient CTL induction. Although TLR ligands caused functional impairment of dendritic cells, we doubt that DC paralysis was solely responsible for CTL suppression. An adoptive transfer of exogenously matured and antigen loaded DCs did not overcome CTL suppression in TLR ligand-treated mice, which further indicated that TLR ligand-induced CTL suppression was rather due to the induction of an inhibitory milieu than due to inability of DCs to activate T cells. In TLR7 ligand-mediated CTL suppression, inhibitory effects could be attributed to type I interferons.

A physiological relevance of TLR-mediated suppression of effector immune responses was shown in mouse models of *Plasmodium ssp*. infection. Cerebral malaria (CM) is a severe immune-mediated complication in *P. falciparum* infection in humans. IFNγ and T cells are among the few factors that are known to be essential in the pathogenesis of experimental CM (ECM) in mice upon *P. berghei* ANKA (PbA) infection. Here, we show by use of knock out mice that TLR2, -3 and -9 and IL-12 are crucial for the development of ECM upon PbA infection. *PbA*-infected mice were also significantly protected against ECM if they were depleted of DCs or were splenectomized. However, a key finding of this thesis was the observation that PbA parasites themselves were able to prevent detrimental effector responses. Wild type mice but not mice deficient in TLR2, TLR9, IL-12p40, IL-10 or iNOS were significantly protected against ECM upon infection with a highly enhanced dose of PbA. Also, systemic application of TLR9 ligand CpG protected PbA-low dose-infected wild type mice but not TLR9 deficient littermates from ECM. CpG blocked the production of pro-inflammatory mediators by DCs and prevented the generation of *Plasmodium* specific $CD8^+$ T cell responses. These results strongly suggest that TLRs fulfill dual functions in regulation of immune responses in *Plasmodium* infection.

Overall, we conclude that the induction of immune suppression represents a sort of "emergency shutdown" that is induced if circumstances of TLR activation or infection due to excessive inflammation endanger the host's life. Prevention of immune pathology ensures survival of the host. Pathogens such as *Plasmodium ssp*. may benefit from these mechanisms of TLR-mediated immune suppression as it offers a possibility of immune evasion or silencing that allows completion of their life cycle.

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3 Abbreviations

α	anti-
A	Ampere
Ab	antibody
ABTA	2,2-Azino-bis-3-Ethylbenzothiazoline-6-sufonic acid
AdOVA	recombinant *Adenovirus* type 5 expressing ovalbumin
AdGFP	recombinant *Adenovirus* type 5 expressing GFP
APC	antigen presenting cell; Allophycocyanin
APC-Cy7	Allophycocyanin-Cy7
B6	C57BL/6 mice
bp	base pairs
BSA	bovine serum albumin
C	Celsius
CD	cluster of differentiation
CFSE	Carboxy Fluoroscein di-amino Succinimidyl Ester
CpG-	Cytosin-phosphat-,Guanin
CM	cerebral malaria
CM-R	cerebral malaria resistant
CM-S	cerebral malaria susceptible
CTL	cytotoxic T lymphocyte
CQ	chloroquine
D	Dalton (1D = 1,6601 x $10e^{-27}$ kg)
DC	dendritic cell
DEPC	Diethylpyrocarbonat
DMSO	Dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	2´-Desoxy-Nukleosidtriphosphat
DOG	CD11c DTR Ovalbumin green fluorescent protein
DTR	Diphtheria Toxin Receptor
DTX	Diphtheria Toxin
ECM	experimental cerebral malaria
E.coli	*Escherichia coli*
EDTA	ethylendiameintetraessigsäure
ELISA	enzyme linked immuno sorbent assay
et al.	et alteres
EtOH	Ethanol
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	Fluorescein
flg	Flagellin
g	gram; acceleration of gravity (9,81m/s2)
GFP	green fluorescent protein
GpC	Guanin, phosphat, Cytosin
h	hour
HE	Hematoxylin-Eosin
HK	heat killed
HSP	heat shock protein

ICAM	intercellular adhesion molecule; CD54
ICS	intra-cellular staining
IDO	Indoleamine 2,3-dioxygenase
IFNα	Interferon alpha
IFNAR	Interferin alpha receptor; type I interferon receptor
IFNß	Interferon beta
IFNγ	Interferon gamma
Ig	Immune globuline
IL	Interleukin
iNOS	inducible nitric oxide synthase
i.p.	intra-peritoneal
IRF	interferon regulatory factor
i.v.	intra-venous
IVIS®200	In vivo imaging system (Xenogen)
kB	kilo bases
kD	kilo Dalton
k.o.	knock out
l	Liter
LiCl	Lithiumchlorid
LM-OVA	recombinant *Listeria monocytogenes* expressing OVA
LPS	lipopolysaccharide
LTA	lipo teichonic acid
luc	luciferase
µ	Micro- (10^{-6})
m	meter, Milli- (10^{-3})
M	molar
mA	Milli-Ampère
MACS®	magnetic activated cell sorter (Miltenyi)
MDP	muramyl dipeptide; NOD2 ligand
min	minute
MHC	major histocompatibility complex
µMT	IgM transmembrane tail exons, deficiency in B cell k.o.
mRNA	messenger RNA
1-MT	1-Methyl-tryptophan
MyD88	myeloid differentiation factor 88
MW	Molecular weight
MZ	marginal zone
n	Nano- (10^{-9}), number
nmol	Nanomol
NaCl	Natrium chloride
NaOH	Natriumhydroxid
NFκB	nuclear factor kappa B
NK	natural killer
NKT	natural killer T cell
NLR	NOD-like receptors
NOD	nucleotide oligomerization domain
Od_x	optical density at x nm
ODN	Oligodeoxynucleotide
Oligo-d(T)	Oligodeoxythymiylacid (= sequence of +thymidin nucleotides)
OT-I	H-2^b restricted $CD8^+$ cells expressing transgenic TCR that recognizes OVA-derived peptide SIINFEKL $AA_{257-265}$ in the context of K^b.

OT-II	H-2^b restricted $CD4^+$ cells expressing a transgenic TCR recognizing OVA-derived MHC class II peptide $AA_{323-339}$ in the context of I-A^b.
OVA	ovalbumin from chicken egg
PbA	*Plasmodium berghei ANKA*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-Cy7
Pf	*Plasmodium falciparum*
PG	Prostaglandin
PGN	Peptidoglycan
pH	*potentia hydrogenii*
p.i.	post infectionem
p.o.	per os
RAG	recombination activating gene
RNA	ribonucleic acid
RNAse	Ribonuclease
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute, cell culture medium developed by G.E. Moore et al.
RT	room temperature
S8L	SIINFEKL, OVA-derived MHC class I peptide
S	supernatant
SA	streptavidin; *Staphylococcus aureus*
s.c.	sub-cutaneous
SDS	sodium dodecyl sulphate
siRNA	short interfering RNA
sec	second
shRNA	short hairpin RNA
Spl-X	splenectomy
sTAg	soluble *Toxoplasma gondii* antigen
TAE	*tris acetic acid EDTA*
Tab.	Table
TCR	T cell receptor
TEMED	N,N,N´, N´,Tetramethylethylendiamin
tg	transgenic
TGFß	transforming growth factor beta
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
TRIF	TIR domain containing adapter inducing Interferon beta
Tris	Tris(hydroxymethyl)aminomethan
U	unit; international enzyme unit
UV	ultraviolet light
V	Volt
VCAM	vascular-cell-adhesion molecule; CD106
v/v	percent by volume
XP	cross-presentation

4 Introduction

4.1 The immune system and the task of defense

The mammalian immune system represents an organization of cells and molecules with specialized roles for the defense against "danger" elicited by viruses, bacteria, or parasites but also by degenerated tumor cells to prevent harm of the organism. In mammals there are two fundamentally different types of responses that are provided by the innate and the acquired (or adaptive) part of the immune system. Innate defense mechanisms reduce pathogen load, whereas final clearance is achieved by the specific adaptive immune response. The innate immune system is the first line of defense characterized by phagocytes including macrophages and dendritic cells. Early detection of potentially harmful microorganisms is facilitated by a limited number of germline-encoded pattern recognition receptors (PRRs) that trigger inflammatory processes to kill the pathogens. Innate defense mechanisms initiate and provide time for the development of highly specific and long-lasting adaptive immune responses that are characterized by high diversity, specificity and memory. Acquired immunity is based on the activation of B and T lymphocytes and develops by clonal selection from a vast repertoire of lymphocytes bearing antigen-specific receptors that are generated via gene re-arrangement. The innate and the acquired immune system cooperate at several points to ensure an optimal protection of the host.

4.2 Pathogens trigger inflammation

Microorganisms causing disease are called pathogens. To establish an infection, a pathogen must first overcome numerous surface barriers, such as enzymes and mucus, that are either directly anti-microbial or inhibit microbial attachment. Generation of an inflammatory response – a complex cascade of events – is the first reaction of an organism to pathogens induced by the infection or tissue injury. Important characteristics are migration of cells with defensive functions, alterations in vascular permeability and secretion of soluble mediators such as cytokines, chemokines and interferons. Inflammatory cells and soluble mediators have strong antimicrobial activities and constitute the effector phase of innate resistance. In turn, further immune cells are recruited and activated, which involves two major groups of cells, antigen-presenting cells and lymphocytes. The subordinate status of the adaptive immune response has been known since the 1960s, when it was shown that mononuclear phagocytic cells were required for an effective lymphoid response to antigens (Dutton, 1967; Unanue and Askonas, 1968). It is now accepted that adaptive immune dependency on innate immune cells is based on the need for antigen presentation, a function performed by antigen-presenting cells (APCs), especially dendritic cells (DCs). DCs are sparsely but widely distributed cells of haemato-poietic origin that are specialized for the capture, transport, processing and presentation of

antigens to T and B cells. At an "immature" stage of development DCs act as sentinels in peripheral tissues, continuously sampling the antigenic environment. Any encounter with microbial products or tissue damage initiates the maturation of DCs. The initial uptake and phagocytosis of microbial antigen by APCs is facilitated by receptor-mediated recognition of microbial molecules. Both scavenger receptors and receptors of the complement system are critical for this process. Upon maturation, DCs migrate to secondary lymphoid organs such as lymph nodes, the spleen and mucosa-associated lymphoid tissue to initiate acquired responses. Antigen-specific interaction of DCs and lymphocytes via cell surface receptors triggers the activation and proliferation of antigen-specific B and T lymphocytes.

A long time, the contribution of innate detection mechanisms that is based on a limited set of non-clonal germline encoded receptors for the generation of an adaptive immune response was underestimated. In contrast, studies from the last decade support an important role of innate detection and effector mechanisms for translating microbial signals to antigen-specific T and B cells and to modulate the quality, strength and duration of the adaptive immune response (Pulendran & Ahmed, 2006).

4.3 Pattern Recognition via Toll-like receptors

Pattern recognition receptors (PRRs) recognize conserved microbial components, described as pathogen associated molecular patterns (PAMPs) that are essential for the survival of the microorganism and are therefore difficult to alter. PRRs are expressed constitutively by the host; they are germline-encoded, non-clonal and expressed on all cells of a given type, and independent of immunologic memory. The discovery of several PRR families and the rapid expansion of knowledge in this field led to a reappraisal of the innate immune system. These studies suggested that pattern recognition receptors evolved to recognize conserved products of microbial metabolism produced by microbial pathogens, but not by the host. Janeway postulated that recognition of PAMPs allows the immune system to distinguish infectious "non-self" from noninfectious "self" (Janeway, 1989). The basic machineries underlying innate immune responses are highly conserved among species from plants and fruit flies to mammals.

The family of toll-like receptors belongs to these detection systems that are critical for the activation of the innate immune system. Toll-like receptors (TLRs) are evolutionary conserved from the worm *Caenorhabditis elegans* to mammals and were originally identified in vertebrates on the basis of their homology with *Toll*, a molecule that was initially identified as a gene product essential for the development of embryonic dorso-ventral polarity in *Drosophila* (Anderson et al., 1985a; Anderson et al., 1985b). Later, experiments with Toll-deficient mutants demonstrated important roles of Toll in anti-fungal responses of the flies (Lemaitre et al., 1996).

4.3.1 Toll-like receptor ligands

So far, 13 members of the TLR-family are known in vertebrates that recognize lipids, carbohydrates, peptide and nucleic acid structures that are broadly expressed by different groups of microorganisms (Figure 1). Some TLRs are expressed at the cell surface and recognize microbial patterns like lipopolysaccharide (LPS) via TLR-4 (Lien et al., 2000; Medzhitov et al., 1997; Poltorak et al., 1998), peptidoglycan and lipopeptides via TLR-2 (Takeuchi et al., 1999), bacterial flagellin through TLR-5 (Hayashi et al., 2001). Further TLRs are expressed on membranes of endocytic vesicles or other intracellular organelles. Among those, TLR-3, -7 and -9 are intracellularly located and are described to recognize nucleic acids. TLR-3 detects double stranded RNA, and poly IC (Alexopoulou et al., 2001). Single stranded RNA and poly U rich sequences are sensed by TLR-7 (Diebold et al., 2004; Heil et al., 2004). TLR-9 is essential for the recognition of invertebrate derived DNA, whose sequences are characterized by unmethylated CpG-rich motifs in contrast to DNA from vertebrates (Hemmi et al., 2000).

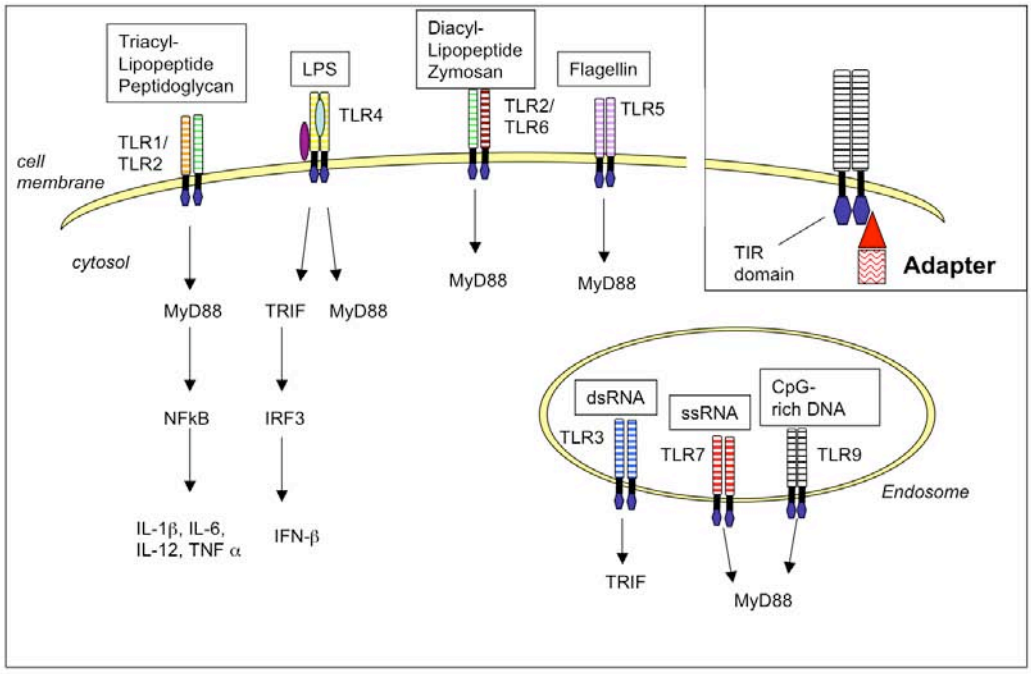

Figure 1 Toll like receptors and their ligands

Toll-like receptor are expressed in cellular membranes at the cell surface or intracellularly. They recognize conserved molecules derived from pathogens. Upon binding, intracellular adapter molecules are recruited and initiate the activation of NF-kB and IRF-dependent target genes.

The first hint that in addition to "non-self" ligands also endogenous ligands are sensed, was the description of TLR4 mediated recognition of heat shock protein 60 (HSP60) (Ohashi et al., 2000). Further on, TLR4-mediated recognition of Fibronectin was described (Okamura et al., 2001) and

Vabulas et al. demonstrated that recognition of HSP70 through TLR2/TLR4 triggered MyD88 dependent signaling (Vabulas et al., 2002). These findings support the "danger" hypothesis postulated by Matzinger (Matzinger, 1994; Matzinger, 2002). According to this, the immune system is activated through "danger" signals released from damaged or stressed tissue rather than through detection of unknown antigens. Thus, if certain criteria of danger are met - i.e. during inflammation - also endogenous ligands can be recognized as appropriate ligands by immune cells and activate immunity.

4.3.2 Toll-like receptors: structure and signaling

TLRs are type I integral membrane glycoproteins characterized by their extracellular amino-terminal domains containing varying numbers of leucin-rich repeat (LRR) motifs that are responsible for the recognition of PAMPs, and a cytoplasmic carboxy-terminal domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie and O'Neill, 2000) that is required for initiating intracellular signaling (Takeda et al., 2003). All TLRs elicit conserved inflammatory pathways, culminating in the activation of transcription factors such as nuclear factor (NF)-kB that act as a master switch for inflammation. Subsequently, the expression of varying response genes is induced and regulated that are involved in immunity and inflammation. Each TLR activates similar signaling pathways, but some TLRs trigger their specific pathways. Cellular localization and composition of the individual TLR are likely to contribute to this kind of specificity. TLRs occur as dimers (Ozinsky et al., 2000). For example, TLR-1 and TLR-2 hetero-dimerize and the resulting dimer senses bacterial triacylated lipo-peptides. TLR-2 hetero-dimerizes with TLR-6, which recognizes di-acylated lipopeptides. In contrast, TLR9 homo-dimerizes. This is presumably also the case for TLR3 and TLR5. It is assumed that the TLR dimers are pre-assembled in a low-affinity complex before ligand binding. Once the ligand binds, a conformational change is thought to occur that brings both TIR domains of each receptor in closer proximity. This complex then recruits TIR domain-containing adapter proteins. Differential induction patterns heavily depend on cytoplasmic adapter molecules that can associate with the TIR domain of TLRs (Akira and Takeda, 2004). The discovery of at least five adapter proteins that are differentially recruited to TLRs has provided important hints for the molecular basis of this specificity (O'Neill and Bowie, 2007). These adaptors are myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (MAL; also known asTIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF; also known as TICAM1), TRIF-related adaptor molecule (TRAM, also known as TICAM2) and sterile α- and armadillo-motif containing protein (SARM).

MyD88 was discovered in 1990 and later described as the first adaptor protein docking to the intracellular domain of an IL-1R and also to the TLR dimer through a TIR-TIR interaction (Lord et al., 1990; Medzhitov et al., 1998; Muzio et al., 1997; Wesche et al., 1997). Upon binding of MyD88,

IRAK-4 is recruited, becomes activated and phosphorylates IRAK-1 that in turn activates Traf-6. A series of ubiquitinylation reactions then occur on Traf-6 itself and on the protein TAK-1, which is a candidate kinase for the activation of the inhibitor of NF-kB kinase (IKK) complex, leading to NF-kB activation, and activation of upstream kinases for p38 and JNK.

Taken together, although the TIR domain is the common signaling domain, differences occur in the utilization of the different TIR-domain-containing adaptors, which might provide specific tailor-made responses to an invading pathogen.

4.4 The adaptive immune response requires innate immune recognition

Unlike antibodies or B cell receptors, T lymphocytes expressing αβ T cell receptors do not recognize pathogens and their antigens as such, but instead recognize only antigen that is processed and presented in the context of molecules encoded by the major histocompatibility complex (MHC). Higher vertebrates express three different classes of MHC encoding genes; two of them are relevant for antigen presentation and can be linked to two subtypes of T lymphocytes. Class I MHC genes encode glycoproteins expressed on the surface of all nucleated cells; class I presented peptides are derived from cytosolic antigens are presented to cytotoxic T cells expressing the CD8 co-receptor. Intracellular proteins are degraded into peptides by a proteolytic system in the cytosol – the constitutive proteasome – present in all cells. Recognition of MHC class I peptides plays an important role in detection and lysis of virus infected cells that display upon intracellular viral replication peptides derived from viral antigens on MHC class I.

In contrast, class II MHC genes encode glycoproteins expressed primarily on antigen-presenting cells (macrophages, dendritic cells, B cells), which present processed antigenic peptides derived from exogenous antigens to $CD4^+$ T helper cells. APCs internalize exogenous antigens by phagocytosis, endocytosis or both processes. Internalized antigen is then degraded into peptides within the compartments of the endocytic processing pathway. Within these acidic compartments, antigen is degraded to oligopeptides of about 13 to 18 residues, which bind to class II MHC molecules and then appear on the cell surface. Dendritic cells are the most effective of the APCs. Because these cells constitutively express MHC class II that is increased upon maturation, and have costimulatory activity, they can activate naïve $CD4^+$ helper T cells.

In addition to the presentation of endogenous antigen in the context of MHC class I molecules via the classical pathway mentioned above, it was recognized that some cells are able to internalize extra-cellular antigen, which is shuttled to the cytoplasm, processed via the proteasome and then loaded on MHC class I molecules. This pathway was called cross-presentation (Bevan, 1976) and is an important process for the induction of $CD8^+$ T cell immunity (called cross-priming) or $CD8^+$ T cell tolerance (called cross-tolerance).

4.5 T cell activation, CD4 help and Memory

Antigen-specific $CD4^+$ and $CD8^+$ T cells exist at very low frequencies in the naïve host. After immunization or infection, naïve T cells are activated and undergo clonal expansion, culminating in a higher frequency of antigen-specific cells that rapidly exhibit effector functions. Upon activation, naïve $CD4^+$ T cells differentiate into different functional subsets called T helper 1 (T_H1) cells that produce predominantly IFNγ and T helper type 2 (T_H2) cells that are characterized by production of IL-4. T_H1 immune responses are essential for the protection against a variety of intracellular infections, whereas T_H2 responses can be protective against certain extracellular parasites. $CD8^+$ T cells that mediate their effector functions through production of cytokines such as IFNγ, TNFα and/or by cytolytic mechanisms are key players in a powerful T_H1 response.

The relevance of $CD4^+$ T cell help and of activation, growth and survival signals provided by other cells such as DCs, in induction of an efficient $CD8^+$ T cell response including clonal expansion, development of effector function and the generation of long-term memory is not entirely understood. A widely accepted theory postulates that the generation of long-lasting CTL responses require antigen-specific interaction of $CD4^+$ T cells and $CD8^+$ T cells with the same DC (Behrens et al., 2004; Castellino and Germain, 2006).

In most cases, naïve $CD8^+$ T cells first encounter antigen being cross-presented by DCs in lymph nodes and the outcome depends on the maturation status of the DC. Immature DCs residing in the periphery are incapable of T cell priming. Presentation of antigen by immature DCs results in tolerance induction by mechanisms involving deletion, induction of unresponsiveness (anergy) or both (Mescher et al., 2007).

According to the "two signal" theory, activation of T cells requires both antigen engagement by the TCR and signals provided by a co-stimulatory receptor such as CD28 binding to its B7 ligands (Janeway and Bottomly, 1994). In addition it has been shown that these classical two signals are not sufficient to support strong clonal expansion, development of effector functions or establishment of a long-lived memory population. The extended model postulates that the basic prerequisites for efficient priming of $CD8^+$ T cells are antigen processing and presentation by activated DCs (signal 1), co-stimulation (signal 2) and the so-called signal 3, which can be provided by IL-12 or IFN alpha/beta (Cousens et al., 1999; Curtsinger et al., 2005; Marrack et al., 1999; Mescher et al., 2007). TLR ligands or CD4 helper cells stimulate dendritic cells to produce these cytokines. Interaction of DCs with $CD4^+$ helper cells via CD40-CD40L conditions these DCs to activate naive CD8 T cells (Ridge et al., 1998; Schoenberger et al., 1998). This process is also called "DC licensing". Engagement of CD40 on DCs stimulates the cells to produce IL-12 and thus provision of the critical third signal for $CD8^+$ T cell activation (Cella et al., 1996). Although IL-2 signals are required for sustained T cell proliferation and clonal expansion, there is currently no evidence to classify IL-2 as a helper cytokine provided by CD4 T cells in vivo as this cytokine is unable to support the development of effector functions in naïve

CD8+ T cells (Curtsinger et al., 2005). The initial activation of $CD8^+$ T cells is followed by the expansion phase that lasts for five to eight days, during which antigen-specific CD8+T cells numbers may increase by > 10,000-fold (Badovinac et al., 2002; Kaech et al., 2002). Clonal expansion is also associated with the differentiation to effector cells that migrate throughout the body. The third phase involves a contraction in number where 90-95% of effector cells are eliminated over the ensuing week (Badovinac et al., 2002; Sprent and Tough, 2001). The final phase is the initiation and maintenance of the $CD8^+$ T cell memory pool by the cells surviving the contraction (Kaech et al., 2002).

Whereas the contribution of CD4 help in the induction of primary effector responses is controversially discussed, an essential role for CD4 help in establishing long-term memory is commonly accepted. IL-12 and IFNα/β provide survival of the responding cells, as they become effector cells. A recent study showed that the death of the majority of the effector cells that occurs following the peak of clonal expansion is more rapid and profound in the absence of a third signal (Curtsinger et al., 2003).

It has been postulated that the expression of costimulatory molecules needs to be inducible by microbial infection (Janeway, 1989). It is now known that PRRs of the innate immune system control many aspects of DC maturation such as expression of costimulatory molecules and cytokine production (Medzhitov, 2001). These stimulatory effects of pathogens are utilized in vaccination strategies by using adjuvants to boost T_H1 immune responses followed by long-term protective memory responses. Discovery of TLR-mediated signaling as the underlying mechanism of infection helped to explain how adjuvants trigger inflammatory reactions to activate the immune system and thereby increase the immunogenicity of the co-administered antigen.

4.6 Interleukin 12 and regulation of innate resistance and adaptive immunity

IL-12 is a heterodimer formed by a 35-kDa light chain (=p35 subunit or IL-12α) and a 40 kDa heavy chain (p40 subunit or IL-12β) (Kobayashi et al., 1989; Stern et al., 1990). It was identified in 1989 as a product of Epstein-Barr virus (EBV)-transformed human B cell lines. that can activate NK cells, generate lymphokine-activated killer cells (LAKs), and induce IFN-γ production and T cell proliferation of pre-activated T cells and NK cells (Kobayashi et al., 1989; Stern et al., 1990). Subsequent studies indicated that IL-12 could boost the generation of cytotoxic T cells by promoting the transcription of genes encoding cytolytic factors including perforin and granzyme (Trinchieri, 1998). Importantly, IL-12-induced IFNγ mediates many of the pro-inflammatory activities of IL-12, whereas the ability to favor a T_H1 response exemplifies its function as an immunoregulatory cytokine that bridges innate resistance and adaptive immunity (Hsieh et al., 1993). In B cells, IL-12 enhances the production of T_H1-associated classes of immunoglobulins such as IgG2a. In 2000 it was discovered that IL-12p40 not only associates with IL-12p35, but also with another molecule, p19, to form a new heterodimer known as IL-23 (Oppmann et al., 2000). The main producers of IL-12 are phagocytes and DCs in response to microbial stimulation (D'Andrea et al., 1992; Macatonia et al.,

1995). In response to viral infections, both IL-12 and IFNα are produced by plasmacytoid DCs. The production of IL-12 is regulated strictly by positive and negative regulatory mechanisms. Microbial products are strong inducers of IL-12 production by APCs. The ability of IFNγ to enhance IL-12 production forms a positive feedback mechanism during inflammation and T_H1 responses. Interestingly, IL-4 and IL-13 –two T_H2 cytokines – are also potent enhancers of IL-12 production. In addition to IFNγ and IL-4, T cells can trigger IL-12 production through direct cell-cell interactions, mostly through ligands of the TNF family such as CD40-CD40L. Negative regulation of IL-12 is performed by several cytokines. TGF β reduces the stability of IL-12p40 mRNA. Although IFNα and IFNβ have some overlapping effects with IL-12 in terms of induction of IFNγ production and T_H1 responses, they are also able to suppress IL-12 production as well as TNFa. Production of IL-12 is inhibited markedly by ligand binding to $G_{\alpha s}$-linked G protein coupled receptors (GPCR), mainly through their induction of cyclic AMP. IL-10 is a potent inhibitor of IL-12 by blocking transcription of both its encoding genes and is often induced when IL-12 is inhibited. It was postulated that the expression of IL-12 and IL-10 is reciprocally regulated which may have important implications on immune regulation.

The IL-12 receptor family consists of IL-12Rβ1, IL-12Rβ2 and IL-23R (Cooper and Khader, 2007). The IL-12p70 receptor is a homo-dimer of IL-12-Rβ1 and IL-12Rβ2, whereas the IL-23 receptor is composed of IL-12Rβ1 and IL23R. The IL-12 receptor is mainly expressed by activated T cells and NK cells (Presky et al., 1996), but has been reported also for DCs (Grohmann et al., 1998) and B cell lines (Airoldi et al., 2000). On most resting T cells, IL-12R is undetectable but it is expressed at a low level by NK cells, which could explain the ability of these cells to respond rapidly to IL-12. T cell activation through the TCR up-regulates the transcription and expression of both IL-12 receptor chains. This up-regulation – in particular of the beta chain – is enhanced by IL-12 itself, IFNα, IFNγ, TNFα and co-stimulation through CD28. Regarding T cells, the expression of IL-12Rβ2 is assumed to be confined to T helper cells and its expression correlates with responsiveness to IL-12 (Rogge et al., 1997; Szabo et al., 1997). Upon binding of IL-12, the heterodimeric IL-12 receptor recruits JAK2 (*Janus kinase*) and TYK2, leading to phosphorylation of STAT-1, -3, -4 and -5 (*signal transducer and activator of transcription*); the specific biological effects of IL-12 are due mainly to activation of STAT-4. IL-23 induces the same JAK/STAT signaling pathway as IL-12, but induces different DNA binding STAT dimers (Parham et al., 2002).

4.7 Type I interferons

Interferons (IFNs), which are only found in vertebrates, are a family of structurally related cytokines with a hallmark function in antiviral activity and. IFNs exhibit a diversity of biological functions such as antiviral activity, antitumor activity and immunomodulatory effects. Type I IFNs are massively produced in most cells in response to viral and other microbial infections. A unique subset of DCs, the plasmacytoid DC, is endowed with a special capacity to produce high amounts of IFNα and IFNβ upon viral exposure or the activation of certain PRRs. By virtue of their potentiating effect on DC maturation, type I IFNs are currently recognized as pivotal cytokines bridging innate and adaptive immune system (Takaoka and Yanai, 2006). Furthermore, type I IFNs are dominating in promoting conditions for IFNγ production by T cells (Cousens et al., 1999).

The type I interferons consist of IFN-α, -β, ω, ε and κ. All IFN α/β subtypes interact with the same receptor complex (IFNAR), which consists of at least two subunits, IFNAR-1 and IFNAR-2. Most cell types bind type I IFNs with a large variation in the number of binding sites and binding affinities. The intracellular domains of these two subunits are associated with JAK1 and Tyk2. Upon binding of IFNs to IFNAR, JAK-1/Tyk-2 are cross-activated and phosphorylate their downstream substrates, STAT-1 and STAT-2. This leads to the activation of transcriptional activator complexes that translocated into the nucleus. The IFN stimulation of promoters containing ISRE results in the transcriptional induction of a large number of target genes to evoke multiple biological activities. IFNARs interact with a number of negative regulatory molecules, including SOCS-1 (*suppressor of cytokine signaling 1*) to limit the extent of signaling.

4.8 Malaria

Malaria is one of the most serious infectious diseases of humans in the world today, infecting nearly 10% of the world population and causing one to two millions deaths, mostly of children, every year. Ninety percent of the deaths occur in sub-Saharan Africa and South East Asia, although malaria is endemic in more than 100 countries. Malaria is caused by infection with various species of the protozoa *Plasmodium* that are transmitted to the human host by the female *Anopheles* mosquito. Four different Plasmodium species - of which *P. falciparum* is the most virulent and prevalent - cause disease in humans. Serious pathological complications such as severe malarial anemia, metabolic acidosis and cerebral malaria (CM) represent life-threatening risks of infection. CM is multi-factorial and a complex syndrome. It is assumed that CM is a consequence of immune-mediated pathology due to overwhelming inflammatory processes.

4.8.1 Life cycle of *Plasmodium*

The *Plasmodium* parasite that causes malaria is transmitted from mosquitos to men. The parasites spend part of their life cycle in the mosquito and part of it in the human host . The infective plasmodial sporozoites enter the bloodstream from the saliva of the female *Anopheles* mosquito during a blood meal. The Kupffer cells of the liver clear the sporozoites from the blood stream and kill many of the microorganisms. Some of the sporozoites escape destruction, and penetrate the hepatocytes where they take up residence. Within the hepatocytes, the parasites transform into a new form called schizonts. The nuclear genetic material in the schizonts replicates until the hepatocytes are totally filled with new forms called merozoites. A single schizont can produce thousands of merozoites. Then, hepatocytes burst and release the merozoites into the bloodstream where they invade circulating erythrocytes. This marks the end of liver-stage replication and initiation of the blood-stage infection, which is the main cause for disease. After penetrating the erythrocytes the merozoites form a ring form called trophozoites. These organisms consume hemoglobin in erythrocytes and enlarge until they fill the cell completely. During their growth, the trophozoites transform into schizonts and produce new merozoites inside the erythrocytes. The red blood cells subsequently lyse and release merozoites that can penetrate new red blood cells and restart the pernicious process.

Some of the trophozoites in the erythrocytes undergo a different developmental pathway and form gametocytes. Gametocytes are the sexual form of the parasite and do no lyse the red blood cells. A mosquito taking a blood meal from a person whose erythrocytes contain gametocytes acquires the malarial parasite. The sexual reproduction cycle then begins in the mosquito. The mosquito subsequently transmits the parasite when it attacks another human host.

4.8.2 Murine models of *Plasmodium* infection

The investigation of CM pathogenesis in humans is limited to analysis of biopsies due to ethical reasons. Post-mortem studies provide end-point findings, whereas the full extent of events leading to different syndromes remains largely unresolved. Therefore, the understanding of CM pathogenesis relies on the use of experimental models. Several animal models exist to study the different pathologies occurring during *Plasmodium* infection in humans. Infection of mice with *Plasmodium berghei ANKA* was established in 1966 (Vincke et al., 1966). The transfer of *P. berghei ANKA* (PbA) infected red blood cells into C57BL/6 mice induces experimental CM (ECM) and ranks among the lethal models of CM. Infected mice develop a neurological syndrome characterized by paralysis, deviation of the head, ataxia, convulsions and coma starting six to 14 days after inoculation with parasitized red blood cells. Infection leads to death in 60-100% of mice with relatively low parasitemia. Mice that do not develop CM die during the second or third week *post infectionem* from hyper-parasitemia and severe anemia (Engwerda et al., 2005a). Most data generated within the model of PbA infection in CM susceptible mice could be validated in humans.

4.8.3 Malaria pathogenesis as a result of immunological processes triggered by *Plasmodium* infection

Severe malaria syndromes are assumed to arise from four basic processes: (1) the site-specific localization of parasitized red blood cells (PRBCs) among target organs, (2) the local and systemic action of bioactive parasite products, such as toxins, on host tissues, (3) the local and systemic production of pro-inflammatory and counter-regulatory cytokines and chemokines by the innate and adaptive immune system in response to the parasite products and (4) the activation, recruitment and infiltration of inflammatory cells (Schofield and Grau, 2005). The contribution of these events to the pathogenesis of CM is controversially discussed.

4.8.3.1 Sequestration of parasitized red blood cells

The mechanical (or sequestration) theory postulates that P. falciparum parasitized erythrocytes (pRBCs) bind to the post capillary venules that in turn causes obstruction of the blood flow, decreased perfusion of the tissue and decreased removal of waste products i.e. lactic acid (van der Heyde et al., 2006). This is supported by the development of lactic acidosis of which the precise causes are not clear. The mechanical hypothesis was expanded by the assumption that pRBCs become less deformable during the progress of *P. falciparum* infection and, consequently, plug the small capillaries (Glenister et al., 2002). Evidence for capillary plugging in CM pathogenesis is provided by the observations that the extent of RBC deformability is an indicator of poor prognosis and that functional capillary density decreases markedly during ECM (Dondorp et al., 2000). However, pBRC sequestration in the brain is controversially discussed, as clinical studies and experimental models indicate only a low correlation between parasitemia and mortality. Individuals with parasitemia are sometimes asymptomatic, and symptomatic infected hosts can have low parasitemia. Furthermore, sequestration is not observed in post mortem analysis performed on tissue from patients who succumbed to *P. falciparum* malaria. Nevertheless, sequestration does occur during malaria, and the adherence of pRBCs probably signals within endothelial cells after binding to CAMs, eliciting a disruption of tight endothelial junctions and increased CAM expression. It is proposed, that the sequestration contributes interdependently from other processes to the development of pathology.

4.8.3.2 Toll-like receptors in *Plasmodium* infection

It is supposed that the diverse organ-specific or systemic disease syndromes are end-stage processes of atypical inflammation cascades that are initiated in target organs by pathogen products. Several studies implicate that *P. falciparum* derived glycosyl-phosphatidyl-inositol (GPI) and hemozoin, a crystalline residue of parasite-mediated hemoglobin digestion are recognized as malarial PAMPs as these components were demonstrated to trigger the expression of pro-inflammatory genes. *Plasmodium*-derived GPI is recognized by TLR2 and TLR4 (Krishnegowda et al., 2005). The finding that that hemozoin stimulates host cells via TLR9 was quite surprising (Coban et al., 2005), as TLR9 had been described as a receptor for CpG-rich DNA. A recent study demonstrated that it is plasmodial DNA instead that stimulates TLR9; the authors suggested that hemozoin may act as a carrier and amplify immune responses to AT-rich plasmodial DNA by targeting the DNA to TLR9 in the endosome (Parroche et al., 2007). TLR recognition activates monocytes to secrete several pro-inflammatory cytokines, including IL-1, IL-6, tumor necrosis factor (TNF) and lymphotoxin, which are suggested to trigger critical processes in malaria pathogenesis. However, the contribution of TLRs in development of ECM is still unclear.

4.8.3.3 Inflammatory and anti-inflammatory cytokines

The inflammation hypothesis, proposed by Maegraith in 1948, states that *Plasmodium* parasites elicit a systemic inflammatory response that causes multi-organ failure and death. Numerous studies support this hypothesis by showing that pro-inflammatory T_H1 cytokines acting in a cascade are important determinants of malaria disease states. In particular, IFNγ and lymphotoxin α are critical factors as depleting antibodies as well as genetic deficiency protected PbA infected mice from ECM. Non-conventional lymphoid populations such as CD1d-restricted natural killer T cells that can recognize GPI as a natural ligand are supposed to account for IFNγ production very early in infection. IFNγ seems to be also the most important cytokine secreted from T cells. In response to TLR ligation, several inflammatory cytokines are produced such as IFNγ, which in turn trigger the secretion of further effector cytokines, such as TNF that are boosting the cytokine cascade. The collection of inflammatory signals during malaria elicits vascular leak and, in combination with signaling through the CAMs, are assumed to contribute to microvascular disruption in several vascular beds, including the brain. The signaling pathways triggering IFNγ effector responses particularly in ECM are not entirely known. IL-12 and TNF are assumed to contribute to the inflammatory response, whereas production of anti-inflammatory IL-10 is supposed to inhibit effector responses. Protective effects of IL-10 were concluded from PbA infection experiments in mice that received IL-10-depleting antibodies, or by treatment with recombinant IL-10 (Kossodo et al., 1997).

4.8.4 Adaptive immune responses in *Plasmodium* infection

CM has been assumed to be a T cell-dependent disease since it was observed that several T cell deficient mice - such as nude, SCID, or RAG-deficient mice - are protected against ECM upon *Plasmodium* infection. This was supported by functional studies using antibodies that depleted T cells (Belnoue et al., 2002; Boubou et al., 1999; Finley et al., 1982; Grau et al., 1986; Nitcheu et al., 2003). In particular, an important role for $CD8^+$ T cells in CM development was concluded from a recent study by Boubou et al., who showed a selective increase of sequestered $CD8^+$ expressing $\alpha\beta$ cells in CM positive mice. CM was prevented if PbA infected mice were specifically depleted of these T cells via antibodies (Boubou et al., 1999). T cells represent a cellular source of IFNγ and contribute to permeability changes of the murine blood-brain barrier through perforin-dependent mechanisms (Nitcheu et al., 2003; Potter et al., 1999). $CD8^+$ T cells sequestered in the brain were proposed to be responsible for the neurological syndrome and for death, but direct evidence is still missing. Furthermore, the induction of parasite-specific $CD8^+$ effector T cells has not yet been shown.

4.9 Regulation of immune responses

To protect the host from succumbing from infection, the innate immune system must rapidly detect and categorize the type of pathogen, which includes extra- or intracellular localization. Immediate activation of innate effector mechanisms ensures early defense and limitation of infection. These immediate decisions set the course for an appropriate adaptive immune response to eliminate the infection and prevent its recurrence. Immune activation is a double-edged sword for the host. It is essential for provoking the immune response and enhancing adaptive immunity against pathogens (Akira et al., 2001; Medzhitov, 2001), which basically aim clearance of infection by destructive mechanisms. The destructive potential requires strict control because the armory used against pathogens is also efficient and detrimental against the host. Self-inflicted damage is therefore inevitable during a protective response, either by direct lysis of infected cells by CTLs or indirectly by the release of antimicrobial factors (cytokines, free radicals) that are toxic to the host. Thus, excessive inflammation must be prevented.

The regulation of immune responses involve multiple points of interaction and cross-regulation between pathogens, antigen-presenting cells (APC) and lymphocytes, which was defined by Bachmann and Kopf by the term “immunological ménage à trois” (Bachmann and Kopf, 2002). This implies several possibilities of interference in immune activation by negative feed back mechanisms induced by the host but also by mechanisms of immune modulation from the parasite.

5 Aims of this thesis

Toll-like receptors (TLR) are crucial for the detection of pathogens by recognition of their conserved molecular patterns. TLR signaling triggers activation of the vertebrate's immune system and generally results in immunity. However, immune activation is a double-edged sword. For instance, while efficient combat of several bacterial and parasitic infections requires IFNγ, regulation of overwhelming IFNγ dependent immune response by induction of anti-inflammatory mechanisms is critical for survival. How excessive inflammation is counter-regulated is still largely unknown.

The aim of this thesis was to determine the role of TLRs in immune regulation. In particular, we were interested

- how TLR stimulation influenced the induction of cytotoxic $CD8^+$ T cell responses in mice infected with recombinant adenovirus, recombinant *Listeria monocytogenes* or *Plasmodium ssp*. and

- how TLRs are involved in the induction and modulation of immuno pathology in the course of *Plasmodium* infection.

6 Material and Methods

6.1 Material

6.1.1 General laboratory equipment

AutoMACS®	Miltenyi, Bergisch Gladbach
Cell culture dishes 25cm2, 80cm2,	Nunc, Wiesbaden
Cell strainer 40μm	BD, Heidelberg
Centrifuge tubes 15ml, 50ml	Greiner Labortechnik, Frickenhauser
Injection needle 25G, 27G	BD, Heidelberg
Metal sieve	University of Heidelberg
Multipipette	Dunn Labortechnik
Microtiter plates 24/48/96 wells	Greiner BioOne
Microwave	Bosch, Stuttgart
Neubauer chamber	Brand, Wertheim
Parafilm®	American National Can TM, Greenwich
Pasteur glass pipettes 150mm, 230mm	Roth, Karslruhe
Pipettes Gilson	Heidelberg
Pipette-boy	Hirschmann Labortechnik, Eberstadt
Reaction tubes 0.5-2mL	Eppendorf, Hamburg
Scalpel	Aesculap, Tuttlingen
Sterile filter 0.2μm	Nunc, Wiesbaden

6.1.2 Machines

Name	Description	Company
AutoMACS®	Magnetic cell separator	Miltenyi, Bergisch Gladbach
Calibur®	Flow Cytometer	BD Biosciences, Heidelberg
Canto®	Flow Cytometer	BD Biosciences, Heidelberg
Canto II®	Flow Cytometer	BD Biosciences, Heidelberg
LSR II®	Flow Cytometer	BD Biosciences, Heidelberg
DiVA®	FACS-Sorter	BD Biosciences, Heidelberg
IVIS®200	*In vivo* Imaging	Xenogen, San Francisco, USA
ELISA reader	ELISA reader	Molecular devices

6.1.3 Analyzing software

Name	Description	Company
CellQuest Pro	Acquisition of FCS files	BD Biosciences, Heidelberg
DiVA	Acquisition + analysis of FCS files	BD Biosciences, Heidelberg
FlowJo	Analysis of FCS files	TreeStar
SPF	ELISA software	Molecular Devices
Living Image 2.5	IVIS Software	Xenogen, San Francisco
Prism	Statistic software	GraphPad Software

6.1.4 Chemicals, reagents and kits

ABTS	Sigma, Deisenhofen
Acetic acid ($C_2H_4O_2$, MW=60,05)	Roth, Karlsruhe
7-Aminoactinomycin D (7-AAD)	Sigma, Deisenhofen
Agarose	Invitrogen, Karlsruhe
Albumin, Bovine serum- (BSA)	Gerbu, Gaiberg
Ammoniumchlorid (NH_4Cl, MW = 53,49)	Merck, Darmstadt
Brefeldin A ($C_{16}H_{24}O_4$, MW = 280,4)	Sigma, Deisenhofen
Bromphenolblue ($C_{19}H_{10}Br_4O_5S$, MW = 670,0)	Sigma, Deisenhofen
Collagenase A	Roche, Mannheim
CFSE	Molecular Probes, Netherlands
Dimethylsulfoxid (DMSO) ($(CH_3)_2OS$, MW = 78,13)	Merck, Darmstadt
Dinatriumhydrogenphosphat (Na_2HPO_4, MW = 142,0)	Merck, Darmstadt
DOTAP®	Roth, Karlsruhe
DMEM	Invitrogen, Karlsruhe
Ethanol, absolute (C_2H_5OH, MW = 46,07)	Merck, Darmstadt
Ethidiumbromid ($C_{21}H_{20}N_3Br$, MW = 394,3)	Sigma, Deisenhofen
Ethylendiamintetraacetate (EDTA)	Sigma, Deisenhofen
($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$, MW = 372,2)	Gerbu, Gaiberg
Foetal calf serum (FCS)	PAA, Cölbe
GBSS (Gey's balanced salt solution)	Invitrogen, Karlsruhe
In vivo JET PEI®	Biomol/Polyplus, Hamburg
L-Glutamine (200mM) ($C_5H_{10}N_2O_3$, MW = 146,1)	Invitrogen, Karlsruhe
GM-CSF	Immunotools
HCl, MW = 36,46)	Sigma, Deisenhofen
Heparin	Amersham Pharmacia, Freiburg

HEPES 1 M ($C_8H_{18}N_2O_4S$, MW = 238,3)	Invitrogen, Karlsruhe
Hoechst 33342	Molecular probes, Netherlands
Isopropanol ($(CH_3)_2CHOH$, MW = 60,1)	Merck, Damrstadt
Lymphoprep	PAA, Cölbe
2-Mercaptoethanol ($HS(CH_2)_2OH$, MW = 78,13)	Sigma, Deisenhofen
Natriumazid (NaN3, MW = 65,01)	Sigma, Deisenhofen
Natriumbicarbonat ($NaHCO_3$, MW = 84,01)	Sigma, Deisenhofen
Natriumchlorid (NaCl, MW = 58,44)	Merck, Darmstadt
Natriumchlorid (NaCl) 0.9%	B.Braun, Melsungen
Natriumdihydrogenphosphat (NaH_2PO_4, MW = 120,0)	Merck, Darmstadt
Natriumhydrogenphosphat (Na_2HPO_4, MW = 142)	Sigma, Deisenhofen
Natriumhydroxid (NaOH, MW = 40,0)	Merck, Darmstadt
Nycodenz	Axishield, Nycomed, Norway
Ovalbumin, Grad V	Sigma, Deisenhofen
Paraformaldehyde (PFA) (H (-OCH_2)n-OH)	Serva, Heidelberg
Penicillin	Invitrogen
PBS	Biochrom, Berlin
Percoll	Amersham Pharmacia, Freiburg
Propidium iodide	Molecular probes, Netherlands
RPMI	Invitrogen, Karlsruhe
Saponin	Sigma, Deisenhofen
Streptomycin	Invitrogen
Sulfuric acid (H_2SO_4)	University of Heiderlberg
Triton X-100	Sigma, Deisenhofen
Trypanblue, 0,4% ($C_{34}H_{24}N_6O_{14}S_4Na_4$, MW = 960,8)	Biochrom, Berlin
Trypsin/EDTA	Invitrogen, Karlsruhe
Türcks solution	Merck

6.1.5 Buffers and media

Cell culture media (DC, T cells)

RPMI 1640 incl. HEPES, 8% (w/v) FCS, 2mM Glutamine, 100 IU/ml Penicillin, 100μg/ml Streptomycin, 50μM 2-Mercaptoethanol was prepared and kept under sterile conditions at 4°C.

CFSE stock:

5,6-Carboxy-Succinimidyl-Fluoresceine-Ester (CFSE) was solved in DMSO (10mM) and stored in aliquots à 10μl at –20°C.

Collagenase A buffer

GBSS ($Ca2^+$ deprived) contained 0.05% (w/v) Collagenase A. 40ml Aliquots were stored at –20°C.

EDTA 0.5M

46,53g Ethylendiamintetraacetat (EDTA) ($C_{10}H_{14}N_2O_8Na_2$ x $2H_2O$, MW = 372,2) was solved in 250 ml ultra-pure water and adjusted to pH 8.0 with NaOH. Solution was autoclaved and stored at room temperature.

ELISA buffers

- Blocking buffer 1x PBS containing 1% (w/v) BSA
- Coating 0,1M $NaHCO_3$, pH 8,2 (stored at 4°C)
- Detection buffer: 1mg ABTS per ml 0,1M citirc acid, pH 4,35 (stored at -20°C)
- Washing buffer 1x PBS containing 0.05% (w/v) Tween prepared freshly

FACS buffer

1x PBS containing 1% (w/v) FCS was stored at 4°C.

MACS buffer

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

2-Mercaptoethanol stock solution

178μl of 14.3 M 2-Mercaptoethanol was diluted in 500ml PBS (=0.005M). Solution was kept sterile and stored at 4°C. It was further diluted 1:100 in media.

Permeabilization buffer incl. Saponin

1x PBS containing 0.5% (w/v) saponin and 2% (w/v) BSA prepared freshly

Permeabilization buffer incl. TritonX100

Triton X-100 0.1 % diluted in 1x PBS, prepared freshly

PBS (phosphate buffered saline)

1x PBS (NaH_2PO_4 20 mM, NaCl 50mM) pH was adjusted to 7.4, then aliquoted à 500ml, autoclaved and stored at 4 °C.

4% (w/v) PFA stock solution

8g of PFA was solved in 200 ml PBS by incubation at 60°C in the water-bath. pH was set to 7.4. Aliquots were stored at –20°C.

10% (w/v) Triton X-100 stock solution

1ml Triton X-100 was mixed with 9ml ultra-pure water and stored at room temperature.

6.1.6 Synthetic oligonucleotides and TLR ligands

CpG-rich Oligonucleotides were obtained from TIB MolBiol, Berlin, Germany. ODNs were delivered as lyophilized powder and dissolved in sterile 0.9% NaCl at a concentration of 1 nmol/μl, and incubated for 30 min at 37°C. Dissolved ODNs were stored at 4°C. Fluorescently labeled CpG-ODN1668-Cy5 was obtained from Sigma-Aldrich.

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

Synthetic RNAs were obtained from Eurogentech® or Biomers® and delivered as lyophilized powder. RNAs were dissolved in sterile 0.9% Nacl (1μg/μl), aliquoted and stored at -80°C.

RNA	Sequence	Target	Reference
9.2s n	AGC UUA ACC UGU CCU UCA A	human TLR9	(Hornung et al., 2005)
9.2s R8A	AGC UUA ACC UGA AAA AAA A	Control to 9.2sn	(Hornung et al., 2005)
siMyD88	AAG GAG AUG GGC UUC GAG UAC dTdT;	MyD88	(Flandin et al., 2006)
siTRIF	GAC CAG ACG CCA CTC CAA C	TRIF	(Li et al., 2005)

Synthetic or purified TLR ligands

Substance	Derived from	Ligand for	Source
Lipopolysaccharide (LPS)	E.coli	TLR4	InVivogen
R848	Synthetic	TLR7	InVivogen, 3M

6.1.7 Peptides

Peptides were obtained from Pineda, solved in DMSO for 20mM stock solutions and stored at -20°C.

Peptide	Amino acid sequence
H-2k^b OVA $_{257-264}$ peptide	SIINFEKL
H-2k^d GFP$_{200-208}$ peptide	HYLSTQSL
HLA A2 MSP-1	
• region 38/42 epitope 291	GLHHLITEL
epitope 609	YLINLKAKI
epitope 437	VIYLKPLAGV
• region 83/30 epitope 313	KLLDKINEI
epitope 674	KLKEFIPKV

6.1.8 Antibodies

- **Antibodies used for cytokine ELISA**

All antibodies used for detection of murine cytokines via sandwich ELISA were purchased from BD, Heidelberg or eBioscience. Matching pairs of antibodies contained a primary unlabeled antibody and a biotinylated detection antibody that were used in previously titrated amounts according to the manufacturers guide. If not otherwise stated, monoclonal antibody derived from the following clones directed against murine cytokines were used:

IL-2	JES6-1A12 (IgG2a, rat) and JES6-5H4 (IgG2b, rat)
IL-6	MP5-20F3 (IgG1, rat) and MP5-32C11 (IgG2a, rat)
IL-10	JES5-2A5 (IgG1, rat) and SXC-1 (IgM, rat)
IL-12p70	C18.2 (IgG2a, rat) and C17.8 (IgG2a, rat)
IFNγ	AN-18 (IgG1 κ, rat) and R4-6A2 (IgG1, rat)

- **Antibodies used for detection of antibody responses**

goat-anti mouse IgM

goat-anti-mouse IgG conjugated with horseradish peroxidase

- **Antibodies coupled with magnetic beads**

Murine antibodies conjugated with magnetic beads (MACS® Beads) were purchased from Miltenyi, Bergisch Gladbach: anti CD4, anti CD19, anti CD11c.

- **Antibodies used for flow cytometry**

For FACS-staining of murine molecules at the cell surface or intracellularly, the following antibodies were purchased from BD Pharmingen or eBiosciences: All antibodies were either biotinylated or labeled with a fluorochrome (FITC, PE, PE-Cy-7, APC, Al488, Al647, Al405) and used in titrated amounts previously determined in the lab. The antibodies were directed against the following murine epitopes (clone names are given in brackets): anti CD4 (GK1.5), anti CD8a (53.-6.7), anti CD11b (M1/70), anti CD11c (HL3), anti CD19 ()1D3, anti CD40 (3/23), anti CD54 (3E2), anti CD69 (H1.2F3), anti CD80 (16-10A1), anti CD86 (GL1), anti MHC class I /H-2kb (34-2-12), anti MHC class II / I-A^b (KH74), anti IFNγ (R4-6A2), anti IL-12p40/p70 (C15.6), anti NK1.1(PK136), anti TNFα (MP6-XT22), anti TCR Vα2 (B20.1), anti TCR Vβ5(MR9-4).

Fc receptors were always blocked by adding 2.4G2 (rat) antibody.

H-$2k^b$/SIINFEKL tetramers labeled with fluorochromes were purchased from ProImmune or provided by Dirk Busch, Munich.

- **Streptavidin conjugates for secondary staining**

Streptavidin (SA) conjugates that were labeled with different fluorochromes were used in combination with biotinylated primary antibodies for flow cytometric analysis. All conjugates were purchased from Molecular Probes, San Francisco, BD Heidelberg or eBiosciences In particular, the following conjugates were used in titrated amounts: SA-Al488, SA-phycoerythrin (SA-PE), SA-PE-Cy7, SA-Al647, SA-APC-Cy7. Dilution was performed according to manufactures guide.

6.1.9 Enzymes

- Collagenase type I A (Clostridium histolyticum) (Boehringer, Mannheim) was used for the digestion of spleens
- Horseradish peroxidase (Invitrogen) was used for sandwich ELISA.

6.1.10 Cell lines

All cell lines were available in the lab or obtained from the DKFZ, Heidelberg.

Line	Description and origin	Reference
B3Z	$CD8^+$ T cell hybridoma, recognizes Ova $_{257-264}$ peptide presented on $H\text{-}2k^b$	(Van Bleek and Nathenson, 1990)
Mo4	melanoma / fibrosarcoma C3H	(Meyvisch and Mareel, 1982; Storme et al., 1981)
RMA RMA-GFP	NK lymphoma ($H\text{-}2k^b$) transfected with GFP	(Gays et al., 2000)

6.1.11 Recombinant viruses, bacteria and parasites

Recombinant adenoviruses are gutless vectors and kindly provided from other scientist: AdOVA, AdLucGFP (Dr. Andreas Untergasser, Prof. Dr. Ulrike Protzer; Cologne), AdLuc-OVA-GFP (Prof. Dr. Thomas Tüting; Bonn) and AdIL-12 (Dr. Volker Schmitz; Bonn). Wild type and recombinant *Listeria monocytogenes* (LM WT, LM-OVA, L-Luc) were provided by Dr. Stefanie Scheu and Prof. Dr. Klaus Pfeffer, Düsseldorf. Recombinant *Escherichia coli* strains (*E.coli*-luc, E. coli-lux) were generated by Timo Schwandt, Bonn. Dr. Natalija Novak, Bonn, provided *staphylococcus aureus*. *Plasmodium berghei ANKA* was available in the lab of Prof. Dr. Achim Hörauf, Bonn. P. berghei ANKA-expressing GFP was a gift from Dr. Friedrich Frischknecht, Heidelberg. All mentioned pathogens were used *in vivo* to study immune responses in wildtype or transgenic mice.

6.1.12 Mouse strains

Wild type strains C57BL/6N, J (H-2k^b), CB F_1(H-2k^b x H-2k^d), B6D2F_1 F_1(H-2^b x H-2k^d) as well as Balb/c (H-2k^d) were obtained from Charles River or Janvier. Mice were bred in animal facilites of the House for experimental Therapy (HET), University of Bonn under specific pathogen free conditions or in the IMMEI. Following knock out (ko) and transgenic (tg) animals backcrossed on C57BL/6 (I-A^b) and Balb/c (I-A^d) were used:

MHC haplotype (H-2k^b /I-A^b)		**Reference**
CD4$^{-/-}$	Deficient in CD4+ cells	(Rahemtulla et al., 1991)
CD40$^{-/-}$	Lack of CD40 expression	
CD11c-DTR-OVA-GFP tg*	Express under CD11c promoter human Diphtheria toxin receptor and express OVA and GFP	N. Garbi, Heidelberg
HLA-A2 tg	Express human MHC class I molecule HLA-A2	(Ureta-Vidal et al., 1999)
I-Ab$^{-/-}$	Lack CD4+ T cells and MHC class II	(Cosgrove et al., 1991; Grusby et al., 1991)
IL-10$^{-/-}$	IL-10 deficient	(Kuhn et al., 1993)
IL-12p35$^{-/-}$	IL-12 deficient	(Cousens et al., 1999)
IL-12p35/p40$^{-/-}$	IL-12 and IL-23 deficient	Provided by G. Alber, Leipzig
IL-23p19$^{-/-}$	IL-23 deficient	Provided by G. Alber, Leipzig
IFNγ$^{-/-}$	IFNγ deficient	Provided by A. Hörauf, Bonn
IFNAR$^{-/-}$	Lack type I IFN receptor	(van den Broek et al., 1995)
iNOS$^{-/-}$	iNOS deficient	Provided by MPI Berlin
OT-I transgenic	$OVA_{257-264}$ specific CD8$^+$ TCR transgenic line	(Hogquist et al., 1994)
OT-II transgenic	$OVA_{323-339}$ specific CD4$^+$ TCR transgenic line	(Barnden et al., 1998)
RAG1$^{-/-}$	Lack B and T cells	Provided from DKFZ
TLR3$^{-/-}$	TLR3 deficient	(Alexopoulou et al., 2001)
TLR4$^{-/-}$	TLR3 deficient	(Hoshino et al., 1999)
TLR7$^{-/-}$	TLR3 deficient	(Hemmi et al., 2002)
TLR9$^{-/-}$	TLR3 deficient	(Hemmi et al., 2000)

6.2 Methods

6.2.1 Experimental treatment of mice

Mice were obtained from Charles River or Janvier and bred under specific pathogen-free conditions in the animal facility service of ZMBH Heidelberg and House for Experimental Therapy (HET), University of Bonn. Water and food were provided ad libitum.

6.2.1.1 Splenectomy

Surgical removal of the spleen was done prior to immunization or during the immune response. During surgery, mice received inhalational anaesthesia with isoflurane. An incision was made in the abdomen over the spleen, and the skin and muscles were pulled back. The spleen was located, and the blood vessels to and around the spleen, including the splenic artery and vein, were clamped or tied off, so that the organ was no longer attached. The spleen was removed and any bleeding was controlled by tying blood vessels. The wound was cleaned, the muscles and skin were closed with stitches.

6.2.1.2 Generation of bone marrow chimeric mice

For the generation of chimeric mice, recipient mice were γ-irradiated with 9 Gy and reconstituted with bone marrow cells from each relevant donor strain. Bone marrow was isolated from hint legs taken from donor mice under sterile conditions, washed and filtrated. Each recipient mouse was injected with ~1x10e7 donor cells i.v. At six to eight weeks after transplantion mice were used for subsequent experiments.

6.2.1.3 Immunization protocols

Reagents or pathogens or cells were diluted in 0.9% NaCl for experimental immunizations. Intravenous injections were performed with a maximal total volume of 250μl, subcutaneous injections with a volume of 100μl.

• Immunization with soluble antigen

For immunization with soluble OVA, C57BL/6 mice were injected i.v. or s.c. (intra ear pinna) with 100μg OVA. H-2k^b SIINFEKL specific cytotoxicity was determined in the spleen (i.v.) or in draining lymph nodes (s.c.). H-2k^b SIINFEKL specific CD8$^+$ T cells were quantified by MHC tetramer staining (see below).

• Immunization with transduced BMDCs

Mice were injected i.v. with 2x 10e6 AdOVA-transduced DC (see below). H-2k^b SIINFEKL specific cytotoxicity was determined in the spleen five days later.

• Infection with recombinant adenoviruses

Mice were infected i.v. with 1x 10e7 PFU of recombinant adenoviruses expressing OVA, Luciferase and GFP or all three antigens. Viral load of AdLucGFP infected mice was monitored via *In vivo* imaging of bioluminescence at day 2 p.i.. H-2k^b SIINFEKL or H-2k^d HYLSTQSAL specific cytotoxicity was determined in the spleen or in the blood (in splenectomized groups). H-2k^b SIINFEKL specific CD8$^+$ T cells were quantified by MHC tetramer staining (see below). Antibodies against adenovirus and OVA were determined at day 21 p.i.

• Infection with recombinant *Listeria monocytogenes*

Recombinant Listeria monocytogenes expressing OVA were cultured at 20°C over night in brain heart infusion medium in a shaker. Mice were infected intravenously with 1x10e5 bacteria. SIINFEKL or H-2k^d HYLSTQSAL specific cytotoxicity was determined in the spleen.

• Inoculation with *P. falciparum* infected red blood cells

Human erythrocytes were cultured and infected with *Plasmodium falciparum* by members of the lab from Hermann Bujard in the ZMBH, University of Heidelberg. After formation of the "ring" stage, the infected erythrocytes were harvested, counted and incubated for 1 hour with chloroquine. HLA-A2 transgenic mice were immunized with 2,8 x 10e8 *P. falciparum* infected erythrocytes i.v. P. falciparum-derived MSP-1 specific cytotoxicity was determined six days later in spleen or blood.

• Inoculation with RMA-GFP tumor cells

Mice were injected i.v. with 5x10e5 RMA-GFP tumor cells. RMA-specific cytotoxicity was determined six days later in the livers by in vivo CTL assay (see below).

• Inoculation with Mo4 tumor cells

Mice were injected i.v. with 5x 10e5 Mo4 tumor cells expressing luciferase. Tumor growth in the lung was monitored by *in vivo* imaging of bioluminescence every other day (see below).

6.2.1.4 Preparation of *P. berghei ANKA* merozoites and infection of mice

All *Plasmodium* infection experiments were performed in the Department of Parasitology, University of Bonn. *Plasmodium berghei* ANKA (PbA) parasites were obtained from a mouse previously infected with sporozoites. Blood was taken from highly parasitemic Balb/c mice. Parasitemia was determined in Giemsa-stained blood smears from tail blood. Giemsa stain is used to differentiate nuclear and/or cytoplasmic morphology of platelets, RBCs, WBCs, and parasites. Blood samples from donor mice were taken daily from day 4 post infection. 2-3 drops of blood were given on a slide and dispersed to obtain a thin blood film. After the blood has dried, the sample was fixed with methanol by dipping the film briefly in a Coplin jar containing absolute methanol. The sample was air dried again and then stained with diluted GIEMSA (1:20, vol/vol) for 20 minutes. It was briefly washed with PBS and then air dried in a vertical position. To determine parasitemia, 500 erythrocytes were counted and analyzed for parasites.

Recipient mice were infected at the age of eight weeks or older with 5x10e4 parasitized red blood cells (merozoites). After infection, mice were monitored for parasitemia and survival. C57BL/6 mice develop 6-8 days cerebral malaria after infection with 5x 10e4 PbA parasites that is characterized by neurological symptoms, unconsciousness, coma and final death.

6.2.1.5 *In vivo* application of TLR ligands

- TLR9 ligand CpG-rich ODN, TLR4 ligand LPS, TLR8 ligand R848

  TLR ligands were diluted in 0.9% NaCl and injected i.v. in a maximal total volume of 250μl. Titration of the individual TLR ligands to determine sublethal doses that induce immune suppression were determined before. If not otherwise stated, the following doses were applied in vivo: TLR9 ligand CpG 100μg; TLR4 ligand LPS 1μg; TLR7 ligand R848 10μg.

- TLR7 ligands single stranded RNA sequences

  siRNA sequences were injected i.v. or s.c. according to the experiment in a dose of 1 or 10μg per animal. siRNA requires complexation to a carrier to avoid premature degradation, if applied in vivo. Two different strategies were available for siRNA application in vivo.

  - Carrier: Cationic liposomes (CL) facilitates delivery into the endosome

    10μg siRNA was incubated 30μl DOTAP® for 20 minutes at room temperature under sterile conditions and then diluted with sterile 0.9% NaCl to obtain the final volume for injection.

  - Carrier: Polyethylene imines (PEI) facilitates siRNA delivery into the cytoplasm.

    This carrier condenses DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis 21. It is acting as a "proton sponge" that buffers the endosomal pH and protects DNA from degradation.

Continuous proton influx also induces endosome osmotic swelling and rupture which provides an escape mechanism for DNA particles to the cytoplasm (Kichler et al., 1995; Remy et al., 1995).

10μg siRNA are diluted in 50μl sterile 5% glucose solution and mixed with 2μl In vivo Jet PEI® diluted in another 50μl 5% Glucose solution. After incubation of 20 minutes at 37°C, the mixture is filled with glucose to obtain a final volume of 250μl per recipient.

6.2.2 *Ex vivo* methods

6.2.2.1 Enrichment of splenic dendritic cells for *ex vivo* analysis

C57BL/6 mice were injected repeatedly with TLR9 ligand CpG. Two hours after the re-challenge, mice were sacrificed and spleens were taken out. To enrich splenic DCs, spleens were perfused in vitro with GBSS containing 0.5% collagenase A, then cut into small pieces and transferred back into a 15 ml reaction tube. Spleens were incubated in 2 ml GBSS-collagenase buffer at 37°C for 20 minutes. Then, MACS buffer was added to prevent cell clumping. Spleen pieces were gently pressed through a metal sieve to obtain a single cell suspension. Splenocytes were washed with MACS buffer, centrifuged for 5 minutes at 4°C, 1500 rpm and resuspended in RPMI medium. Cells were counted and plated into 24 wells in a concentration of 5x10e6 cells per ml. To determine production of inflammatory cytokines, Golgi Plug® and Golgi Stop® was added.

6.2.2.2 *In vitro* generation of myeloid dendritic cells from bone marrow and transduction with recombinant adenovirus

C57BL/6 mice were immunized with AdOVA-transduced BMDCs to determine the OVA-specific CTL response *in vivo*.

Preparation protocol

Hind legs from mice were cut at the pelvis and disinfected with ethanol. Skin and muscle tissue were removed, blank bones were washed in sterile PBS. Bone ends were cut with a scissor, bone marrow was flushed with a PBS-filled syringe into a sterile 50 ml tube. Cells were filtrated through a 40 μM mesh and washed with PBS, spun down for 10 min at 4°C 1500 rpm. Cells were re-suspended in IMDM medium complemented with GM-CSF; the total cell number was determined. Cells were plated on sterile Agar plates with 5x10e6 cells per plate in a volume of 8 ml. Day 4 after preparation cells were washed and replated. Cells in suspension were transferred into a 50 ml tube. Adherent cells were incubated for 2 min with 2 mM EDTA at 37°C, then removed from the plates by PBS washing and transferred into 50 ml tubes. Cells were washed with PBS and re-plated in fresh IMDM medium at

a number of 5x10e6 per plate. At day 7 of culture, BMDCs were harvested and stained for CD11c, CD11b, CD80 and CD86.

***In vitro* transduction of BMDCs**

BMDCs were cultured for 7 days in Petri dishes (ø 6 cm).

Transduction with recombinant adenovirus was performed at an multiplicity of infection (MOI) of 250 (i.e. 2.5 x 10e8 pfu/well). Cells were incubated for 4 h at 37°Cand 5% CO_2. After this time, 1.5 ml of DC medium with 10% FCS was added. Transduction efficiency and expression of MHC class II *(*I-A^b*)* and CD86 was assessed using flow cytometry. Mice were immunized with 2x 10e6 Ad-transduced DC which were harvested, washed and resuspended in 250 µl of 0.9% NaCl.

6.2.2.3 Isolation and adoptive transfer of splenic T cells

- **Isolation of splenic $CD4^+$ T cells from OVA $CD4^+$ TCR transgenic donors**

C57BL/6 mice received via adoptive transfer splenic $CD4^+$ T cells isolated from OT-II transgenic donor mice that possess OVA-specific $CD4^+$ TCR- transgenic T cells two days before immunization with recombinant adenovirus expressing OVA.

Procedere

A spleen of one OVA-specific CD4+ TCR transgenic (OT-II) was taken out, harvested and filtrated to obtain a single cell suspension. Splenocytes were diluted in 15 ml RPMI medium transferred into a 75cm2 cell culture flask. To activate OVA-specific CD4 T cells, 1mg OVA per ml was added to the culture. The cells were maintained for 7 days at 37°C under sterile conditions and supplemented with new medium, if required. At day seven, the cells were washed with MACS buffer, centrifuged at 1500 rpm for five minutes. The cell pellet was resuspended in 1 ml MACS buffer and filtrated. Isolation of $CD4^+$ T cells was performed in a two-step selection process. First, CD19, CD11b and CD8+ contaminating cells were separated by MACS. The negative flow through was stained with CD4+ MACS beads and then positive selected. Purity of cell was confirmed by flow cytometry. For adoptive transfer of the cells, cells were resuspended in 0.9% NaCl, counted and titrated. Every recipient mouse was injected i.v. with 2,5 x 10e5 $CD4^+$ cells.

- **Isolation and adoptive transfer of splenic T cells from WT donors**

RAG1-deficient mice were reconstituted via adoptive transfer with splenic $CD4^+$ T cells and $CD8^+$ T cells isolated from wildtype donor mice seven days before immunization with recombinant adenovirus expressing OVA or infection with *P. berghei ANKA*.

Procedere

We calculated one donor spleen for one recipient spleen. Spleen of wild type mice were taken out, harvested and filtrated to obtain a single cell suspension. The cells were washed with MACS buffer and centrifuged at 1500 rpm for five minutes. The cell pellet was resuspended in 1 ml MACS buffer and filtrated. Isolation of $CD4^+$ T cells and $CD8^+$ T cells was performed in a positive selection process. The cells were stained with CD4+ MACS beads and CD8+ MACS® beads for 15 minutes at 4°C, then washed with MACS buffer and then positive selected with the help of the Auto®MACS. Purity of cell was confirmed by flow cytometry. For adoptive transfer of the cells, cells were resuspended in 0.9% NaCl, counted and titrated. Every recipient mouse was injected i.v. with 2,5 x 10e5 CD4+ cells.

6.2.3 Analysis of innate and adaptive immune response

6.2.3.1 Flow cytometric analysis of surface and intracellular molecules

Principle of method

The flow cytometer was designed to automate the analysis and separation of cells stained with fluorescent antibodies. The instrument uses a laser beam and light detector to count single intact cells in suspension. Every time a cell passes the laser beam, light is deflected from the detector, and this interruption of the laser signal is recorded. Those cells having a fluorescently tagged antibody bound to their cell surface antigens are excited by the laser and emit light that is recorded by a second detector system located at a right angle to the laser beam. The simplest form of the instrument counts each cell as it passes the laser beam and records the level of fluorescence intensity as the abscissa. More sophisticated versions of the instrument are capable of sorting populations of cells into different containers according to their fluorescence profile. Use of the instrument to determine which and how many members of a cell population bind fluorescently labeled antibodies is called analysis; use of the instrument to place cells having different patterns of reactivity into different containers is called cell sorting. It is possible to determine the absolute number of cells as well the percentage of cells within a certain population expressing the target antigen passing the beam. Analysis of stained cells allows also the distribution of cells in a sample population according to antigen densities as determined by fluorescence intensity. Information about the cell size is derived from analysis of the light-scattering properties of examined cells. Multi-color analysis facilitates differentiated conclusions about phenotype and functions of the examined cells.

Surface staining:
Cells were transferred into a 96 round-bottom plate (circa $1x10^6$ cells/well) and washed with FACS buffer (5 min 1500 rpm 4°C). Antibody mastermix was prepared in FACS buffer for staining in a volume of 50μl per sample. Cell pellet was carefully re-suspended in 50μl aliquot of antibody mastermix and incubated on ice for 15 minutes while covered with tin foil. Cells were washed with FACS buffer and stained if necessary with secondary antibodies to biotinylated primary antibodies again on ice. Cells were centrifuged and washed twice in FACS buffer before analyzing with a flow cytometer.

Intracellular staining of cytoplasmic proteins (cytokines)
Staining of intracellular antigens was always performed after cell surface staining and subsequent fixation of the cells. In case of intracellular cytokine staining, the cells were incubated in vitro for 5 hours in RPMI containing 0.8μl each Golgi Plug® and Golgi Stop® per ml RPMI. Ex vivo analysis of splenic dendritic cells was performed under semi-sterile conditions.

After completion of surface staining, cells were fixed with 2% (w(v) PFA for 10 min on ice. PFA was immediately washed off by centrifugation of the cells at 1800 rpm for 10 minutes. Cells were permeabilized with saponin buffer during an incubation of 20 min at RT. Cells were centifuged and re-suspended in 50μl antibody solution (incl. Fc block) diluted in saponin buffer. Cells were incubated for 20 min at RT, then 150 μl of saponin buffer was added and cells were left for another 5 minutes at RT to allow unbound antibody to diffuse out of the cell. Cells were centrifuged and washed twice in FACS buffer before analyzing with flow cytometry.

SIINFEKL Tetramer staining
H-$2k^b$ –OVA (SIINFEKL)-specific CD8+ T cells were stained in the spleens of mice five days after immunization with AdOVA. A splenocyte single cell suspension was generated. The cells were washed in FACS buffer, centrifuged for five minutes 4°C 1500 rpm. The cell pellet of ~1x10e6 splenocytes was resuspended in 30μl FACS buffer containing 2.5μl Fc blocking antibody and 5μl fluorochrome labeled SIINFEKL-MHC class I tetramers and incubated for 20 minutes at room temperature. Then, 20μl FACS buffer containing diluted fluorescently labeled anti-CD8a antibody was added and incubated for another 15 minutes on ice. Then, the cells were washed with FACS-buffer, centrifuged five minutes 4°C 1500 rpm before analyzing with flow cytometry.

Data acquisition and analysis
Flow cytometry was performed on a FACS-Calibur®, FACS-Canto-II® or LSR II (BD). Doublets were excluded in a FCS-W/SSC-A gate. Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

6.2.3.2 *In vivo* cytotoxicity assay

Principle of method

Cytotoxicity of antigen-specific $CD8a^+$ T cells can be measured at day 5 following immunization. Intravenously applied splenocytes (= target cells) from syngenic donors which are labeled with antigen-specific class I peptide are killed by antigen-specific CTLs of the immunized animals. Labeling of these target cells with CFSE allows visualization of lyse via flow cytometry.

Procedure:

As target cells, splenocytes from syngenic donors were either labeled with the specific class I peptide (2μM, 30min 37°C; peptide see below) and with a high concentration of CFSE (1μM CFSE 15 min 37°C; $CFSE^{high}$). In parallel, reference cells were labeled with a low dose of CFSE without prior peptide loading (0.1μM CFSE 15 min 37°C; $CFSE^{low}$). After CFSE labeling, cells were washed three times with PBS. Cell numbers were determined and mixed at a 1:1 ratio. Each mouse received $5x10^6$ target cells and $5x10^6$ reference cells intravenously. Four hours later, organs (spleen, lymph nodes) were taken out under semi-sterile conditions and homogenized with the help of metal sieves. Cells were washed with FACS buffer and filtrated. Lyse of target cells and ratio of target ($CFSE^{high}$) and reference cells ($CFSE^{low}$) was determined by flow cytometric acquisition of CFSE positive cells. Data were analyzed using FlowJo®, Microsoft Excel® and Prism® software. To calculate specific lyses the following formula was used:

specific cytotoxicity [%] = 100 – (($CFSE^{high}$ / $CFSE^{low}$) of immunized animal / ($CFSE^{high}$ / $CFSE^{low}$) of naïve control) x 100).

Target cells were pulsed according to the immunization protocol with the following peptides:

OVA	(H-2kb)	SIINFEKL
GFP	(H-2kd)	HYLSTQSAL
MSP-1	(HLA-A2)	GLHHLITEL, VIYLKPLAGV, KLLDKINEI, KLKEFIPKV, SLEVSDIVKL

6.2.3.3 ELISA (enzyme linked immuno sorbent assay)

Principle of method:

Enzyme-linked immuno-sorbent assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. The “sandwich ELISA” is used to determine the antigen concentration in unknown samples and requires two antibodies that bind to epitopes that do not overlap on the antigen. Plate-bound “capture” antibody immobilizes antigen of a sample. Then the binding of the “detection” antibody facilitates via biotinylation detection and quantification of antigen. Strepavidin-coupled horseradish

peroxidase binds to biotin and converts a colorimetric substrate to a soluble colored product. Substrates used with peroxidase include 2,2'-azo-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 3,3'5,5'- tetramethylbenzidine base (TMB), which yield green and blue colors, respectively.

Material and solutions

96 well "Maxisorb" micro-titer plate, flat bottom (NUNC Immunosorb); Multi-channel pipette, ELISA reader,

Solutions

coating buffer (0,1M Na_2HPO_4, pH 8,5), washing buffer (PBS, 0,05% Tween 20); blocking buffer (PBS, 1% (w/v) BSA); ABTS buffer (0,1M Na_2HPO_4, pH 4,2); detection solution ABTS buffer, (1mg/ml ABTS, 2ml/ml 30% H_2O_2) or TMB and 0.18M H_2SO_4.

Buffers and samples were added in a volume of 50μl/well unless otherwise stated.

Antibodies and enzymes

Purified capture antibody, biotinylated secondary antibody, horseradish peroxidase

Procedure of sandwich ELISA (cytokine detection)

The micro-titer plate was coated with unlabeled capture antibody (1μg/ml) that was diluted in coating buffer and incubated at 4°C at least four hours or over night. Then unbound antibody was removed by extensive washing with washing buffer. To prevent unspecific binding, the micro-titer plate was saturated by incubation with blocking solution (100μl/ well) for 30 minutes at RT. Wells were washed three times with washing buffer. Antigen-containing samples and defined dilution series of the appropriate recombinant protein standard were added to the wells and incubated over night at 4°C. Cytokine standards were diluted in complete medium and covered a range from 80 ng/ml to 0.078 ng/ml in ¼ dilution steps. Wells were washed three times with washing buffer. The secondary biotinylated antibody was diluted in blocking buffer (1μg/ml) and incubated for four hours at 4°C. Wells were washed three times with PBS/1% Tween. Horseradish peroxidase was diluted in PBS and incubated for one hour at RT. Wells were washed three times with washing buffer. ABTS substrate was diluted in ABTS buffer (1mg/ml) and added to the wells. After suggested incubation time has elapsed, optical densities at 405 nm could be measured on an ELISA plate reader. Alternatively, TMB was used as a substrate. In those cases, commercial TMB solution was added to the well. Enzymatic products were quantified at 650 nm. The enzymatic reaction was stopped by addition of 50μl 0.18 M H_2SO_4/well. The end-point reaction delivered a yellow product, which could be read at 450 nm. Data analysis was performed with SPF software.

Sandwich ELISA for determination of antibody responses

Serum titers of IgG specific for adenovirus or for OVA were quantified by coating plates with adenoviral particles or OVA, respectively and using POX-conjugated goat-anti-mouse-IgG and goat-anti-mouse IgM antibodies (BD, Heidelberg; Jackson)

6.2.3.4 *In vivo* detection of bioluminescence

The AdLucGFP load in C57BL/6 mice and tumor load of mice inoculated with Mo4-luciferase tumor cells was quantified by *in vivo* bioluminescence using the real-time IVIS Imaging System 200 (Xenogen Corp., Alameda, CA, USA). Mice were analyzed for bioluminescence measurement 24 h after infection or tumor cell inoculation. Five minutes before quantification of bioluminescence, mice were injected intraperitoneally with 2.5 mg luciferine (S039; Synchem, Kassel, Germany). Analysis was performed under inhalational anaesthesia with isoflurane. Data were analyzed using Living Image 2.50 software (Xenogen Corp.).

6.2.3.5 Statistical analysis

Graphs displaying specific cytotoxicity show always the results are expressed as mean ± standard deviation (SD). Comparisons were drawn using a two-tailed Student's t-test (Prism 4, Graphpad Software Inc., San Diego CA USA). Statistical analyses for survival curves was performed using the Kaplan-Meier-Test. Each group was compared to the others to investigate statistical differences in survival. *P* values of less than 0.05 were considered significant and are signed with asterisks in the graphs. Significance of one group refers to all other groups, unless otherwise mentioned.

7 Results

7.1 TLR ligands suppress adaptive immune response

7.1.1 Characterization of TLR9 ligand induced CTL suppression

7.1.1.1 Systemic injection of TLR9 ligand inhibited antigen-specific CTL response

It is well established, that local application of TLR ligands – e.g. TLR9 ligand CpG-rich DNA – enhances the immunogenicity of co-administered antigens. This is utilized in vaccination strategies also already in humans for generation of efficient immune responses against infectious antigens (Krieg, 2006).

In the present study, immune stimulatory effects of CpG-ODN1668 (CpG) were confirmed in a murine model of immunization against the soluble model antigen ovalbumin (OVA) *in vivo*. For this purpose, C57BL/6 mice were injected subcutaneously (s. c.) with 100μg OVA with or without 50μg CpG. The activity of OVA-specific cytotoxic lymphocytes (CTLs) was determined 5 days after immunization in the draining lymph nodes by performance of an *in vivo* cytotoxicity assay.

The elimination of syngeneic target cells (H-2k^b) loaded with OVA-derived class I peptide SIINFEKL (S8L) was ten times more efficient in CpG/OVA-treated animals than in mice that received OVA alone (Figure 2 A). Thus, co-administered CpG strongly enhanced OVA-specific CTL responses in draining lymph nodes of C57BL/6 mice following subcutaneous (s.c.) immunization. In contrast, only weak CTL responses were obtained in OVA-immunized mice without CpG (Figure 2**A left panel**).

To analyze whether systemic immunization may also be influenced by TLR ligands, wild type mice were immunized with OVA intravenously (i.v.) with or without co-administration of CpG. Five days later, cytotoxic activity of OVA-specific CTLs was assessed with the help of an *in vivo* cytotoxicity assay in the spleen. Surprisingly, the cytotoxic activity of OVA-specific CD8a$^+$ T cells in the spleen was markedly decreased by CpG application in comparison to control-immunized animals (Figure 2A, **right panels**). Thus, systemic co-injection of CpG and antigen inhibited antigen-specific cytotoxicity.

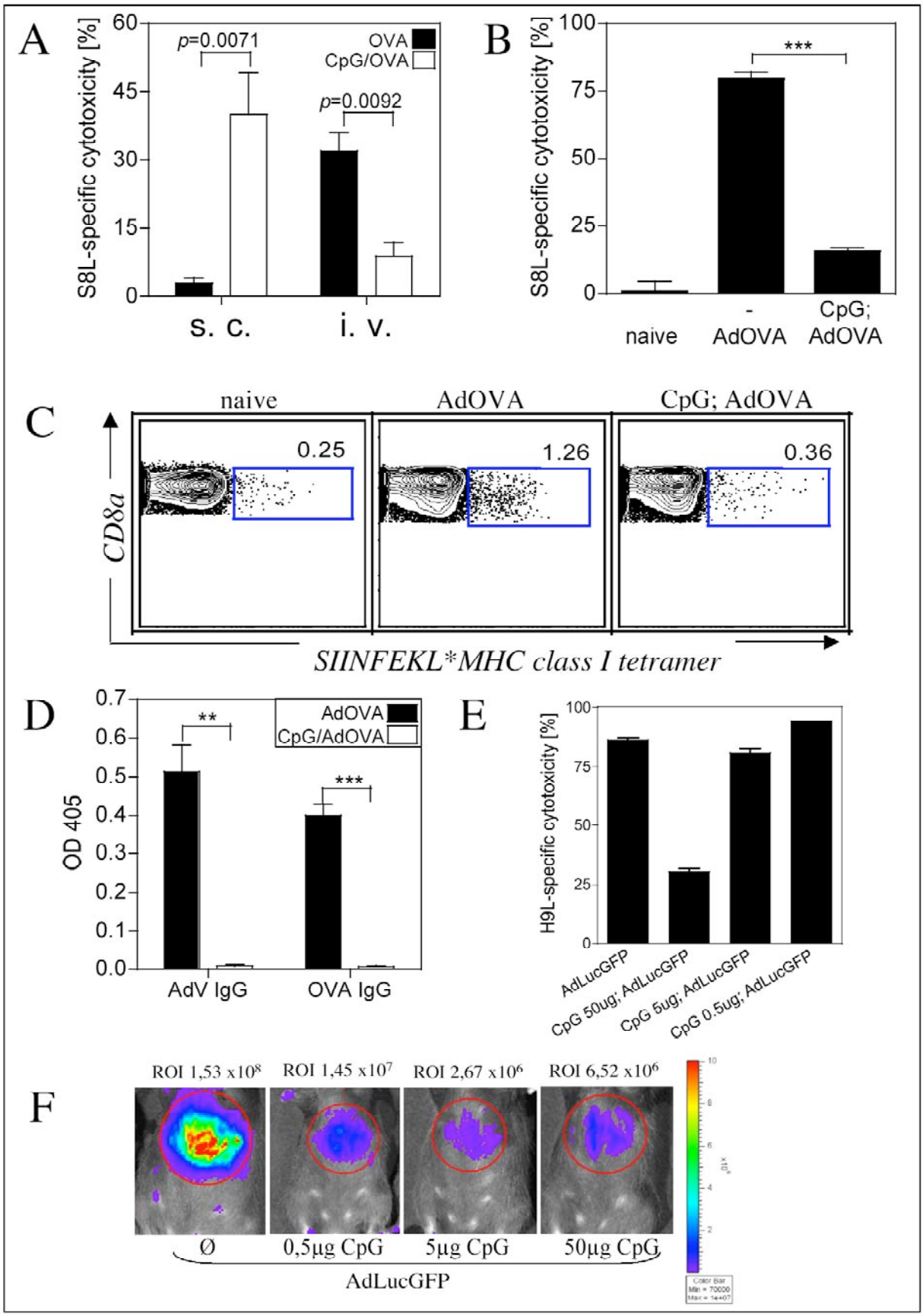

Figure 2: Systemic application of CpG-ODN1668 suppressed adaptive immune response and generation of antigen-specific T cells towards subsequent immunization.

(see next page for figure legend)

- Figure legend for figure 2 -

(a) C57BL/6 mice were immunized with 100µg OVA either s. c. or i. v. with (white bars) or without (black bars) CpG pretreatment (50µg). OVA specific CTL activity was analyzed 5 days p. i. in the draining lymph node (s. c.) or in the spleen (i. v.). (b, c) C57BL/6 mice were immunized with 1x10e7 PFU recombinant AdOVA i. v. with or without CpG pretreatment (100µg i. v.). OVA specific cytotoxicity was determined in vivo 5 days p. i. in the spleen (b). (c) Binding of splenic CD8a positive T cells to SIINFEKL-loaded MHC class I tetramers was assessed with flow cytometry. Numbers indicate percent of double positive cells. Data are representative results from more than three independent experiments with three or more mice per group. students *t* test: two asterisks represent p values <0.005, three asterisks represent p values <0.0001. (d) Determination of adenovirus specific IgG and OVA-specific IgG in sera of C57BL/6 after infection with recombinant AdOVA with or without CpG pretreatment according to (b). (e, f) C57BL/6 x BALB/c F1 mice were injected i.v. with the indicated doses of CpG-ODN 1 day before infection with 1x10e7 PFU recombinant AdLucGFP. (e) Determination of HYLSTQSAL-specific cytotoxicity in vivo 5d p.i. in spleens of AdLucGFP-infected animals. (f) Luciferase-related bioluminescence was quantified 3 days after infection with AdLucGFP to determine viral load in the host. ROI= region of interest; ROI numbers indicate light intensity in the ROI. Results are expressed as mean ± standard error of the mean (SEM). Representative data are shown.

7.1.1.2 Systemic injection of TLR9 ligand CpG prevented adaptive immune responses *in vivo* against AdOVA

Systemic application of CpG inhibited cellular immune responses towards the soluble antigen OVA (Figure 2A). It was investigated next, whether CpG also influenced cellular immune responses towards viral infections. To study this question, mice were infected i. v. with replication-deficient recombinant adenoviruses with or without systemic CpG-pretreatment. Recombinant adenoviruses were chosen as immunization model because recent studies by Harui et al. demonstrate the induction of strong CTL and antibody responses towards adenoviral expressed transgenes (Harui et al., 2004).

Here, experiments were performed primarily with recombinant adenoviruses expressing OVA (AdOVA) or luciferase and green fluorescent protein (GFP, AdLucGFP). OVA-derived MHC class I peptide S8L is presented on H-2k^b that allows determination of cytotoxic activity of S8L-specific CTLs in C57BL/6 after infection AdOVA. Infection with AdLucGFP allows monitoring of luciferase-caused bioluminescence via *in vivo* imaging as well as determination of cytotoxic activity of GFP-specific CTLs recognizing the GFP-derived MHC class I peptide HYLSTQSAL (H9L) presented on H2-k^d.

In the present study, mice were injected i. v. with 100μg CpG one day prior to infection with recombinant AdOVA. Antigen-specific CTL responses were determined five days post infection with the help of an *in vivo* cytotoxicity assay in the spleens of the infected mice.

Systemic pre-treatment of mice prevented generation of antigen-specific CTL responses in C57BL/6 mice after i. v. infection with AdOVA (Figure 2**B**). In addition, we used the MHC class I tetramer technology to quantify S8L specific CTLs by flow cytometry. In spleens of CpG treated C57BL/6 mice, the amount of S8L-specific T cells was reduced by factor 3 compared to control-infected littermates (Figure 2**C**). Furthermore, systemic CpG injection affected also the generation of virus-specific antibodies. In the sera of control-infected mice, high levels of OVA- and adenovirus-specific

immunoglobulins were detected 21 days after AdOVA immunization, whereas both types of antibodies were lacking in CpG-treated animals (Figure 2**D**).

It is well described, that CpG can stimulate innate immune cells resulting in antiviral effects, which may cause reduced viral load. It is possible that the lack of both CTL and antibody responses were the result of CpG-induced activation of innate phagocytic mechanisms that may cause removal of the antigen. To rule this out, the infection rate was investigated in mice 24 hours after infection with AdLucGFP by *in vivo* imaging of luciferase bioluminescence. In addition, activity of H9L-specific CTLs in spleens of the infected (C57BL/6 x Balb/c) F1 mice was assessed by an *in vivo* cytotoxicity assay 5 days post infection and compared to the infection rate. To determine influences of CpG application, mice received 0,5μg, 5μg or 50μg CpG 24 hours before infection with AdLucGFP. All three doses of CpG strongly reduced bioluminescence (Figure 2**F**).

However, CpG-induced inhibition of CTL responses was dose-dependent; only high doses equal or above 50μg of CpG suppressed the GFP-specific CTL response, whereas low doses were immune stimulatory and enhanced GFP-specific CTL responses (Figure 2**E**).

Thus, inefficient T cell priming cannot be explained by complete clearance of antigen due to increased activation of innate immune mechanisms. The anatomic site of CpG application influenced (and determined) the outcome of the subsequent immune response. CpG injection prior to immunization with soluble or virally expressed antigen led to a dose-dependent inhibition of antigen-specific CTL responses. Thus, systemic application of CpG in high doses did not amplify the adaptive immune response, but rather prevented the generation of antigen-specific T cells towards adenoviral infection.

7.1.1.3 CpG induced immune suppression is dependent on TLR9

TLR9 recognizes CpG-rich DNA as a natural ligand (Hemmi et al., 2000). To investigate whether CpG-mediated suppression is dependent on TLR9, experiments were performed in mice that were either competent or deficient in TLR9 expression. To answer the additional question, whether CpG-induced suppression is dependent on TLR9-expression by cells of the hematopoietic system, TLR9-bone marrow chimeric mice were generated. For generation of TLR9-chimeric mice, sub-lethally irradiated wild type mice were reconstituted with bone marrow (BM) purified from TLR9-deficient mice. As a control, wild type mice were reconstituted with BM from wild type littermates.

All mice were infected with AdOVA with or without systemic CpG pretreatment; OVA-specific CTL responses were determined 5 days p. i. in the spleen.

TLR9-deficient animals generated a CTL response against OVA equally well compared to wild type littermates (Figure 3**A**). Importantly, CpG-induced inhibition of the OVA-specific CTL response was abrogated in TLR9-deficient mice (Figure 3**A**). Analysis of TLR9-BM chimeric animals revealed that CpG-induced suppression was dependent on TLR9 expressed by BM-derived cells. Wild type recipients that were reconstituted with $TLR9^{-/-}$ BM generated strong OVA-specific CTL responses

whether or not CpG was injected before immunization (Figure 3**B**). In contrast, wild type recipients reconstituted with BM from wild type littermates, CpG treatment prior to AdOVA immunization suppressed OVA-specific CTL responses (Figure 3**B**).

Thus, CpG mediated suppression was dependent on TLR9 expressed by BM-derived cells.

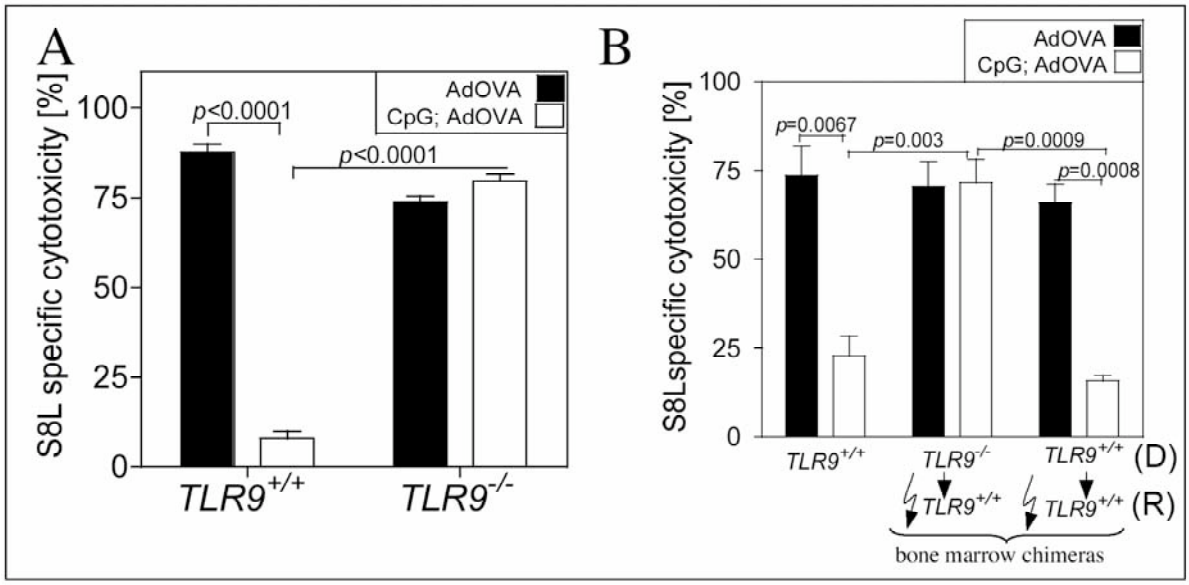

Figure 3 TLR9-dependent suppression induced by CpG

(a) TLR9 deficient and wild type littermates were pretreated with 50µg CpG before infection with 1x10e7 PFU recombinant AdOVA. Cytotoxic function of OVA class I peptide S8L-specific CTLs was determined five days after immunization in a 4h *in vivo* cytotoxicity assay. Specific lysis of target cells in the spleens was calculated and is shown as percentage. ***(b)*** TLR9-BM chimeric mice were generated and analyzed for suppression. WT recipients received either BM from syngenic TLR9 deficient donors (middle) or from TLR9 competent donors (right), Representative data of two independent experiments are shown with three or more mice per group. (D) donor (R) recipient. BM bone marrow

7.1.2 Mechanistic requirements for the induction of CTL responses against antigens expressed by recombinant adenovirus

7.1.2.1 Importance of the spleen and CD4 help for the generation of CTL responses

Observations in patients who underwent surgical removal of the spleen imply that the spleen is of major importance for the generation of protective and functional adaptive immune responses towards several infections (Mebius and Kraal, 2005). Indeed, experiments performed in our laboratory in splenectomized mice supported this hypothesis for the mouse model. C57BL/6 mice were splenectomized either one day before or four days after immunization with AdOVA and an OVA-specific *in vivo* cytotoxicity assay was performed 5 days after infection. Antigen specific CTL

responses against OVA were lacking in all splenectomized animals, as determined in blood and liver (Figure 4**A, data not shown**). This is remarkable, as recombinant adenovirus is reported to primarily infect hepatocytes of the liver in mice and to induce activation of liver DCs (Zhang et al., 2001). This result strongly implies that despite high infection rate of the liver and low infection rate of the spleen, antigen-specific CTL responses were not generated in the liver, but required the spleen.

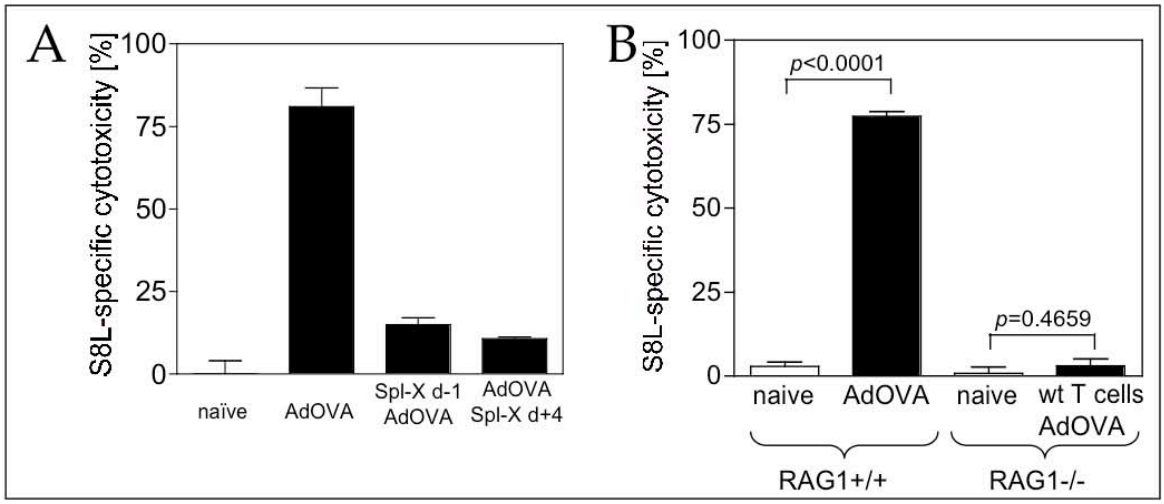

Figure 4 The induction of functional immune responses towards AdOVA infection is dependent of the spleen

(***a, b***) *In vivo* SIINFEKL-specific CTL responses in mice were determined five days after AdOVA infection. (**a**) Comparison of naïve C57BL/6 mice and splenectomized littermates. Splenectomy was performed either prior to or after i. v. immunization with 1x10e7 PFU AdOVA, analysis of blood. (n=3 per group). (**b**) Analysis of S8L-specific cytotoxicity in the spleen of wild type and RAG1$^{-/-}$ mice. RAG1$^{-/-}$ received CD4+ and CD8a+ splenocytes purified from syngenic naïve wild type donors seven days before AdOVA infection.

It was investigated next, whether the generation of a productive CTL response towards AdOVA required intact spleen architecture. Due to its localization in the circulatory system and its unusual structure of its lymphoid compartments, the spleen is a unique lymphoid organ. Functional disruption of the RAG-1 gene by homologous recombination leads to the absence of B and T cells in RAG-1$^{-/-}$ mice, and thus to a disorganized micro-architecture of the spleen. (Mebius and Kraal, 2005). We investigated, whether RAG1$^{-/-}$ mice were able to generate CTL responses towards AdOVA infection after reconstitution with splenic T cells from syngeneic donors. CD4^{+} and CD8a^{+} T cells cells were isolated from naïve C57BL/6 and purified via MACS technology, then pooled and adoptively transferred into RAG1$^{-/-}$ recipients. All mice were infected with AdOVA seven days after transfer. It was not possible to detect cytotoxic activity of S8L-specific CTLs in RAG1$^{-/-}$ mice after substitution with splenic CD4^{+} and CD8^{+} T cells isolated from naïve wild type donors (Figure 4**B**).

These results indicate that not only the lack of certain cell populations, but also a disintegrated architecture of the spleen was responsible for inefficient generation of adaptive immune responses.

7.1.2.2 Induction of antigen specific CTL responses in AdOVA infection requires CD4 help and CD40 signaling

Additionally, we investigated the role of CD4 help in the model of AdOVA infection. It is well known, that for generation of CD8 responses towards some viral infections CD4 help is required (Ahmed et al., 1988; Cardin et al., 1996; Kast et al., 1986). Therefore, the generation of OVA-specific CTLs was investigated in wild type mice that were injected i. p. with a CD4 depleting antibody before AdOVA infection. In addition, $CD4^{-/-}$ or MHC class $II^{-/-}$ (=I-$Ab^{-/-}$) deficient mice that lack $CD4^{+}$ cells (Bevan, 2004) were infected with AdOVA and examined in this experiment. Five days after infection, the cytotoxic activity of OVA-specific CTLs was determined in spleens of these mice. Neither of the groups generated productive OVA-specific CTL responses (Figure 5 A,B).

CD4 help requires a productive interaction between DC and $CD4^{+}$ T cell via CD40-CD40L (Schoenberger et al., 1998). We investigated whether CD40 deficient mice were able to induce an OVA-specific CTL response upon AdOVA infection. OVA-specific CTL responses were determined five days after infection in the spleens, but were not detected in CD40-deficient mice (Figure 5C).

Thus, CD4 help was critical for the induction of functional immune responses towards AdOVA infection.

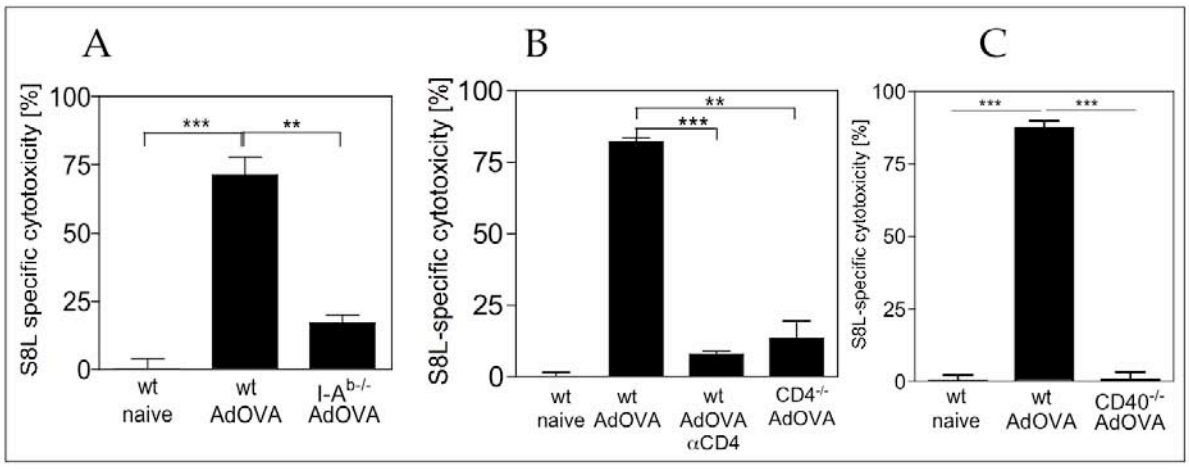

Figure 5 CD4 help and CD40 signaling are required in CTL induction

S8L specific cytotoxicity in spleens of AdOVA infected mice at day 5. (**a**) Analysis of spleen of AdOVA-infected wild type and MHC class II deficient mice (I-$A^{b-/-}$). (**b**) Analysis of spleen of C57BL/6 wild type, CD4-depleted wild type and CD4 deficient littermates (c) . Analysis of spleen of AdOVA-infected wild type and CD40 deficient mice n=4 per group. Results are median ±SEM.

7.1.3 Cellular phenomena of CpG induced suppression

7.1.3.1 Injection of CpG induced an initial immune activation

Production of inflammatory cytokines is a hallmark during immune activation and described to be a consequence of TLR ligation *in vivo* and *in vitro* (Kawai and Akira, 2005). Upon ligation, TLRs trigger the production of NF-kB-dependent gene products like IL-6 and IL-12 (Doyle and O'Neill, 2006). In the present study, the question arose whether the dose of CpG that induced suppression of CTL responses towards subsequent infections, initially also leads to activation of immune cells.

To examine immune activation due to CpG application, mice were injected i. v. with 100μg CpG. Sera and splenocytes were analyzed for production of IL-6 and IL-12. Furthermore, splenic DCs were stained for the surface expression of molecules that are important for antigen presentation and co-stimulation and indicate DC maturation.

Indeed, whereas in sera of naïve C57BL/6 mice neither IL-6 nor IL-12 could be measured, sera of CpG-injected littermates contained elevated levels of IL-12p70 and IL-6 2 hours after single CpG injection (Figure 6**A**). In addition, increased levels of IL-12p40p70 were detected in $CD8^+CD11c^+$ splenocytes of CpG-injected mice but not samples of naïve mice as determined by intracellular staining (ICS) and analyzed via flow cytometry (Figure 6B). Flow cytometric analysis 24 hours after CpG injection showed further that splenic dendritic cells of CpG-injected mice expressed enhanced levels of CD40, CD80 and $I\text{-}A^b$ in comparison to naïve mice (Figure 6**C**). Thus, systemic application of TLR9 ligand CpG induced a phenotypic and functional activation of splenic dendritic cells.

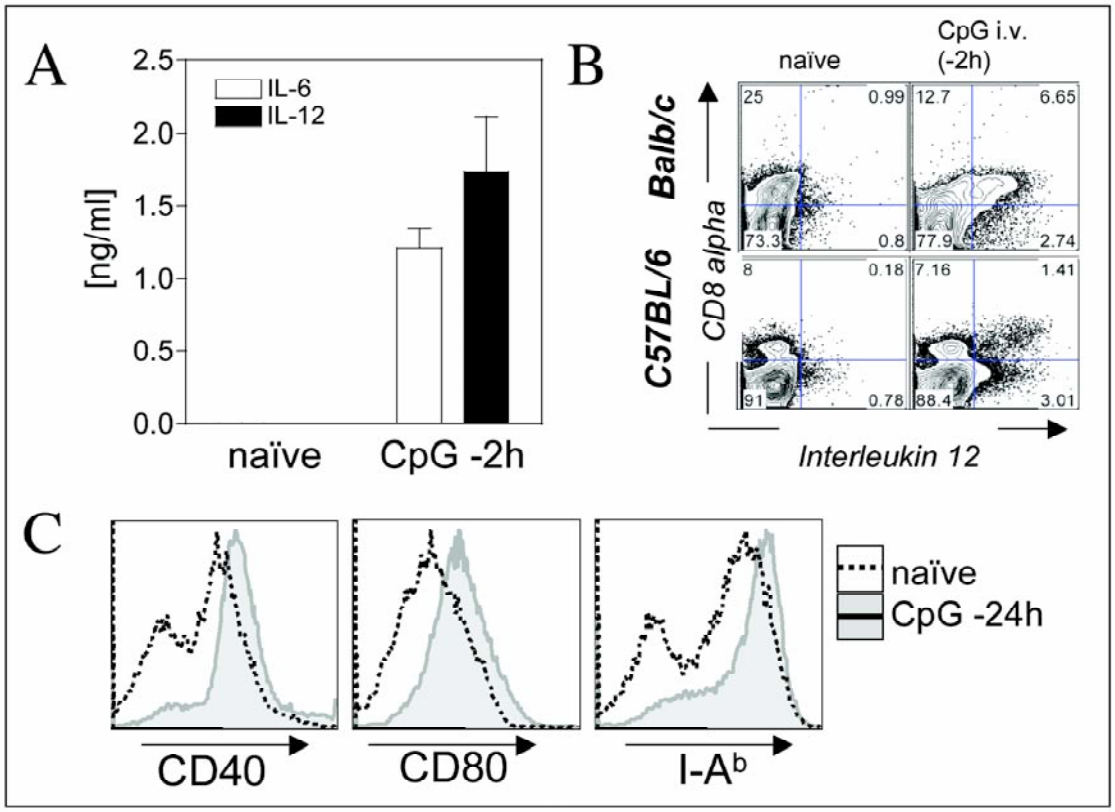

Figure 6 Systemic CpG application activated splenic dendritic cells

(a) Sera taken from ± CpG (100µg i.v.) injected C57BL/6 were analyzed for IL-6 and IL-12 via sandwich ELISA. n=2. (b) Intracellular detection of IL-12 produced by splenic $CD8a^+$ $CD11c^+$ cells isolated from Balb/c (upper row) and C57BL/6 (lower row). Mice received 100µg CpG i. v. and were sacrificed 2 hours later. n=2 per group, representative data from one mouse from more than 3 independent experiments are shown.(c) Splenic dendritic cells isolated from C57BL/6 24 hours after systemic injection of 100µg CpG. Cells were gated on CD11c (open histograms = naïve control; tinted histograms = TLR-L treated). n=2 per group, representative data from one mouse are shown.

7.1.3.2 Multiple TLR stimulation is followed by paralysis and altered composition of DC subpopulations

The primary finding showed that systemic injection of TLR9 ligand CpG inhibited the generation of CTL responses towards subsequent AdOVA infection. This contrasts the observation that CpG treatment triggered systemic immune activation and maturation of splenic dendritic cells (Figure 6). Therefore, we asked whether a second TLR stimulation could re-elicit production of pro-inflammatory cytokines in mice previously injected with CpG in comparison to single CpG-injected mice. To mimic CpG injection and subsequent AdOVA infection, mice were given two CpG injections of 100μg each 24 hours apart. All mice were sacrificed 2 hours after the last injection. Systemic levels of IL-12p70 were determined in the sera of all mice by sandwich ELISA. The production of inflammatory cytokines was examined by intracellular FACS staining for IL-12p40p70, TNFα and IFNγ. To distinguish different splenocyte subsets, $CD11c^+$ splenocytes were gated for CD8α, CD11b and NK1.1

(Figure 8). Figure 8 illustrates the amount of cells that were stained positive for each cytokine and for the subset-marker. The relative amount of double positive cells was calculated and stated as percent corresponding to the individual subset (Figure 8).

In splenocytes of mice that received a single injection of CpG briefly before analysis, strong signals for IL-12p40p70, TNFa and IFNg were detected. In particular, almost 50% of $CD8a^+CD11c^+$ were stained for IL-12p40p70, whereas in the same splenic DC subset of naïve controls no reasonable amount of this cytokine was detected (left column #1 and #2, Figure 8A). In addition, more than 7% of splenic $CD11b^+CD11c^+$ splenocytes were stained for TNFα (middle column #1 and #2, Figure 8 B) and almost 30% of $NK1.1^+CD11c^+$ splenocytes were stained for IFNg (right column #1 and #2, Figure 8C). Again, in splenocytes of naïve mice, signals for TNFα and IFNγ were almost undetectable or very low. Systemic activation as a result of CpG injection was concluded from increased IL-12p70 levels detected in sera of CpG-injected mice compared to lacking signals in sera of naïve mice (D). These results corresponded to the data shown in 7.1.3.1 where a primary immune activation was observed following a single injection of CpG.

In contrast, in CpG re-injected mice, levels of IL-12p40p70 detected in $CD8a^+CD11c^+$ splenocytes were greatly impaired, as determined by ICS (Figure 7 left column). Compared to splenocytes from single injected mice, the amount of $CD8a^+CD11c^+$ splenocytes stained positive for IL-12p40p70 was reduced by 80% following repeated CpG treatment. In addition, production of TNFα by $CD11b^+$ $CD11c^+$ as well as IFNγ production by $NK1.1^+CD11c^+$ was greatly impaired in this group (Figure 7 middle and right panel). A quantification of ICS results revealed, that only 50% of $CD11b^+CD11c^+$ were positive for TNFα and only 20% of $NK1.1^+CD11c^+$ were positive for IFNγ (Figure 8). Most importantly, the expression of all examined splenic subset marker molecules was remarkably reduced in these splenocyte-samples of mice that received repeated CpG injection within 24 hours. CD8a and NK1.1 were decreased about twofold whereas expression of CD11b was only marginally reduced. To determine whether the induction of DC paralysis in the spleen results also in systemic suppression, levels of IL-12 were measured in the sera of CpG treated or untreated mice. Samples of mice that received CpG prior to a secondary CpG stimulus contained markedly reduced levels of IL-12 compared to single-injected mice (Figure 8D).

Thus, primary CpG application induced paralysis of splenocytes and changed the composition of cellular subsets in the spleen. This resulted in abrogated cytokine production in response to a secondary TLR stimulation in the spleen, but was also detected systemically.

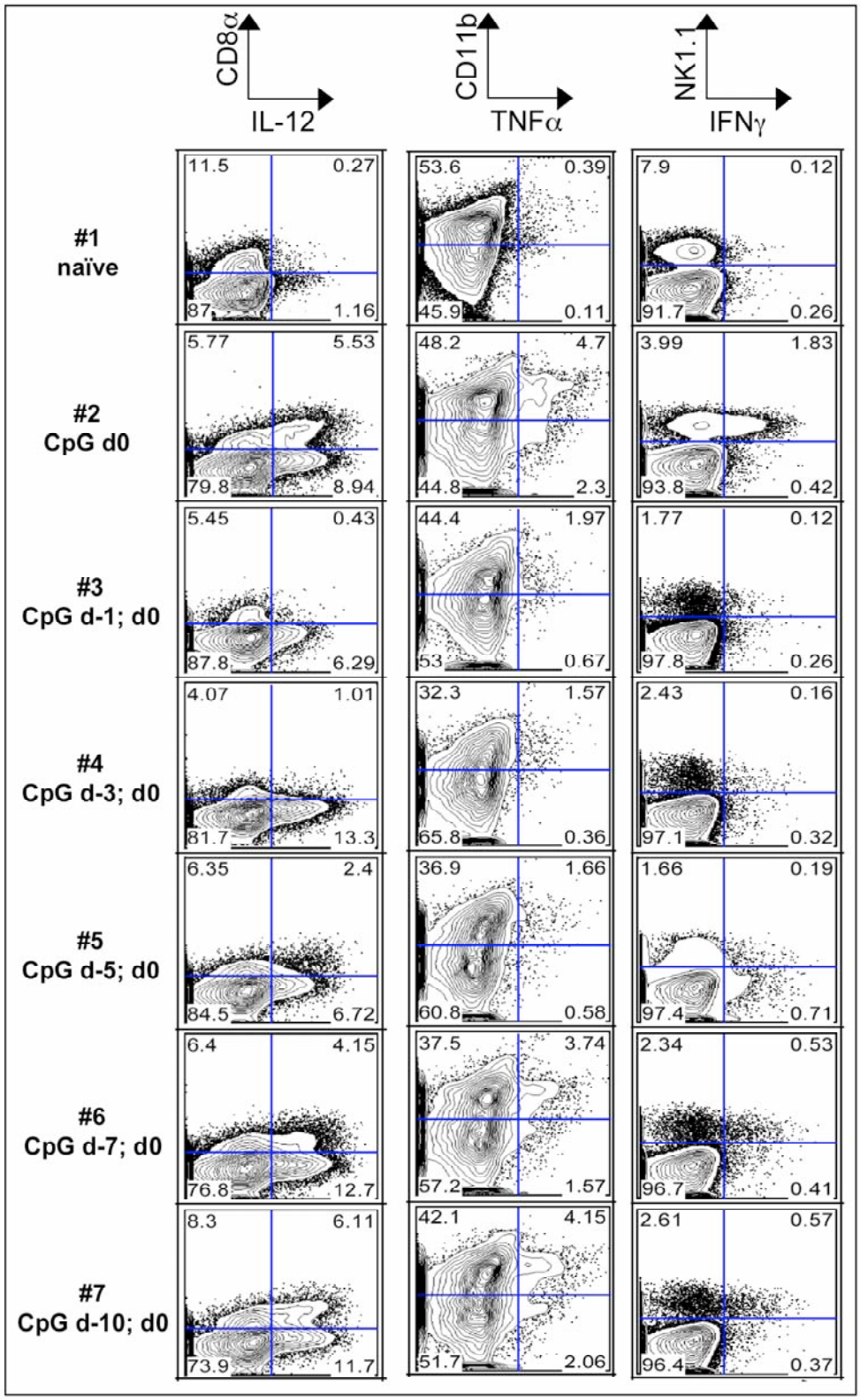

Figure 7 Systemic CpG injection rendered splenic DC inactive

C57BL/6 were pretreated *in vivo* with 100µg CpG i. v. at indicated time points. 2 hours before analysis, mice were restimulated *in vivo* with 100 µg CpG i.v. Then spleens were isolated and digested with collagenase. 5x10e6 cells were incubated *in vitro* for 4 hours with 0.8 µl Golgi Plug® and Stop® per ml medium. Production of IL-12p40p70/ TNFα/IFNγ was assessed by ICS. $CD11c^+$ gated splenocytes are displayed. Representative data from 1 out of 2 mice are shown.

In order to determine the duration of CpG induced paralysis, the kinetic of restoration of the response to CpG was studied in previously paralyzed animals. Table 1 gives an overview about the treatment schedule. Mice received a first systemic injection of CpG on different days prior to CpG re-challenge and were compared to littermates injected once with CpG as well as naïve mice.

Group of C57BL/6 (n=2)	Treatment 100μg CpG i.v.
#1	-
#2	d0
#3	d-1; d0
#4	d-3; d0
#5	d-5; d0
#6	d-7; d0
#7	d-10; d0

Table 1 Treatment schedule for ex vivo analysis

C57BL/6 were injected twice with 100µg CpG-ODN1668 i. v. at the indicated time points. The individual groups are indicated. Exceptions: Naïve mice did not receive CpG treatment (=group #1) and group #2 received only one-time CpG injection 2 hours before analysis. 2 hours after the last CpG injection, mice were sacrificed for analysis. (# =group).

Complete abrogation of IL-12 production by $CD8a^+$ DCs lasted for 3 days (Figure 7 left column, Figure 8A). Five days after the initial challenge, a slight restoration of the response towards CpG was observed as an increased amount of $CD8a^+CD11c^+$ splenocytes that were positive for IL-12p40p70 was detected. The full response to CpG was restored in CpG-re-challenged mice if the initial CpG injection dated back longer than seven days. Similar results were obtained by the determination of intracellular levels of TNFα in $CD11b^+CD11c^+$ and of IFNγ in $NK1.1^+CD11c^+$ cells. The amount of splenic $CD11b^+CD11c^+$ cells that were stained positive for TNFα, was strongly decreased when CpG injection was repeated within 24 hours but was completely recovered, when the initial CpG injection was given seven days prior to CpG re-challenge (Figure 8B). Similarly, less $NK1.1^+CD11c^+$ splenocytes were stained for IFNγ immediately upon CpG re-challenge, but increased the more primary CpG application dated back (Figure 7 middle and right panel). This indicates a re-population of these splenic $CD11c^+$ subsets into the spleen.

Kinetics of systemic paralysis reflected the results obtained from the spleen as sera of CpG-treated mice contained less IL-12p70 after consecutive CpG treatment. Systemic levels of IL-12p70 triggered by CpG increased concomitantly and were comparable to values from the positive controls if the primary challenge dated back seven days or more (Figure 8D). These data suggest, that TLR ligation alters splenic $CD11c^+$ subpopulations as they are temporarily refractory towards repeated microbial signals. This is not only detected locally but also systemically.

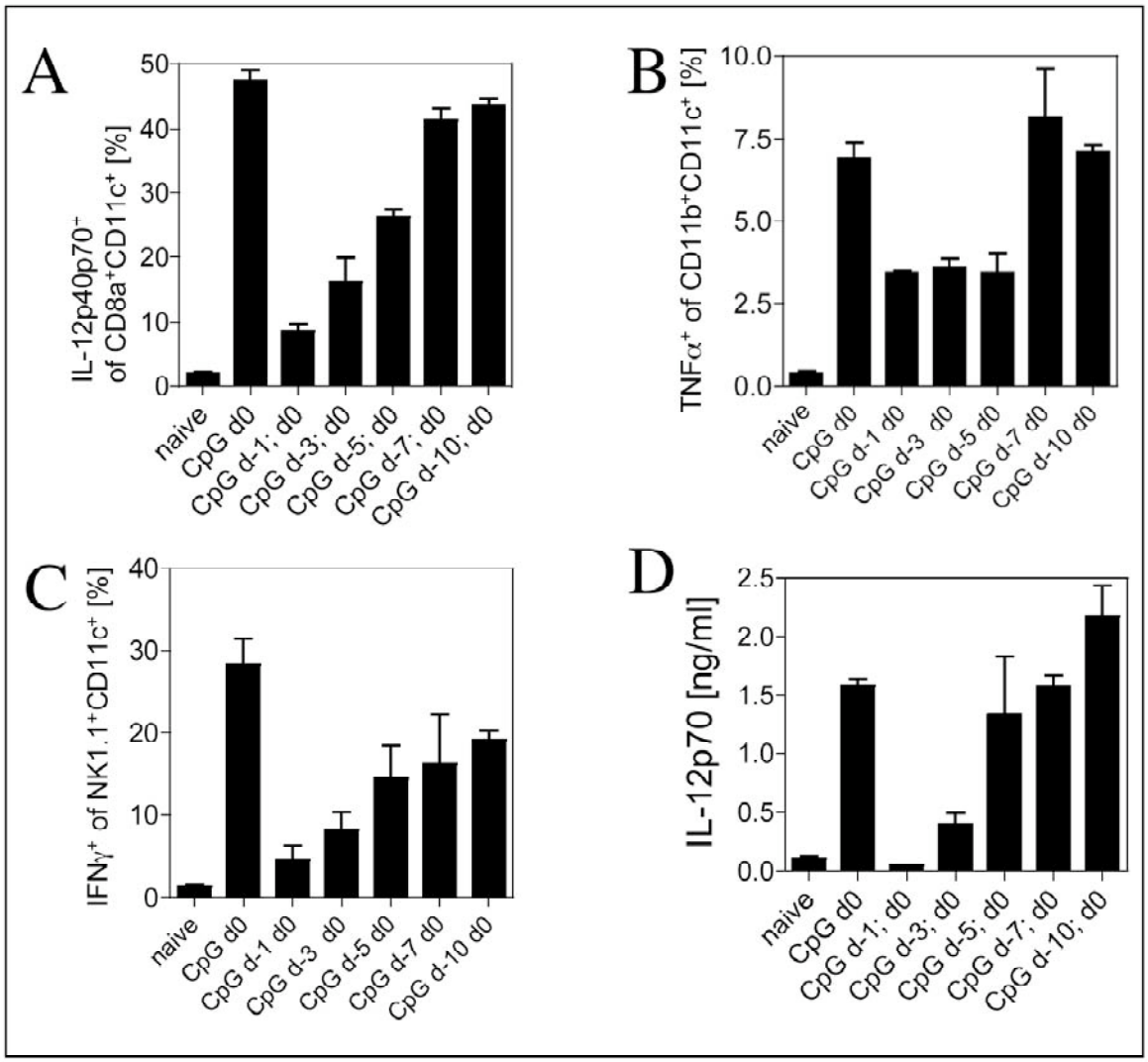

Figure 8 Quantification of CpG-induced DC paralysis.

(a, b, c) Calculation of cytokine production by CD11c+ splenocyte subsets, all data and groups correspond to figure 7 (a) Calculation of $CD8a^+CD11c^+$ splenocytes stained for IL-12p40p70 (b) Calculation of $CD11b^+CD11c^+$ splenocytes stained for TNFα (c) Calculation of $NK1.1^+CD11c^+$ splenocytes stained for IFNγ (d) Determination of systemic production of IL-12p70 detected in sera of CpG treated mice corresponding to figure 7 via Sandwich ELISA.

As mentioned above, in CpG treated mice subpopulations of $CD11c^+$ splenocytes were fundamentally altered regarding the cellular composition. This remarkable change was also observed during the kinetic experiment. Particularly splenic $CD11c^+$ subsets expressing CD8α or NK1.1 were markedly decreased upon repeated CpG injection, whereas the fraction of $CD11b^+CD11c^+$ was decreased to a lesser extent. The amount of $CD8\alpha^+CD11c^+$ was mostly decreased in spleen of mice that were injected with CpG at d-3 and d0. The longer the time-span was between primary and secondary injection of CpG, the higher the amounts of $CD8\alpha^+CD11c^+$ splenocytes were detected by ICS. If more than 5 days elapsed between primary and secondary CpG application, levels of $CD8\alpha^+CD11c^+$ splenocytes reached numbers that were comparable to those determined in spleens of naïve controls. Similar findings were made for $NK1.1^+CD11c^+$ splenocytes. Here, the lowest amount of $NK1.1^+CD11^+$ was

detected in spleens of mice that received the primary CpG injection at d-5, d-3 or d-1. Importantly, the amount of this splenic subpopulation did not recover until a period of 10 days to levels that were equivalent to naïve controls. This suggested, that not only production of pro-inflammatory cytokines by individual splenocyte subsets was decreased but also the relative amount of this specific splenocyte subpopulation was reduced.

To examine whether this loss of distinct splenocyte subpopulations was a result of primary CpG injection, mice were injected with CpG and splenocytes were examined 24 hours later in respect to their expression of CD11c and CD8a, NK1.1, CD11b or CD19. The amount of the different subsets were determined by flow cytometric analysis and calculated as relative proportion of $CD11c^{+}$ cells. $CD11c^{high}$ cells were almost absent in spleens of CpG-treated mice, which constituted a clearly confined population in spleens of naïve animals (Figure 9A). CD11c expression by splenocytes was rather decreased to intermediate levels. Furthermore, whereas the fraction of $CD11c^{+}$ splenocytes stained positive for CD8α or NK1.1 was decreased by factor 3 and 4, respectively, the proportion of $CD11b^{+}CD11c^{+}$ splenocytes differed only marginally (Figure 9B). Interestingly, the amount of $CD19^{+}CD11c^{+}$ splenocytes was more than twofold increased in CpG treated mice (Figure 9D).

These findings show that systemic CpG injection does not only impair DC function but also results in loss /disappearance of certain DC subpopulations. We assume that this will contribute to the impaired immune responses towards further microbial challenge *in vivo*.

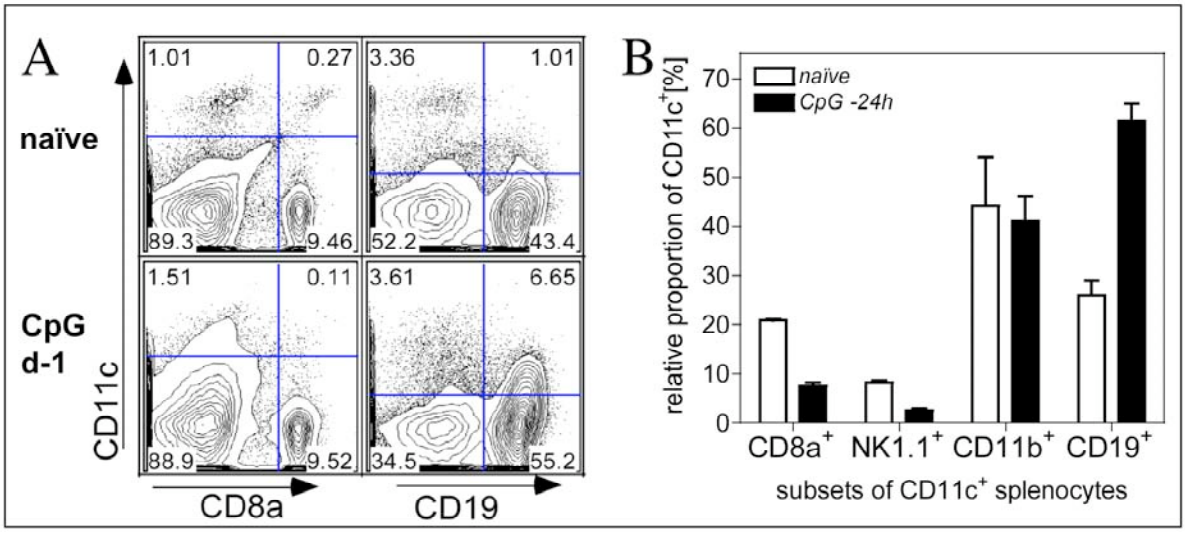

Figure 9 CpG altered composition of splenic DC subpopulations

(a) Staining of CD11c+ splenocytes regarding CD8a (left column) and CD19 (right column) expression of naïve C56BL/6 (upper panel) and 24h after CpG (100µg i.v.) lower panel). Representative data of one mouse out of two are shown. (b) Quantification of relative proportion of splenic CD11c+ subsets isolated in naïve mice compared to CpG-injected littermates after flow cytometric analysis (100µg CpG-ODN1668 i.v. 24 hours before analysis) Mean ± standard deviation of n= 2.

7.1.3.3 Exogenously matured DCs are unable to restore CTL response in CpG pretreated recipients

The previously presented data described the inhibition of adaptive immune responses induced by CpG (Figure 2**B**). This suggested two different possibilities of CpG-induced suppression. CpG could either directly inhibit DC maturation and antigen presentation or indirectly by induction of an inhibitory local milieu. To distinguish between these two possibilities, an adoptive transfer of *in vitro* infected bone marrow-derived DCs (BMDC) was performed. Efficient activation and re-localization of transferred BMDC into the spleen could be confirmed *in vivo* recently by Schweichel et al. (Schweichel et al., 2006).

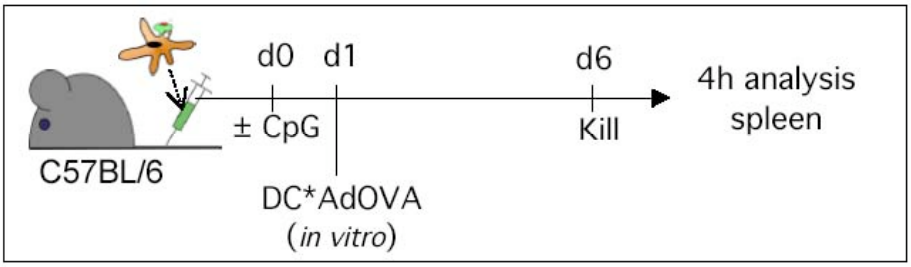

Figure 10 Treatment schedule for adoptive transfer of AdOVA- transduced BMDCs into syngenic naïve or CpG-1668 pretreated recipients

Therefore, BMDC derived from syngenic donors were transduced with AdOVA *in vitro*. 24 hours later, the transduced DCs were adoptively transferred into C57BL/6 that were either naïve or were systemically injected with 100μg CpG-ODN1668 at the previous day according to Figure 10. An OVA-specific cytotoxicity assay was performed six days after transfer and clearly showed a strong CTL response in the control group, whereas in spleens of mice that were systemically injected with CpG before transfer of AdOVA-transduced BMDC, the OVA-specific CTL response was significantly reduced (Figure 11**A**). In a second approach, mice received BMDCs that were incubated *in vitro* with CpG or not one day before *in vitro* transduction with AdOVA. Cells were adoptively transferred another 24 hours later in naïve syngeneic C57BL/6 recipients. An *in vivo* cytotoxicity assay was performed 5 days after transfer and revealed that *in vitro* incubation of BMDC with CpG did not inhibit OVA-specific CTL responses (Figure 11B).

Thus, insufficient maturation and/ or antigen presentation possibly caused by CpG cannot exclusively be made responsible for impaired T cell responses.

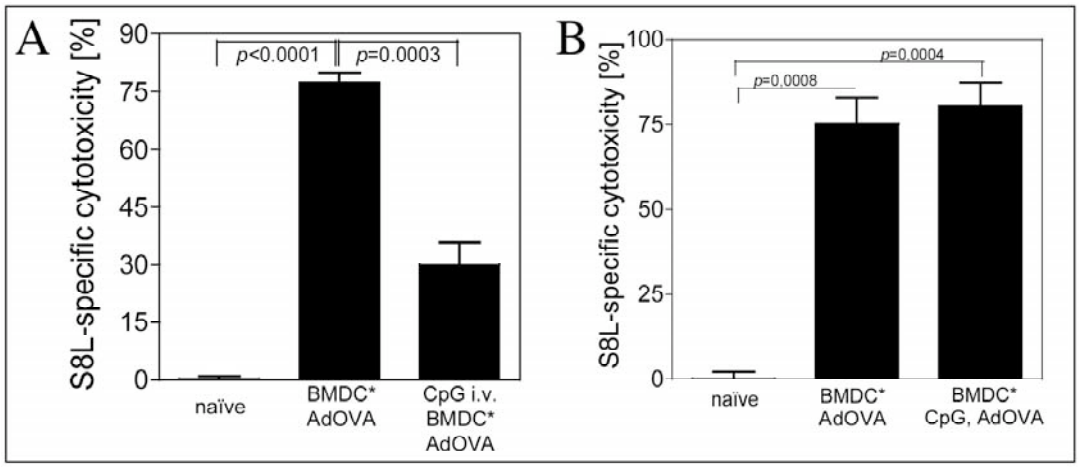

Figure 11 Systemic CpG pretreatment prevented efficient T cell priming by exogenously matured DCs

(a) S8L-specific CTL response was determined in spleens of C57BL/6 mice after immunization with 2x10e6 AdOVA-transduced BMDCs at day 6. One group of mice was pretreated with 100µg CpG-ODN1668 24 hours before immunization. (b) C57BL/6 received BMDCs, that were incubated over night with 10nmol CpG *in vitro* or not 24 hours before *in vitro* transduction with AdOVA. 2x10e6 BMDCs were injected per mouse i.v. 24 hours later into naïve recipients. Cytotoxic activity of S8L specific CTLs were determined in the spleen at day 5 p.i. and calculated in %.

7.1.3.4 Exogenous CD4 help abrogated CpG-induced CTL suppression

It was shown that for an efficient generation of CTL responses towards AdOVA infection spleen and CD4 help are required. In addition, systemic CpG treatment prevented the generation of immune responses. As adoptive transfer of fully matured DCs could not compensate for the inhibitory effects of previously administered CpG, this led to the suggestion, that provision of exogenous activated CD4 helper cells might circumvent the suppression. Therefore, experiments were performed with the help of OT-II transgenic mice, which express a transgenic CD4 T cell receptor specific for OVA MHC class II derived 323-339 peptide presented on I-A^b (Barnden et al., 1998).

To examine the influence of naïve or activated antigen-specific CD4+ T cells, naïve or *in vitro* activated MACS-purified $CD4^+$ splenocytes from OT-II transgenic mice were adoptively transferred into naïve wild type recipients. Indicated groups received CpG 24h later and were infected with AdOVA according to Figure 12.

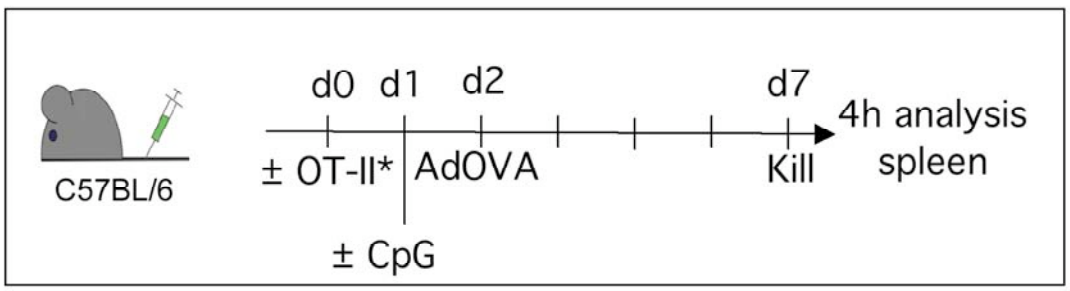

Figure 12 Time schedule of adoptive T cell transfer

A CTL response was lacking in spleens of mice that received naïve $CD4^+$ cells from OT-II mice and CpG, whereas transfer of activated $CD4^+$ T cells from OT-II mice completely restored the activity of S8L-specific CTL (Figure 13).

Thus, CpG-induced inhibition of CTL responses can be abrogated by the provision of exogenously activated antigen-specific CD4 T cells. These results further support the hypothesis, that the amount of antigen presented by DCs is sufficient for T cell priming.

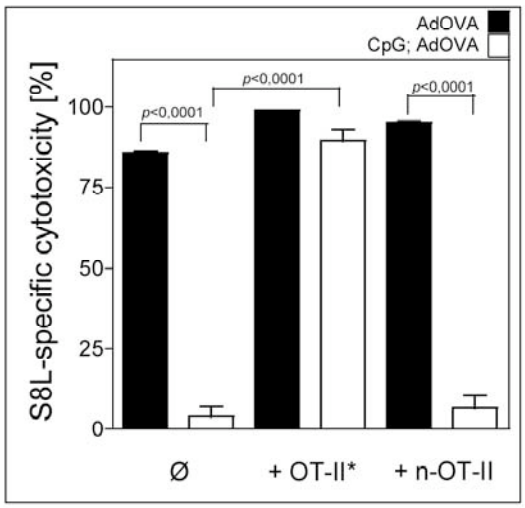

Figure 13 Transfer of Exogenously activated CD4+ helper cells restored CTL activity in CpG pretreated mice

Transfer of *in vitro* activated CD4+ cells from OT-II transgenic mice restored CTL activity in CpG treated mice. C57BL/6 mice received either 2,5 x10e5 *in vitro* activated OT-II (OT-II*) or naïve OT-II (n-OT-II) or were left untreated (Ø) 24 hours before i.v. injection with 100µg CpG-1668. n=3 per group. Mean ±SEM of S8L specific lysis is shown.

7.1.4 Summary of chapter 7.1

The data presented so far showed that systemic, but not local injection of high doses of CpG suppressed the generation of innate and adaptive immune responses. CD8 and CD4 T cell responses against the model antigen OVA were completely abrogated by CpG application in a dose-dependent manner. Furthermore, the generation of specific CTL responses against specific model antigens expressed by recombinant adenoviral vectors required the spleen, CD4 help and CD40 signaling. CpG-induced suppression could not solely be attributed to a direct inhibition of DC maturation or antigen presentation. Nevertheless, CpG treatment induced paralysis of splenocytes that resulted in strongly reduced secretion of pro-inflammatory mediators and fundamental alteration of $CD11c^+$ splenocyte subsets. We assumed that CpG rather induced an inhibitory milieu that resulted in block of CD4 help. This hypothesis was further supported by the observation that CpG-induced suppression was abrogated through adoptive transfer of activated antigen-specific $CD4^+$ T helper cells.

7.2 The role of the TLR system for the control of host immune responses induced by infection with *Plasmodium ssp.*

Cerebral malaria is a fatal neurological complication in humans infected with *Plasmodium falciparum* (Pf) and is mainly caused by the host immune response. Infection of C57BL/6 with *Plasmodium berghei ANKA* (PbA) infected red blood cells (iRBC) induces experimental cerebral malaria (ECM) and ranks among the acknowledged experimental models of malaria (Greenberg and Kendrick, 1957a; Greenberg and Kendrick, 1957b).

It has been shown by experiments in genetically deficient mice or by use of depleting antibodies, that inflammatory cytokines (IFNγ, lymphotoxins) and activated CD8 T cells recognizing parasite-derived antigens are essential for ECM development (Belnoue et al., 2002; Yanez et al., 1996) Production of inflammatory cytokines and activation of T cells implies requirement for detection of parasite-derived molecular patterns by sensors of the host immune system and subsequent DC activation.

Recently, immune activation upon recognition of *Plasmodium*-derived GPI was linked to TLR2 and recognition of hemozoin/*Plasmodium* ssp.-derived DNA to TLR9. However, a role of TLRs for development of ECM was not determined until recently. Therefore, the question was addressed whether TLRs, in particular TLR2 and TLR9 are critically involved in ECM development. Furthermore, as the first part of the present thesis revealed that systemic injection of TLR9 ligand CpG suppressed TLR9-mediated immune responses, it was hypothesized that CpG application could prevent development of ECM in PbA infected mice. These questions were investigated with the help of transgenic mice that were genetically deficient for relevant/individual TLRs.

7.2.1 Mechanistic requirements for ECM development in PbA-infected C57BL/6 mice

7.2.1.1 Importance of TLR2, TLR3 and TLR9 for ECM induction

The role of TLR during PbA infection was unclear in the beginning of this project. However, basic necessity for sensors that detect the presence and trigger the response towards *Plasmodium* infection in the host was assumed. As mentioned earlier on, recent studies by other groups describe recognition of *Plasmodium*-derived components GPI and hemozoin /Plasmodium-derived DNA by TLR2 and TLR9. Until then, recognition of bacterial lipopeptides by TLR2 and TLR9-specific detection of CpG-rich DNA was known.

To decipher the relevance of TLRs for development of ECM upon infection with PbA in the present study, survival after PbA infection was compared between mice that were genetically deficient for TLR2 or TLR9 and wild type littermates. In addition, mice that were deficient for TLR4 or TLR3 were infected with PbA to investigate possible relevance of those receptors for ECM induction. TLR3

is until now described to bind nucleic acids like double-stranded RNA or poly IC and is intracellularly located in the endosome like TLR9 (Alexopoulou et al., 2001). Among the multitude of TLR4 ligands that were discovered so far, lipopolysaccharide is the most prominent example. Both TLR3 and TLR4 were not yet associated with Pf or PbA infection.

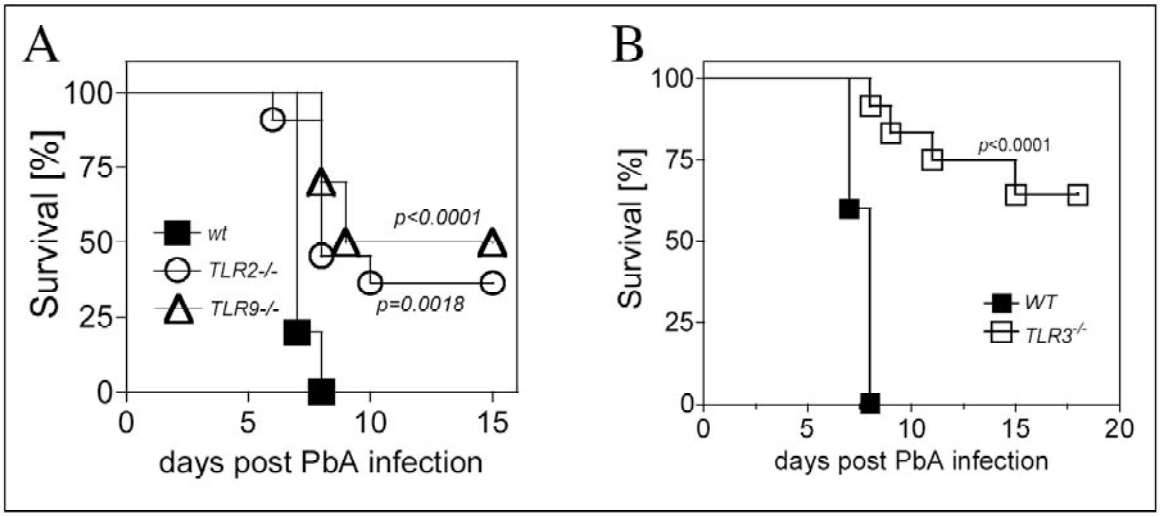

Figure 14 TLR2$^{-/-}$, TLR9$^{-/-}$ and TLR3$^{-/-}$ mice are significantly protected against ECM after PbA infection

Survival of C57BL/6 and TLR-deficient littermates after i. v. infection with 5x10e4 PbA-iRBC is shown. n=6-10 per group. Statistical analysis by Kaplan-Meier.

C57BL/6 mice developed ECM 6-8 days after infection with 5x10e4 PbA-iRBCs (Figure 14A). TLR2$^{-/-}$ mice and TLR9$^{-/-}$ mice developed significantly less often ECM after infection with 5x10e4 PbA iRBC than wild type littermates (Figure 14**A**).

TLR4 deficient mice showed no difference in survival after PbA infection compared to WT (data not shown), whereas TLR3$^{-/-}$ survived PbA infection significantly better than WT controls (Figure 14B).

These results provide strong evidence, that the TLR system is essential for induction of ECM after PbA infection; in particular TLR2, TLR3 and TLR9 are critically involved.

7.2.1.2 ECM induction after PbA infection is dependent on the spleen

The spleen is the central organ involved in the generation of immune responses towards systemically circulating antigens. Several studies postulate that the spleen is a crucial site for removal of parasitized erythrocytes as well as for generation of parasite-directed immune responses during infection with different *Plasmodium* parasites (Engwerda et al., 2005b). To investigate the importance of the spleen for ECM development during PbA infection, two approaches were conducted. In the first instance RAG1$^{-/-}$ mice were examined regarding ECM development upon adoptive transfer of splenic T cells from wild type donors. Mice deficient in RAG1$^{-/-}$ or RAG2$^{-/-}$ exhibit an abnormal a disorganized architecture of the spleen (Mebius and Kraal, 2005). Furthermore, RAG2$^{-/-}$ mice are described to be

resistant against ECM development, which is mainly ascribed to lack of effector T cells. In a study from Nitcheu et al., RAG1$^{-/-}$ mice were rendered ECM susceptible by transfer of effector T cells purified from PbA-infected immuno-competent wild type donors after induction of ECM (Nitcheu et al., 2003).

We analyzed whether reconstitution of RAG1$^{-/-}$ mice with splenocytes derived from naïve WT littermates prior to PbA infection was capable to induce host immune responses that result in ECM. For this purpose, CD4^{+} and CD8a^{+} cells were isolated via MACS® from spleens of naïve WT donors and adoptively transferred into RAG$^{-/-}$ recipients seven days before PbA infection. It was impossible to induce immune-mediated damage in reconstituted RAG1$^{-/-}$ mice as all mice were still protected against ECM (Figure 15**A**). In part 4.1 of this study it was shown, that RAG1$^{-/-}$ did not generate CTL responses towards AdOVA infection despite reconstitution with splenic T cells derived from wild type donors seven days prior to AdOVA infection **(**Figure 4**B).** This suggested that simple reconstitution of RAG1$^{-/-}$ with naïve T lymphocytes may be insufficient for induction of host immune responses also during PbA infection.

It was investigated next whether surgical removal of the spleen could influence the development of ECM during PbA infection. C57BL/6 mice were infected with 5x10e4 PbA-iRBCs two days after surgical removal of the spleen. Compared to intact littermates infected with PbA, splenectomized animals did not develop ECM **(**Figure 15**B**). Taken together, these data support a major relevance of the spleen and its unique architecture for generation of host-derived immune responses causing severe pathologies during the course of PbA infection.

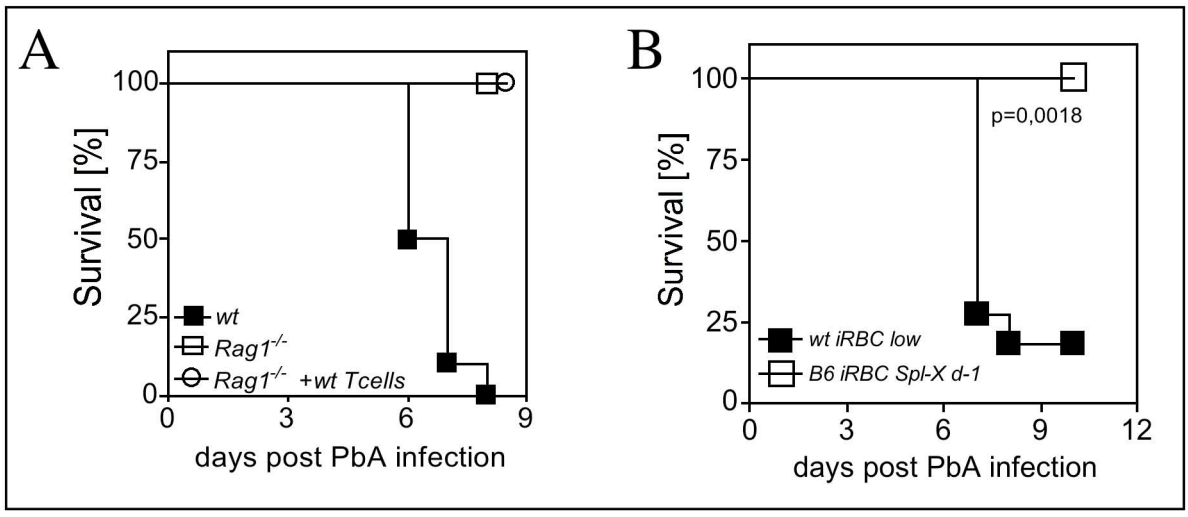

Figure 15 Intact spleen is required for generation of ECM pathology

(a) Survival of C57BL/6 and RAG1-/- after infection with 5x10e4 PbA-iRBC i.v. 7 days before experimental infection with 5x10e4 PbA iRBCs, one group of RAG1$^{-/-}$ was reconstituted with splenic CD4+ and CD8a+ from wild type donors (1 donor/1 recpipient); *n*=10 mice per group (b) Survival of splenectomized C57BL/6 and intact littermates after infection with 5x10e4 PbA-iRBC i.v. Splenectomy was performed 2 days before infection. *n*=10 mice per group.

7.2.1.3 Depletion of DCs prevents ECM in PbA infected C57BL/6 mice

DCs link innate and adaptive parts of the immune system during an infection. Recognition of pathogens via TLRs leads to activation and subsequent production of pro-inflammatory mediators and up-regulation of co- stimulatory molecules that amplify antigen presentation towards T cells and T cell priming. During infection, DCs are permanently exposed to parasite-derived antigen, as parasite-infected red blood cells are mainly trapped in the spleen. It was assumed that targeted elimination of DCs could prevent ECM, as they maintain a key role during early phases of infection in T cell priming, but may contribute to pathology during late stages by production of inflammatory cytokines. Furthermore, mice that were genetically deficient for TLR2, TLR3 or TLR9 showed increased survival towards PbA infection, which supports a critical role for DCs in induction of PbA-directed immune responses, because expression of several TLRs is a particular characteristic of this cell subset. Jung et al. recently described efficient depletion of DCs in CD11c transgenic mice that express the human Diphtheria toxin receptor under control of the CD11c promoter. Intra-peritoneal injection of diphtheria toxin (DTX) is followed by almost complete elimination of DCs in the spleen (Jung et al., 2002). Natalio Garbi (DKFZ Heidelberg) generated similar mice that express the human Diphtheria toxin receptor under control of the CD11c promoter together with OVA and GFP. These CD11c-DTR-OVA GFP transgenic mice were designated as DOG mice[1].

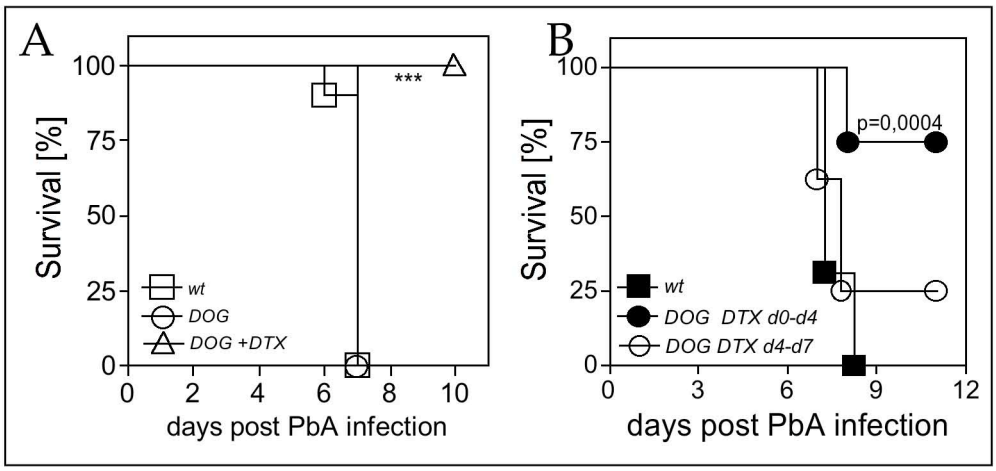

Figure 16 Depletion of DCs prevents ECM in PbA-infected DOG mice

(a) Survival of C57BL/6 and CD11c-diphtheria-Toxin-Receptor transgenic mice after PbA infection. DTR transgenic mice received either daily injections of 40ng DTX/g mouse or not. n=5 mice per group (b) Analogous to (a) with different DTX treatment schedule: Mice received DTX either from d0 until d+4 post PbA infection or from d+4 until d+7 post PbA infection. n=10 mice per group

To investigate the relevance of DC elimination for ECM induction, PbA infection experiments were performed in DOG mice. Daily intra-peritoneal application of 40ng Diphtheria toxin (DTX) per gram animal weight is followed by almost complete depletion of DCs in the spleen (data not shown)

[1] DOG transgenic mice= CD11c-Diptheria toxin receptor-Ovalbumin-green fluorescent protein (Natalio Garbi)

Importantly, DOG mice can be injected repeatedly with DTX to prevent presence of DCs. In the first approach, DTX was given daily during PbA infection of DOG transgenic mice and compared to WT controls as well as non-depleted DOG tg mice infected with PbA. DTX treatment led to increased survival of DOG mice (Figure 16**A**). In the second approach it was determined whether the presence of DCs was critical in the early or the late stage of infection. For this purpose, DOG tg mice were depleted either from day 0 to day 4 post infection or from day 4 till the end of the experiment. Only early DTX treatment resulted in significantly enhanced survival of infected mice, whereas late DTX treatment only partially protected mice from ECM (Figure 16**B**). These experiments provide evidence, that dendritic cells are key players during the early phase of murine PbA infection for induction of pathogenic immune responses.

7.2.1.4 IL-12 and IL-23 confer ECM susceptibility

So far, mice that were deficient in single TLRs or were injected with DTX to deplete DCs, did not develop ECM after infection with PbA. An important result of TLR ligation is the production of the pro-inflammatory cytokine IL-12, which is a prerequisite for T cell activation. Therefore it was hypothesized that abrogated production of IL-12 may result in impaired generation of host responses directed against PbA and consequently give rise to increased survival of IL-12 deficient mice after PbA infection.

Bioactive IL-12 is a hetero-dimer p70 consisting of a p35 and a p40 subunit. In addition, p40 is part of the hetero-dimer p19p40 (Trinchieri, 2003). In the present study, survival of IL-12p35-deficient animals and IL-23p19-deficient animals after PbA infection was monitored. IL-12p35-deficient animals were significantly protected against ECM; only 30% of PbA infected mice died in contrast to WT control-infected mice that all succumbed to ECM (Figure 17). In comparison, IL-23p19 deficient mice died after PbA infection, but death was significantly delayed in comparison to WT controls (Figure 17). Thus, pro-inflammatory mediators IL-12 and IL-23 are also involved in ECM induction after PbA infection.

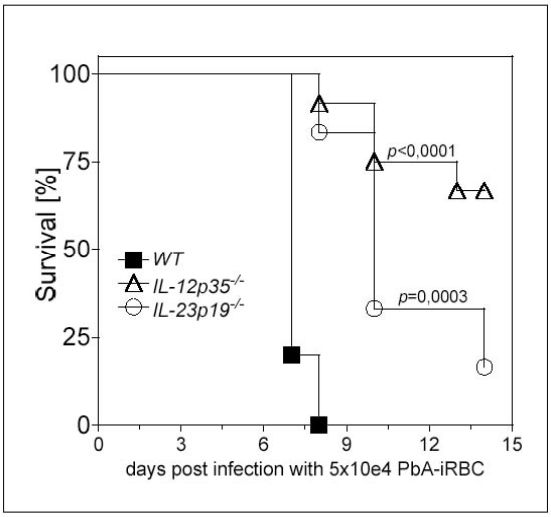

Figure 17: Lack of IL-12p35 or IL-23p19 leads to increased survival of PbA infected mice

Survival of C57BL/6 WT mice or littermates deficient in IL-12p35 or IL-23p19 after infection with 5x10e4 PbA iRBC. N=10 per group.

7.2.2 CpG prevented ECM in PbA infected mice

7.2.2.1 Prophylactic CpG injection before PbA infection prevents ECM development in C57BL/6 mice

In section 7.1 of this thesis it was shown, that systemic application of CpG inhibited the generation of CTL responses towards AdOVA and led to paralysis of DCs. Accordingly, Reis e Sousa et al. demonstrated in a model of parasite infection that repeated triggering of the innate immune system by components of *Toxoplasma gondii* induced DC paralysis. They concluded that limitation of the inflammatory responses by DC paralysis led to prevention of tissue damage (Reis e Sousa et al., 1999). Based on these observations, we hypothesized that systemic injection of mice with CpG could inhibit the generation of detrimental innate and adaptive effector molecules and cells, thereby preventing ECM upon PbA infection.

To investigate possible suppression of host immune responses towards PbA infection, C57BL/6 mice received 100μg CpG i.v. 1 day before (d-1) PbA infection and survival was monitored in comparison to control-infected littermates. Most importantly, CpG-treated mice survived and did not develop ECM (Figure **18**). Thus, systemic CpG treatment inhibited cytopathological consequences of PbA infection.

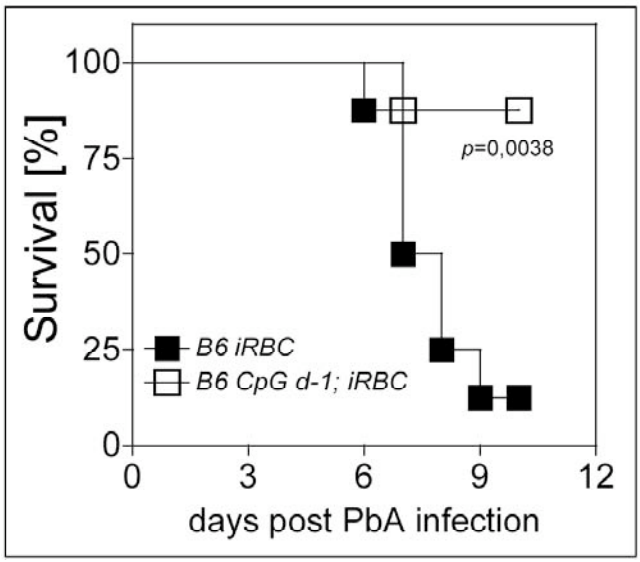

Figure 18: CpG treatment prevented ECM development in PbA infected C57BL/6 mice

Survival of C57BL/6 mice after PbA infection (5x10e4 PbA iRBC i.v.) with or without systemic pre-treatment with 100µg CpG-ODN1668 i.v. n=8 per group

7.2.2.2 Early CpG treatment prevents ECM, whereas late CpG injection accelerates ECM in PbA-infected C57BL/6 mice

To dissect time-dependent consequences of TLR ligation and subsequent activation of DCs, C57BL/6 mice were injected intravenously with 100μg CpG either 1 or 5 days after PbA infection (d+1, d+5) and survival was monitored (see Figure 19). D+1 CpG treated mice survived and did not develop ECM (Figure 20**A**). In contrast, CpG application five days post infection had detrimental consequences and led to an accelerated development of neurological symptoms, coma and death (Figure 20**A**).

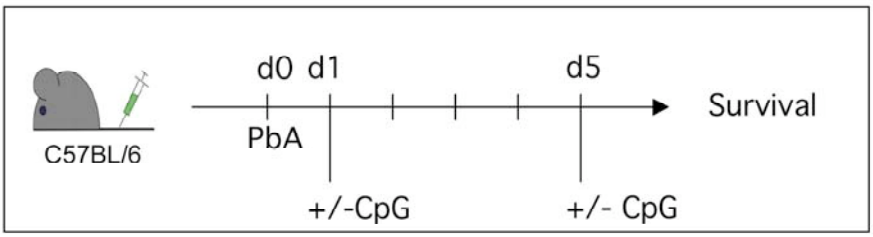

Figure 19: Time schedule for CpG-treatment of PbA-infected mice

Additionally, parasitemia of early treated mice was significantly lower than in control-infected and late treated animals (Figure 20 **B**). Sera were taken from different groups and analyzed for the presence of inflammatory cytokines. CpG treatment elicited an immediate IL-12 peak, regardless of the time point of application after PbA infection (Figure 20 **C**). Strict dependence of CpG-induced protection on TLR9 was confirmed in TLR9 deficient animals. CpG-treatment prevented induction of ECM only in WT mice but not TLR9$^{-/-}$ (data not shown).

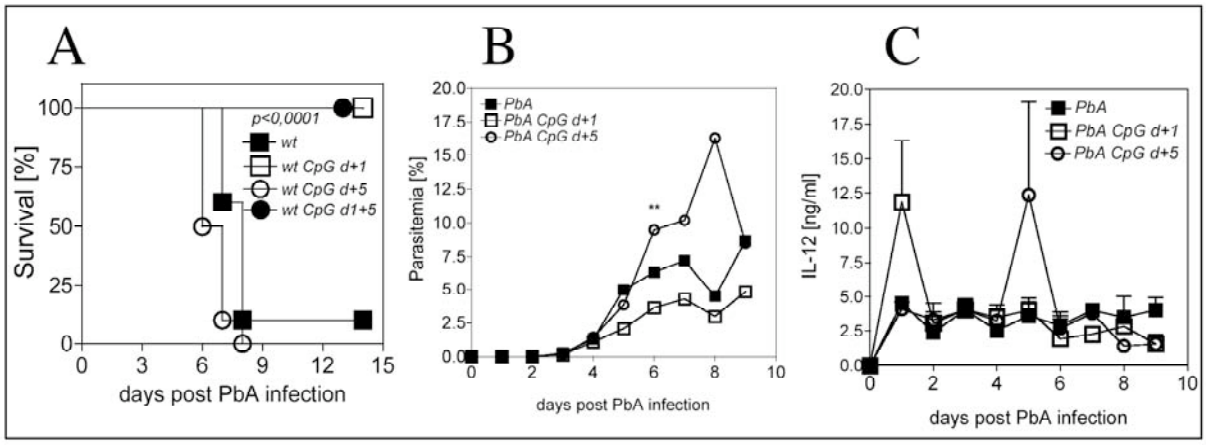

Figure 20 CpG treatment of infected mice protected mice from ECM in a TLR9 dependent manner

(a, b) Survival of C57BL/6. (a) Mice were injected i.v. with 100µg CpG either at d+1, d+5 or d+1 and d+5 following injection of 5x10e4 PbA iRBCs. n=10 mice per group (b) Parasitemia of C57BL/6 mice with or without CpG treatment. C57BL/6 mice were CpG treated at indicated time points. ** p<0.005 to CpG d+1 (c) IL-12 ELISA from sera of PbA infected C57BL/6 with or without CpG treatment. Serum samples were taken every day and analyzed for cytokines by ELISA. Analysis of survival is shown and calculated as percentage.

To examine whether protection against ECM by d+1 post infectionem (p.i.) CpG application could be abrogated, PbA-infected C57BL/6 that were already injected with CpG i. v. at day 1 after PbA infection received an additional CpG injection at d+5 p.i. However, twice-injected mice were still protected against ECM and survival was comparable to CpG d+1 p. i. sinlge injected mice (Figure 20**A**). In conclusion, early CpG treatment prevented ECM, which was dependent on TLR9, whereas late CpG application accelerated pathological incidences resulting in death. Furthermore, primary CpG application prevented death of PbA-infected mice that were re-challenged with CpG at day 5.

7.2.2.3 Role of IL-10 in CpG induced protection from ECM

We speculated, that CpG-induced prevention of ECM in PbA-infected mice may be due to induction of anti-inflammatory mediators i.e. interleukin 10 (IL-10). To investigate a possible role of IL-10 during CpG-induced protection against ECM, survival of PbA-infected IL-10-deficient mice that were injected with CpG one day before infection was monitored. Importantly, IL-10-deficient PbA infected mice died within the following hours after CpG injection (Figure 21), whereas those IL-10-deficient mice, that did not receive CpG application, developed to a similar time ECM as WT littermates (Figure 21).

Thus, we could not exclude a role for IL-10 during CpG-induced prevention of ECM after PbA infection.

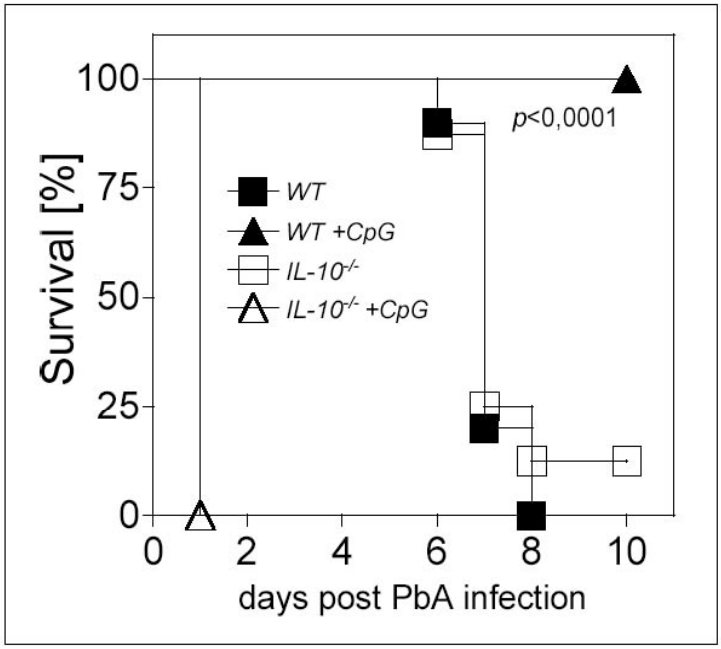

Figure 21 Subsequent death of PbA-infected IL-10-deficient mice after systemic injection of CpG

Survival of IL-$10^{-/-}$ and WT littermates after i.v. infection with 5x10e4 PbA-iRBC; indicated groups received 100µg CpG-ODN1668 i.v. 24 hours after infection. IL-$10^{-/-}$ deceased within 6 hours after CpG injection. n=10 per group.

7.2.2.4 CpG injection prevents generation of Pf-specific CTLs in the spleen of HLA A2 transgenic mice

Systemic injection of C57BL/6 with TLR9 ligand CpG suppressed CTL responses towards subsequent infections (Figure 2**B**). During *Plasmodium* infection, T cell responses are generated and are supposed to be a major cause of immune pathology as depletion of $CD8a^+$ cells by injection of neutralizing antibodies protected mice from death, even if depletion was performed proximate to ECM (Hermsen et al., 1997; Yanez et al., 1996).

So far, direct investigation of *Plasmodium*-specific T cell responses *in vivo* was impossible due to the lack of an appropriate experimental model. The laboratories of Hermann Bujard and Hans-Georg Rammensee identified merozoite-surface protein 1 (MSP-1)-specific CTL epitopes derived from *Plasmodium falciparum* (Pf) that bind to human MHC class I molecules HLA A2 (personal communication). This allowed the design of specific MSP-1-derived peptide sequences and facilitated the exploration of MSP-1 specific CTL response in humanized transgenic mice expressing HLA A2 (HLA A2 tg) (Ureta-Vidal et al., 1999). Therefore, it was speculated that inoculation of HLA A2 tg mice with Pf-infected iRBC should induce a MSP-1 specific CTL response. The HLA A2 tg mice received 2,8 x 10^8 Pf iRBC i. v. Six days later, the activity of MSP-1 specific CTL was examined by an *in vivo* cytotoxicity assay. Indeed, in the blood of immunized animals significant lysis of syngenic target cells loaded with MSP-1 derived MHC class I peptides was measured (Figure 22). The data show that pre-treatment of mice with CpG or splenectomy significantly reduced the activity of MSP-1 specific CTL as measured in the blood (Figure 22). Thus the generation of specific CTLs against Pf-iRBC in HLA-A2 tg mice was dependent on the spleen and could be inhibited by systemic CpG injection.

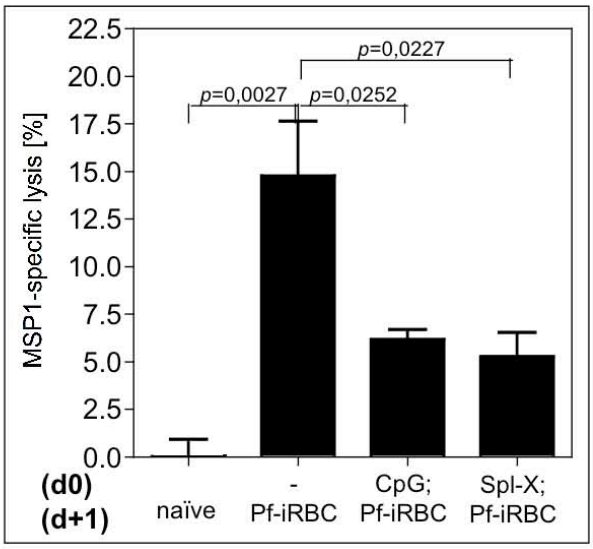

Figure 22 CpG inhibits Pf-specific CTL responses

In vivo cytotoxicity assay against *P. falciparum* derived MSP1 in the blood of HLA-A2 transgenic C57BL/6. Mice were injected with 2.8x10e8 Chloroquine-treated *P. falciparum* infected human red blood cells; one group was pre-treated with 100µg CpG-ODN1668 at d-1. MSP-1 specific kill was performed 6 days later. n=5 per group. students t test

7.2.3 Role of TLRs for ECM development and induction of immune suppression by PbA parasites

7.2.3.1 High dose infection with PbA prevents ECM in C57BL/6 mice

C57BL/6 mice developed within 6-8 days after infection with $5x10^4$ PbA-iRBC ECM. In order to accelerate ECM, WT mice were infected with $1x10^7$ – a greatly increased number – of PbA-iRBC. Interestingly, WT mice infected with high dose PbA did not develop ECM but were rather protected from death (Figure 23**B**). This provided strong evidence that PbA itself was able to induce immune suppression, probably to evade detection of the immune system and subsequent induction of inflammatory responses. To decipher a possible role for TLRs during Plasmodium-induced immune suppression, survival after infection with PbA high infection was studied in mice that were genetically deficient in TLR2 or TLR9 in comparison to WT controls. Importantly, 30% of TLR2-deficient mice as well 30% of TLR9-deficient mice died following PbA^{high} infection, whereas WT littermates survived PbA^{high} infection completely (Figure 23**A**). This suggested that several TLRs collaborate with and/or compensate for each other. To investigate whether PbA^{high} induced protection against ECM might also be dependent on expression of TLR2/3/9, survival of triple-deficient mice was monitored. Upon infection with PbA^{high}, circa 40% of TLR2/3/9-triple deficient mice survived and did not develop ECM (Figure 23**B**). From these results, a dual role of TLRs during PbA infection was concluded. On the one hand, TLR2, TLR3 and TLR9 were involved in development of ECM after infection with a low dose of PbA, whereas on the other they also play a role during induction of immune suppression elicited by high dose infection.

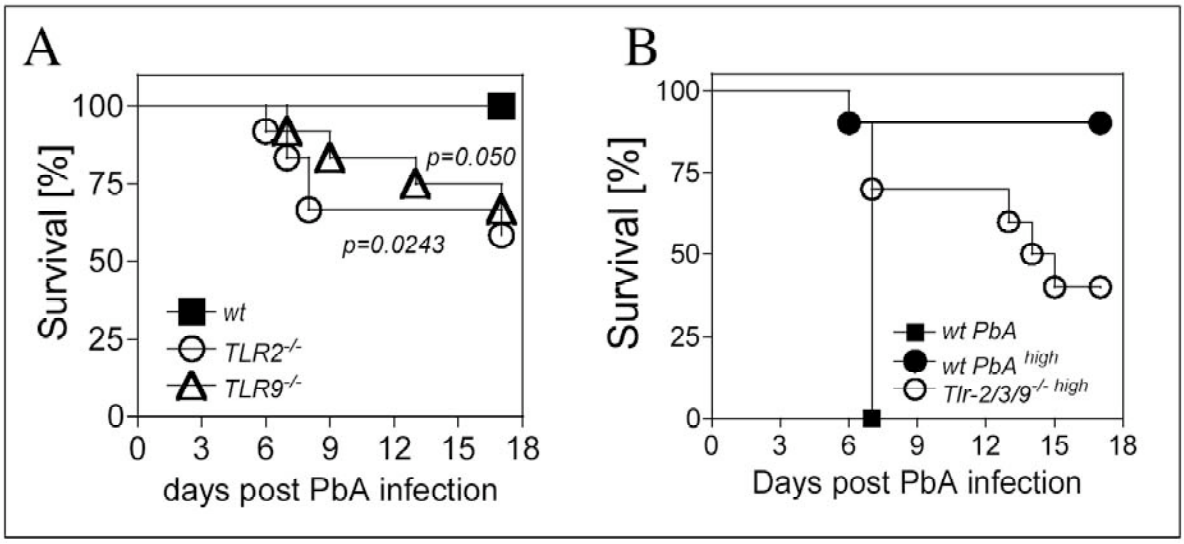

Figure 23 TLR2, TLR3 and TLR9 are involved in PbA-induced immune suppression

Survival of C57BL/6 wild type and TLR deficient littermates upon PbA infection (a) Survival of wild type, $TLR2^{-/-}$ and $TLR9^{-/-}$ littermates following i.v. infection with 1x10e7 PbA-iRBC (=high), n=10 per group. (b) Survival of wild type and $TLR2^{-/-}/3^{-/-}/9^{-/-}$ mice after infection with 5x10e4 or 1x10e7 (=high) PbA-iRBC; n=10 per group.

7.2.3.2 PbA-induced immune suppression required IL-12 and IL-23

To examine a possible negative role of IL-12 or IL-23 in induction of immune suppression by PbA^{high} infection, survival and ECM development after PbA high infection was monitored in IL-12p35p40 double-deficient mice. Importantly, PbA^{high}-induced protection against ECM was completely abrogated in IL-12p35p40 double deficient mice (Figure 24A). In contrast, mice that lacked expression of either IL-12p35 or IL-23p19 were still protected against ECM (Figure 24B). This suggested involvement of the p40 subunit that is present in both IL-12 and IL-23. Thus, IL-12/IL-23 were important for induction of protective factors that prevented ECM after PbA^{high} –infection.

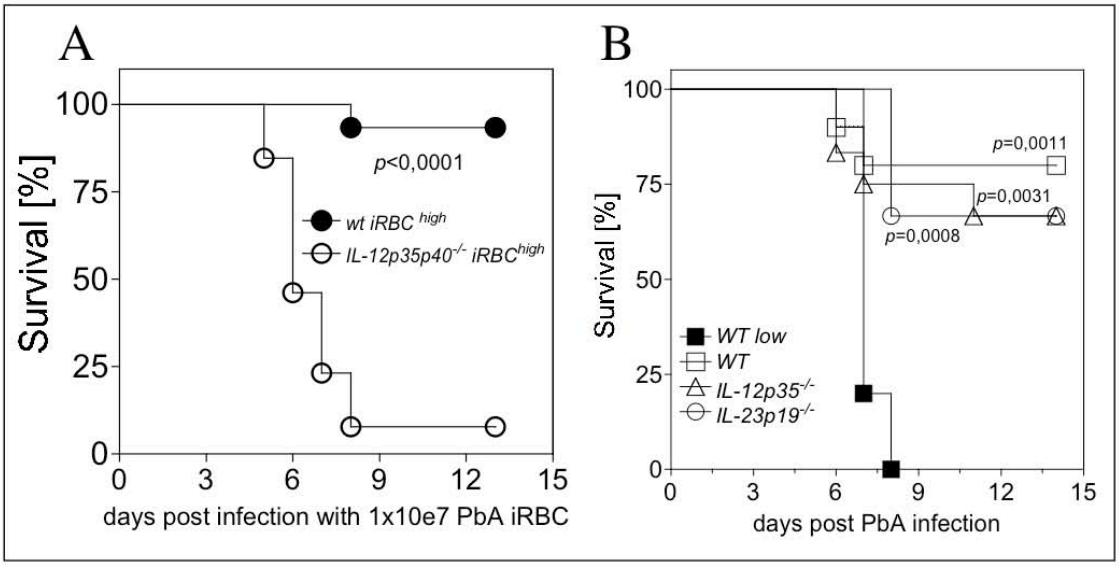

Figure 24 IL-12 and IL-23 are critically involved in protection against ECM induced by PbA high dose infection.

(a) Survival of C57BL/6 WT and IL-12p35$^{-/-}$p40$^{-/-}$ mice after infection with 1x10e7 PbA-iRBCs i.v. n=10 per group (b) Survival of IL-12p35-/- or IL-23p19-/- mice compared to WT after infection with 1x10e7 PbA-iRBCs i.v. n=10 per group. Statistics refer to WT controls.

7.2.3.3 Relevance of IL-10 and iNOS in PbA high-dose induced protection from ECM

IL-12 was required for the induction of protective mechanisms that prevented ECM development upon infection of mice with a high dose of PbA. IL-12 is described to induce IL-10 and inducible nitric oxide synthase (iNOS) that triggers production of nitric oxide (NO) (Gerosa et al., 1996; Huang et al., 1996; Koblish et al., 1998). Both IL-10 and iNOS are suggested to represent negative regulators in immune activation and were also linked to protection of PbA-infected mice against ECM by induction of neuro-protective factors and by blocking of neuro-toxic factors (Hunt and Grau, 2003; Pamplona et al., 2007). Negative regulation is assumed to result in stabilizing the integrity of the blood-brain barrier by neutralizing inflammatory mediators. This finally prevents infiltration of parasitized RBC into the brain and subsequent attack by parasite-specific effector cells. Therefore, it is assumed that

survival of PbA infection depends on the ratio between neuro-protective versus neuro-toxic factors. To determine the relevance of IL-10 and iNOS regarding the capacity to mediate protection against ECM in PbA^{high}-infected mice, survival of IL-10-deficient mice and iNOS-deficient mice was monitored following infection with a high dose of PbA. Circa 50% of IL-10-deficient and 40% of iNOS-deficient mice were protected against ECM after infection with PbA^{high} dose, whereas 75% of WT mice survived PbA^{high} dose infection (Figure 25). From this experiment a critical role of IL-10 and iNOS for the induction of mechanisms that prevented ECM was concluded.

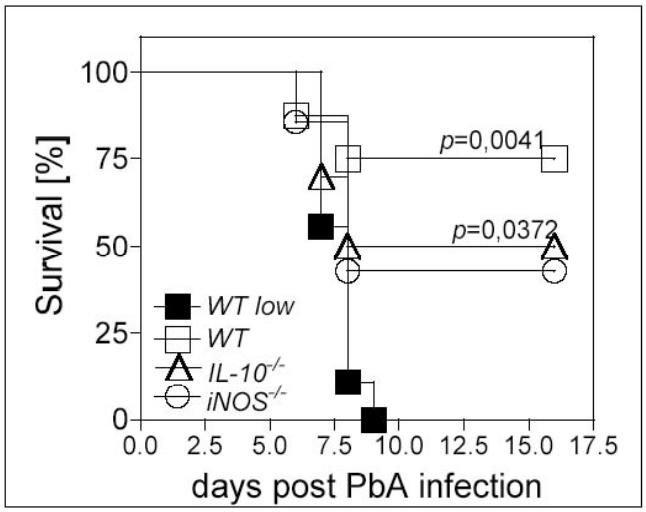

Figure 25 Participation of iNOS and IL-10 in PbA-high dose induced protection against ECM.

Survival of WT C57BL/6, IL-10-/- mice and iNOS-/- mice after PbA infection. 1 group of WT mice was infected with 5x10e4 PbA iRBC as a control (indicated as "low"), all other groups reveived 1x10e7 PbA iRBC i.v. n= 10 per group. Statistics refer to WT PbA low group.

7.2.4 Summary of chapter 7.2

Induction of inflammatory innate and adaptive immune responses towards Plasmodium infection is assumed to cause immune pathologies that may result in life-threatening complications like cerebral malaria. Detrimental immune responses causing ECM were less prevalent in mice deficient in TLR2, TLR3 or TLR9. Complete prevention of ECM was observed if PbA-infected mice that were splenectomized or depleted of DCs.

Systemic application of CpG also protected PbA-infected mice against ECM. This was attributed to DC paralysis and suppression of parasite-specific T cells. Importantly, infection of C57BL/6 WT with a high dose of PbA prevented ECM as well. The regulation of this protection required several factors. Detection via TLR2 and TLR9 were involved that mediated protection via IL-12. Finally, IL-10 and iNOS were suggested to have negative regulatory roles in inflammatory pathways.

7.3 TLR7 ligands inhibit antigen-specific CTL responses via type I interferon

Systemic application of TLR9 ligand CpG induced suppression of innate and adaptive immune responses towards a subsequent AdOVA infection in C57BL/6 mice. In particular, prevention of antigen-specific CTL responses was one of the basic findings. It might be reasonable that the induction of immune suppression by several TLR ligands is a general mechanism to limit overwhelming immune activation thereby prevention of tissue damage. On the other side it was hypothesized that TLR-ligand-induced inhibition of CTL responses might also result in lack of protection against infectious agents. As TLR ligands are used therapeutically in progressive rate because of their immune-stimulatory effects, this implies that *in vivo* application of TLR ligands harbor substantial risks of subsequent immune suppression.

Therefore, questions were addressed whether apart from TLR9 ligand CpG also ligands of other TLRs have the potential to inhibit CTL responses towards subsequent infections. In addition, the question was addressed whether TLR ligation after AdOVA infection influenced also the generation of immune responses. Finally, it was investigated, whether therapeutic application of TLR ligands in two different tumor models in mice can interfere with the generation of antigen-specific CTL responses.

7.3.1 TLR7 ligand siRNA induces suppression of adaptive immune responses

7.3.1.1 Other TLR ligands also suppress adaptive immune responses

It was hypothesized that apart from TLR9 ligand CpG also other microbial-derived patterns that are recognized as TLR ligands might influence the generation of immune responses directed against AdOVA infection in mice. Lipopolysaccharide (LPS) is recognized by TLR4 expressed on the cell surface of many immune and non-immune cells, whereas the imidazoquinoline derivate R848 – also known as Resiquimod – was identified as ligand for TLR7. Like TLR9, TLR7 is also intracellularly expressed in the endosome of immune cells but its expression is limited to few DC subsets (Heil et al., 2003; Kadowaki et al., 2001).

In addition, as application of TLR9 ligand prior to AdOVA infection resulted in inhibition of immune responses, the consequence of TLR ligation after AdOVA infection was investigated.

Here, ligands for TLR4, TLR7 or TLR9 were compared with respect to the generation of OVA-specific CTL towards AdOVA infection. C57BL/6 mice were injected i. v. with LPS, R848 or CpG and then infected with AdOVA 24 hours later. OVA-specific CTL responses were determined five days after AdOVA infection by an *in vivo* cytotoxicity assay in the spleen. LPS and R848 also induced suppression of OVA-specific CTL responses in the spleen, if they were injected i. v. into C57BL/6 mice before AdOVA immunization (Figure 26**A, B**). Furthermore, suppression of OVA-specific CTL responses by LPS and R848 was induced in a dose-dependent manner as it was already observed for

CpG. Only high doses inhibited the generation of OVA-specific CTLs whereas low doses did not influence the CTL response (Figure 26B, data not shown). In contrast to TLR ligand injection before AdOVA infection, the OVA-specific CTL response was not inhibited but rather enhanced, if CpG or LPS were injected i. v. one day after AdOVA infection (Figure 26**A**).

Suppression of OVA-specific CTL responses by LPS or R848 was abrogated in TLR4-deficient and TLR7-deficient mice, respectively (Figure 26**C, D**).

Importantly, TLR ligation after AdOVA infection elicited immune stimulation rather than suppression.

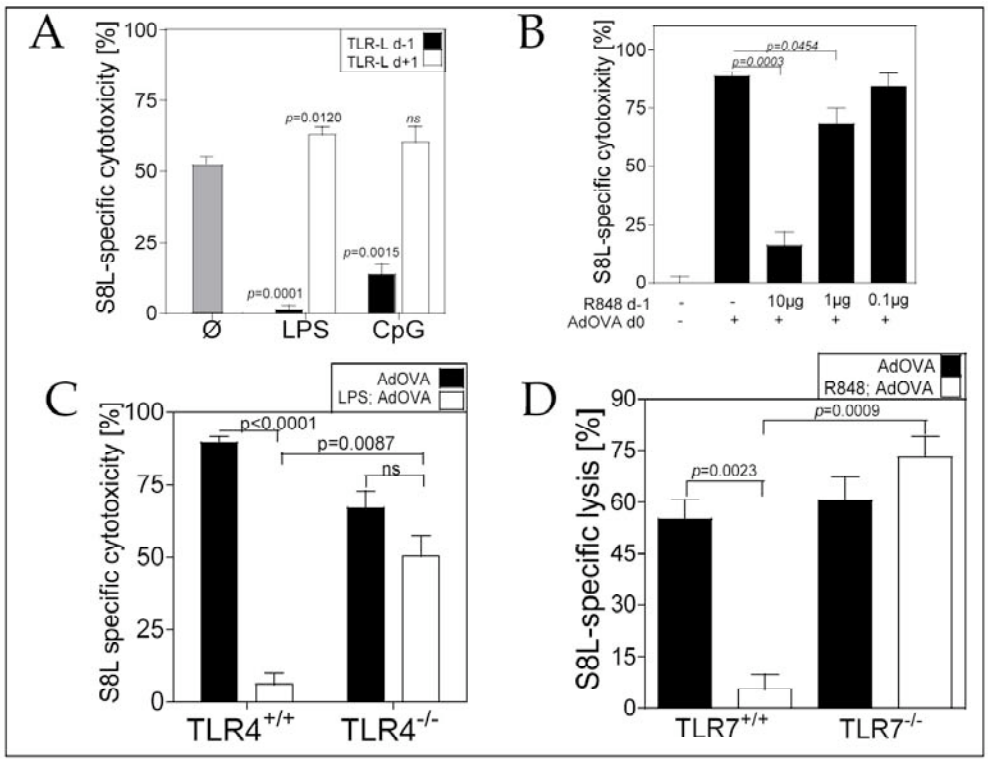

Figure 26: Several TLR ligands induce suppression of adaptive immune responses

(a, b, c, d) Mice were infected with 1x10e7 PFU AdOVA and subjected to an *in vivo* S8L-specific cytotoxicity assay; spleen was analyzed. **(a)** C57BL/6 wild type mice were injected i. v. 24h before (black bars) or 24h after (white bars) AdOVA immunization with 1µg LPS or 100µg CpG. Grey bar represents AdOVA control.**(b)** Dose-dependent suppression elicited by R848. C57BL/6 received 0.1µg, 1µg or 10µg R848 i.v. 24 hours before AdOVA infection. **(c)** TLR4 deficient and wild type littermates were injected with 1µg LPS (white bars) or not (black bars) before immunization. **(d)** TLR7 deficient mice and wild type littermates received 10µg R848 i v. (white bars) or not (black bars) 24 hours before AdOVA infection. n=3 or 4 animals per group

7.3.1.2 Inhibition of immune responses induced by small-interfering RNA

TLR7 is an intracellular pattern recognition receptor and binds in addition to primary identified ligands R848 and R837 also uridin-rich single-stranded (ss) RNA derived from human immune deficiency virus (HIV) and influenza virus (Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2002). In addition, TLR7 recognizes poly U sequences and certain small-interfering RNA (siRNA) (Hornung et al., 2005). As several TLR ligands were shown here to elicit immune suppression, it was hypothesized that also TLR7-mediated recognition of siRNA harbors the risk to inhibit immune responses.

We analyzed possible inhibitory effects of systemically injected siRNAs concerning generation of CTL responses against *Listeria monocytogenes (L.M.)* or adenovirus *in vivo*. Therefore, three different siRNA sequences were investigated. First, a previously published siRNA-sequence targeting MyD88 (siMyD88) was chosen (Flandin et al., 2006). For generation of immune responses towards both bacterial and viral infection *in vivo*, MyD88 represents a central adapter molecule for the transmission of TLR signals and triggering adaptive immune responses (Lord et al., 1990; Muzio et al., 1997; Wesche et al., 1997). Secondly, we chose the siRNA sequence designed as 9.2s that targets human TLR9 and was previously shown by others to elicit immune-stimulatory effects in mice in a TLR7 dependent manner (Hornung et al., 2005). Finally, the control sequence 9.2s-R8A, lacking any immune stimulatory qualities was used (Hornung et al., 2005). These experiments were done in cooperation with the group of Gunther Hartmann.

OVA-specific CTL response was determined by an *in vivo* cytotoxicity assay in the spleens five days post infection. OVA-specific CTL responses were markedly decreased in spleens of mice treated with MyD88-specific siRNA before AdOVA or LM-OVA infection (Figure 27**A, B**). This indicated that application of MyD88-siRNA caused gene silencing that prevented transduction of TLR signal and subsequent generation of an immune response. However, systemic injection of 10μg 9.2s siRNA suppressed OVA-specific CTL responses in C57BL/6 mice equally well compared to siMyD88 (Figure 27A, B) and was also observed after i. v. injection of 5μg; this dose was reported by Hornung et al. to exert immuno-stimulatory effects (data not shown) (Hornung et al., 2005). It was concluded, that inhibition of OVA-CTL depended on the presence of immune stimulatory motif, as the sequence 9.2s-R8A lacking that motif did not influence the CTL response towards AdOVA infection. These results suggested that the siRNA-induced suppression did not (only) result from target-specific gene silencing, but must have been due to intrinsic properties of single stranded immune-stimulatory RNA.

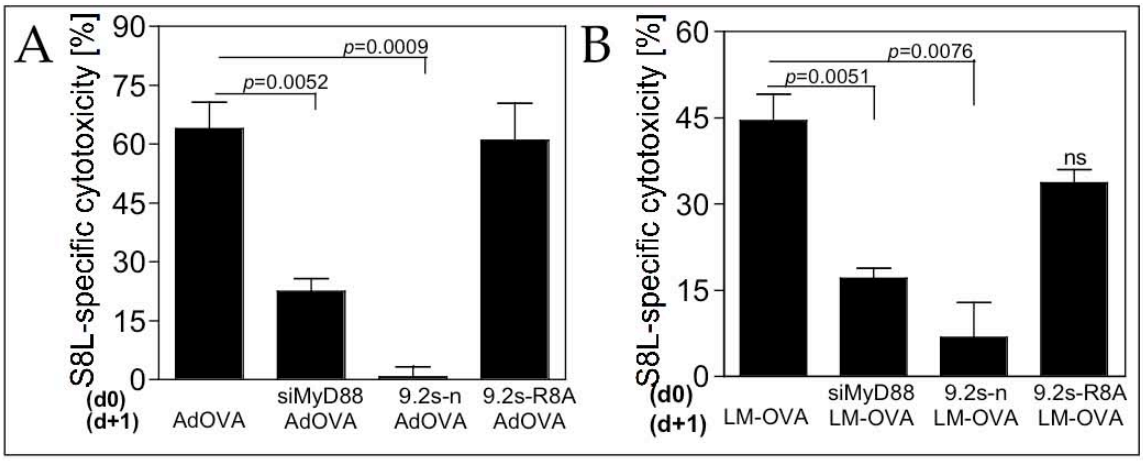

Figure 27 siRNA mediated suppression

S8L specific cytotoxicity was determined in spleens of C57BL/6 mice at day five post infection with (a) AdOVA or (b) *L. monocytogenes* expressing OVA. Indicated groups of mice received 24 hours before infection (1x10e7 PFU AdOVA; 2x10e4 LM-OVA) 50µg siMyD88, 10µg 9.2s or 10µg 9.2s-R8A. RNA was complexed with 30µl CL per animal. n=4 per group.

7.3.1.3 Immune stimulatory RNA inhibited CTL responses in a TLR7-dependent manner

So far, 9.2s-n siRNA was demonstrated to strongly stimulate type I interferon responses in a TLR7 dependent manner as work by Hornung et al. showed, but was not yet associated with immune suppression (Hornung et al., 2005).

Inhibition of OVA-specific CTL responses towards AdOVA infection by prior injection of 9.2s RNA could be the result of three different scenarios. First, human and murine TLR9 show 75.5% homology regarding the amino acid sequence (Hemmi et al., 2000). Thus, inhibitory effects due to gene silencing could not be completely excluded in the previous experiment. Secondly, if the suppression was caused by TLR7-mediated recognition of 9.2s RNA, this should be abrogated in TLR7 deficient mice. Accordingly, if CTL suppression was due to silencing of TLR9, suppressive effects elicited by 9.2s application should be still observed in TLR7 deficient mice. Thirdly, recognition of siRNA-duplex structures by TLR3 represents another possibility for TLR-mediated interference.

Therefore, WT, TLR3-deficient and TLR7-deficient mice were injected i. v. with 9.2s siRNA one day prior to infection with AdOVA. The OVA-specific CTL response was determined 5 days later in the spleens by an *in vivo* cytotoxicity assay. Importantly, in spleens of both WT and TLR3-deficient mice that were pre-treated with 9.2s RNA before AdOVA infection OVA-specific CTL response was completely absent. In contrast, 9.2s-induced suppression of CTL responses was abrogated in TLR7-deficient mice (Figure 28**A**).

These results provide strong evidence, that 9.2s-induced immune suppression of AdOVA-directed CTL responses in mice cannot be attributed to siRNA-caused gene silencing of TLR9 but rather by recognition via TLR7. In addition, these results suggested that systemically circulating nucleic acids in general are able to induce immune suppression.

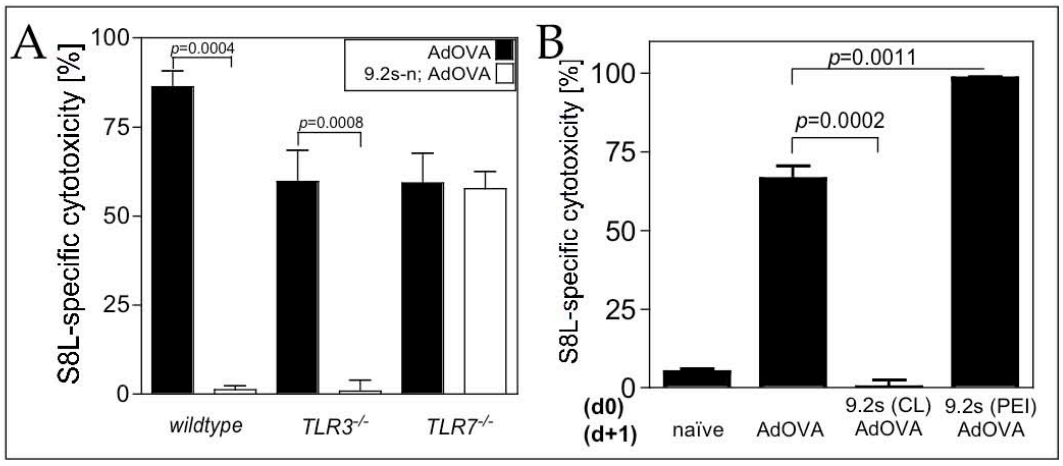

Figure 28 9.2s-induced immune suppression requires TLR7 and targeting to the endosome

S8L specific cytotoxicity was assessed in the spleens of mice 5 days post immunization. (a) Indicated groups of C57BL/6 WT, TLR3$^{-/-}$ or TLR7$^{-/-}$ littermates, respectively, received 10µg 9.2 24 hours before AdOVA infection (1x10e7 PFU i.v.). All RNAs were complexed to 30µl cationic liposomes (CL) (=DOTAP) per 10µg RNA per mouse (b) Indicated groups of C57BL/6 WT mice received 10µg 9.2 siRNA 24h before i. v. infection with 1x10e7 PFU recombinant AdOVA. siRNA was either complexed to 30µl CL per 10µg RNA or to PEI. n=3-4 per group, representative data of two or more independent experiments are shown.

7.3.1.4 Intracellular targeting of TLR7 ligand siRNA has stimulatory or suppressive consequences

TLRs that bind nucleic acids are predominantly localized intracellularly in the endosome. Efficient delivery of RNA into different sub-cellular compartments requires association to vehicles, particularly in cases of non-stabilized nucleic acids to prevent premature degradation. For *in vivo* therapies, RNA samples are either complexed with cationic liposomes (CL) or polyethylene imines (PEI), in case they lack special stabilization. RNA complexed to CL is exclusively targeted to the endosome whereas PEI facilitates delivery into the cytoplasm after osmotic rupture of the endosome (Kichler et al., 1995). Application of siRNA in the previous experiments was performed with CL complexation.

The relevance of siRNA application bound to different vehicles regarding the influence on the generation of CTL responses towards AdOVA infection was investigated *in vivo*. C57BL/6 mice were injected with 9.2s RNA either complexed with CL or PEI one day before AdOVA infection. OVA-specific CTL responses were determined 5 days after infection by an in vivo cytotoxicity assay in the spleen. Only 9.2s RNA complexed to CL induced immune suppression, whereas binding to PEI did not inhibit CTL responses towards subsequent AdOVA infection (Figure 28**B**). Thus, delivery of nucleic acids into the cytoplasm prevented induction of suppression.

7.3.2 Experimental therapeutic application of TLR7 ligand 9.2s induces suppression of adaptive immune responses towards subsequent AdOVA infection

7.3.3

The association of siRNA with immune suppression was unexpected as siRNA application is commonly expected to induce immune stimulation due to its capability to induce high amounts of type I interferon. Therefore, we asked whether experimental therapeutic application of isRNA as cancer treatment could exert adverse effects on subsequent infections in murine models. This is of major interest as the connotation of RNA-caused gene silencing with its type I interferon-inducing capability is currently supposed to be favorable in anti-tumor therapy.

In particular, three questions were addressed. First, does systemic application of siRNA after inoculation of mice with tumor cells influence the anti-tumor CTL response or tumor growth? Secondly, does siRNA treatment inhibit a CTL response directed against a subsequent infection with recombinant AdOVA? Thirdly, does anatomical site of application as well as the dose of siRNA determine the outcome of the OVA-specific CTL response towards the subsequent AdOVA infection?

In the present study, experimental siRNA treatment was applied in two different tumor models to analyze whether siRNA-induced anti-tumor effects were accompanied by an inhibition of adaptive immune responses towards subsequent infection with recombinant adenoviruses.

7.3.3.1 9.2s-n siRNA enhances CTL response against RMA tumor but suppressed immune responses against subsequent AdOVA infection

To study the influence of siRNA application on tumor-specific CTL responses experimentally, C57BL/6 mice received i. v. RMA tumor cells expressing eGFP (RMA-GFP); RMA tumor cells predominantly localize in the liver upon systemic injection, whereas local i.e. subcutaneous injection of tumor cells leads to local growth.

To mimic therapeutic application of siRNA in an experimental tumor treatment, tumor-inoculated mice received 9.2s RNA i. v. two days after injection of RMA-eGFP. The tumor-specific CTL response was determined 6 days after tumor cell injection by an *in vivo* cytotoxicity assay in the liver (see Figure 29 for overview about the treatment schedule). As shown in Figure 30, RMA-GFP-specific CTL responses in siRNA-treated mice were minimally stronger than in tumor-bearing mice that did not receive siRNA. Thus, siRNA-treatment did not inhibit CTL responses against RMA tumor cells.

We next addressed the questions, whether in RMA-tumor bearing mice local versus systemic application or the doses of 9.2s siRNA were decisive for generation of OVA-specific CTL towards a subsequent AdOVA infection. The treatment schedule is shown in Figure 29.

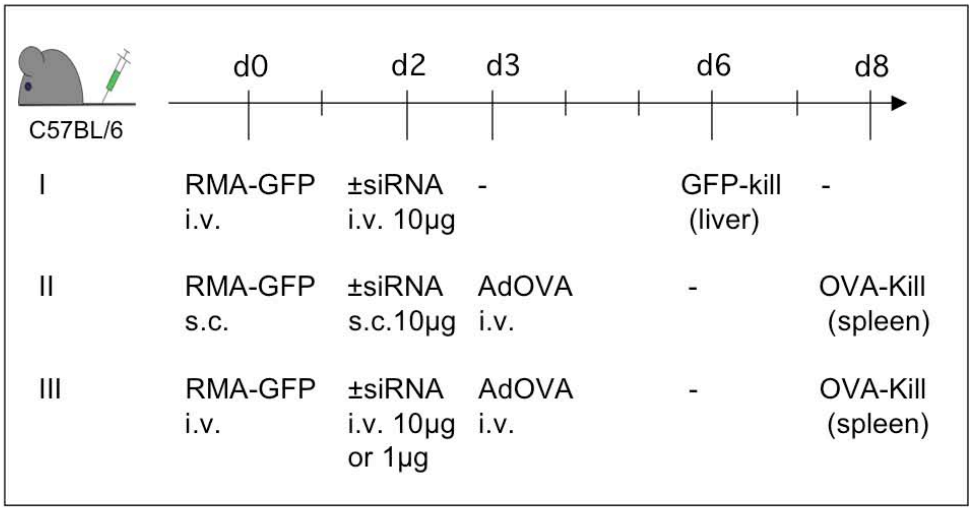

Figure 29 Time schedule of RMA-GFP tumor experiments

C57BL/6 mice received RMA-eGFP tumor cells either s.c or i. v. Two days later, mice received experimental treatment with siRNA 9.2s. 9.2s siRNA was injected either s. c. in mice that had received RMA-GFP tumor cells also s. c., or i. v. in mice that were injected with RMA-GFP tumor cells i.v., respectively. To decipher the role of high or low dose 9.2s, mice were injected either with 1μg or 10μg siRNA.

Three days after tumor inoculation mice were infected i. v. with recombinant AdOVA to mimic a secondary infection. OVA-specific CTL responses were determined five days after AdOVA infection in the spleen. OVA-specific CTL responses against AdOVA were strongly reduced in mice that had received high doses of 9.2s isRNA i.v. In contrast, low dose or s.c. injection of 9.2s isRNA did not inhibit OVA-specific CTL responses (Figure 30**B**).

Taken together, our findings show that systemic (i.v) rather than local (s.c.) application of high doses of CpG or siRNA induced immune suppression (Figure 2**A**), (Figure 30**B**). This indicated that immune suppression was only associated with the systemic route and was not dependent on the TLR-ligand itself. Importantly, the time point of TLR ligation in respect to antigen application was also critical whether the induced CTL response was stimulated or suppressed.

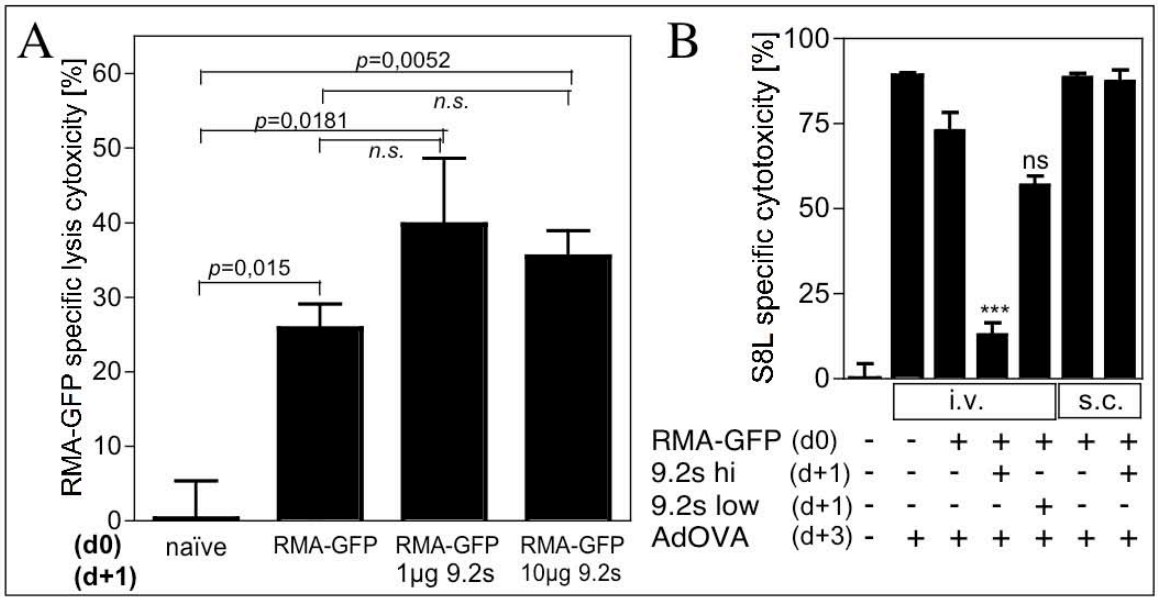

Figure 30 Systemic injection of 9.2s siRNA into RMA-GFP tumor bearing mice prevented the generation of CTL responses towards subsequent AdOVA infection

(a) C57BL/6 received 5x10e5 RMA-GFP cells i. v. and were injected with TLR ligands 2 days later. RMA-specific cytotoxicity assay was performed in vivo 6 days later and lysis of target cells was determined in the spleen. (b) S8L-specific cytotoxicity of AdOVA-infected C57BL/6 that received three days before AdOVA 5x10e5 RMA-GFP cells i. v. and were injected with 1µg 9.2s or 10µg 9.2s RNA 2 days later. Spleen was analysed, n=4 per group.

7.3.3.2 TLR7 ligand 9.2s-n siRNA inhibits growth of melanoma but also cellular immune responses towards AdOVA infection

Systemic application of Mo4 cells, which is a weakly immunogenic B16 melanoma, leads to tumor growth in the lung (Meyvisch and Mareel, 1982). In the present study, Mo4 transfected with luciferase (Mo4-luc) was utilized, which allowed monitoring of tumor growth with the help of *in vivo* imaging of bioluminescence. Here, the question was addressed whether experimental treatment with siRNA 9.2s reduced tumor growth and concomitantly influenced CTL responses against subsequent infection with recombinant AdOVA. To investigate this question, C57BL/6 WT mice were injected with Mo4-Luc i. v. and received experimental siRNA treatment two days later by i.v. injection of either with a low dose or a high dose of 9.2s siRNA (see Figure 31 for treatment schedule). At day 3, mice were infected i. v. with recombinant AdOVA. *In vivo* imaging of bioluminescence of anaesthetized mice was started at day 4 and then performed every other day to monitor tumor growth. OVA-specific CTL responses were determined at day 8 in the spleen by an *in vivo* cytotoxicity assay. Treatment of Mo4-luc tumor cell-injected mice with a low dose of 9.2s siRNA did neither induce significant reduction in tumor growth nor inhibition of OVA-specific CTL responses towards AdOVA infection (Figure 32**A, B**). In contrast, injection of high dose 9.2s siRNA caused a significant reduction in tumor growth but prevented OVA-specific CTL responses towards AdOVA infection (Figure 32**A, B**).

Thus, these results support the relevance of 9.2s siRNA dose. Although systemic application of high doses of 9.2s siRNA reduced growth of Mo4-luc tumor cells, it also inhibited CTL responses directed against subsequent infection with recombinant AdOVA.

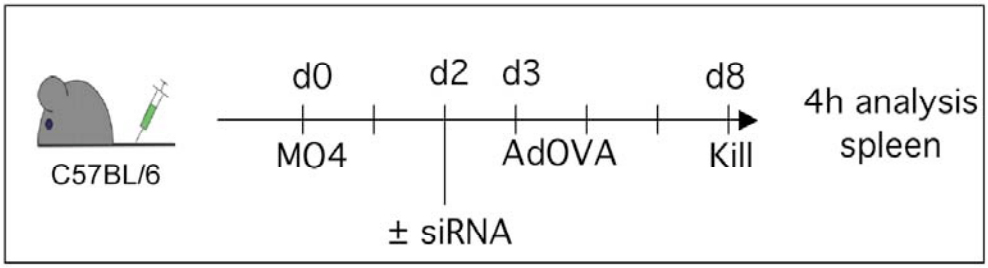

Figure 31 Time schedule for inoculation of mice with Mo4 tumor cells and subsequent AdOVA infection

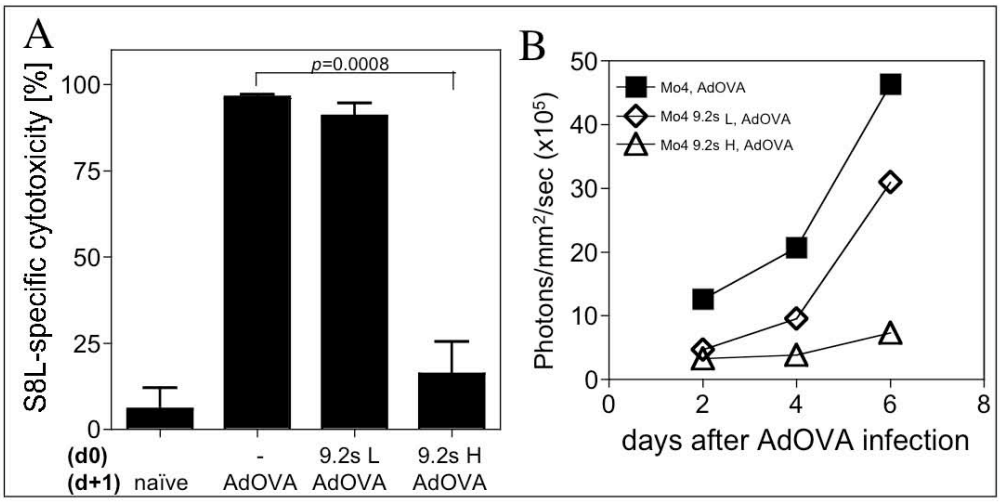

Figure 32 Systemic injection of 9.2s siRNA inhibited growth of Mo4 tumor, but suppressed also CTL response towards subsequent AdOVA infection.

(a) S8L specific cytotoxicity was determined 5 days in the spleen after infection of C57BL/6 with 1x10e7 PFU AdOVA. Before, C57BL/6 had received x Mo4-luciferase expressing tumor cells i.v. and were treated 2 days later with 1µg (L) or 10µg (H) 9.2s siRNA complexed to CL. AdOVA infection was performed at d3. (b) Multiplication of luciferase expressing tumor cells was monitored via In vivo Imaging (IVIS®200) every other day starting 2 days after inoculation of mice with 9.2s siRNA.

7.3.4 TLR7 ligand siRNA induced suppression of adaptive immune responses can be abrogated by exogenous CD4 help and is dependent on type I IFN

7.3.4.1 Restoration of cytotoxicity by provision of CD4 help

In the present study it was figured out, that OVA-specific CTL responses were very weak or almost absent in AdOVA-infected CD4 cell-deficient mice, suggesting that $CD4^+$ T cells are necessary for primary CD8 T cell responses towards infection with recombinant AdOVA (Figure 4C). Moreover, CpG-induced suppression of CTL responses towards infection with recombinant AdOVA was abrogated by provision of exogenously activated antigen-specific $CD4^+$ cells purified from TCR-transgenic OT-II mice (Flandin et al., 2006). To investigate the capacity of exogenous CD4 help to abrogate 9.2s siRNA-induced CTL suppression, two groups of C57BL/6 mice were adoptively transferred with *in vitro* activated $CD4^+$ cells that were isolated before from CD4-TCR transgenic OT-II mice. Indicated groups received 9.s siRNA 24h later and were infected with recombinant AdOVA. OVA-specific CTL responses were determined in all groups five days after AdOVA infection in the spleen. A strong OVA–specific CTL response was detected in spleens of AdOVA-infected mice that had received *in vitro* activated OVA-specific $CD4^+$ T cells from OT-II mice by adoptive transfer. 9.2s siRNA treatment did not abrogate S8L-specific cytotoxicity when activated CD4+ T cells were present (**Figure 33**). Thus, provision of exogenously activated antigen-specific helper cells rescued the CTL response suppressed by 9.2s siRNA (**Figure 33**). This result strengthened the role of CD4 T cells in siRNA induced suppression and provided evidence that TLR-ligand induced inhibition of CTL response cannot be explained exclusively by insufficient antigen-presentation.

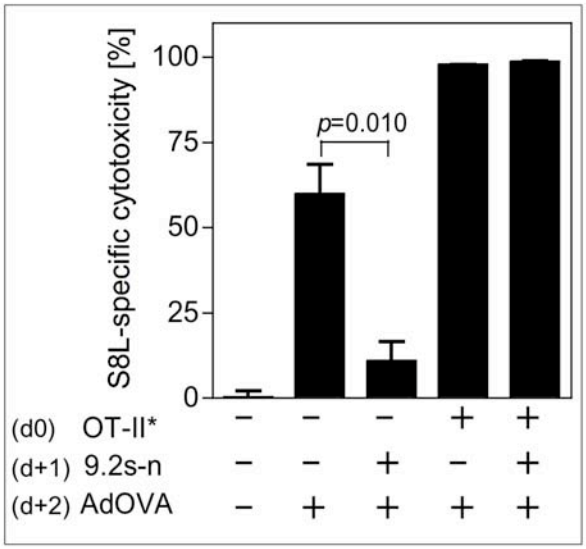

Figure 33 Adoptive transfer of exogenously activated CD4 cells abrogated 9.2s siRNA-induced CTL suppression

S8L specific cytotoxicity was measured in spleens of AdOVA infected C57BL/6 mice d+5 p.i. 2x10e5 *in vitro* activated CD4+ cells from transgeneic OT-II donors were adoptively transferred into indicated groups (+ OT-II*) 2 days before AdOVA infection (1x10e7 PFU i.v.). Indicated groups were injected with 10µg 9.2s per mouse at d-1 and infected i. v. with 1x10e7 PFU AdOVA at d0.

7.3.4.2 siRNA-induced suppression is dependent on type I interferon

Hornung et al. reported induction of high levels of type I interferon upon systemic injection of 9.2s siRNA (Hornung et al., 2005). Induction of immune activation is one of the main characteristics that are attributed to type I IFNs (Curtsinger et al., 2005). In contrast, other studies demonstrate inhibitory functions of type I IFNs (Billiau, 2006). We asked whether the generation of antigen-specific CTL responses might be inhibited through the induction of type I IFN. This was investigated in IFNAR-deficient mice, which cannot respond to type I interferons alpha and beta due to a lack of the receptor (van den Broek et al., 1995).

To further investigate the influence of 9.2s siRNA on viral load, mice were infected with recombinant adenovirus expressing luciferase, OVA and GFP (AdLOG) that allowed *in vivo* analysis of luciferase expression by determination of bioluminescence. WT and IFNAR-deficient mice were injected with 9.2s siRNA or not one day before infection with recombinant AdLOG. One day after infection, luciferase-caused bioluminescence was determined in all groups by *in vivo* imaging and OVA-specific CTL responses were determined 5 days after infection in the spleen.

Most importantly, siRNA did not inhibit OVA-specific CTL responses in IFNAR deficient mice (Figure 34**A**); this demonstrated clearly a negative regulatory role of type I interferons. Analysis of viral load by determination of bioluminescence revealed that the application of 9.2s siRNA did not significantly influence luciferase-expression in WT mice in comparison to control-infected littermates. In contrast, bioluminescence was 4 fold stronger in AdLOG infected $IFNAR^{-/-}$ mice than in WT controls. siRNA treatment reduced luciferase expression in AdLOG infected $IFNAR^{-/-}$ mice compared to control-infected IFNAR-/- mice (Figure 34**B, C**). As the OVA-specific cytotoxicity was not influenced by siRNA in $IFNAR^{-/-}$ mice, these results strongly support irrelevance of TLR-L induced reduction of antigen dose for efficient generation of CTL responses.

To specify the contribution of interferon beta to the suppressive effect of type I IFNs, IFN-$\beta^{-/-}$ ($H2\text{-}k^{d}$) mice were infected with AdLOG after pretreatment with 9.2s siRNA. Five days later, the GFP-specific CTL response was determined in the spleens of those animals. 9.2s-induced suppression of CTLs was still present in IFN $beta^{-/-}$ mice (Figure 34**D**). Thus, IFN-β can be excluded to participate in type I IFN-mediated immune suppression. This suggests that IFN-α is critically involved in siRNA-mediated immune suppression.

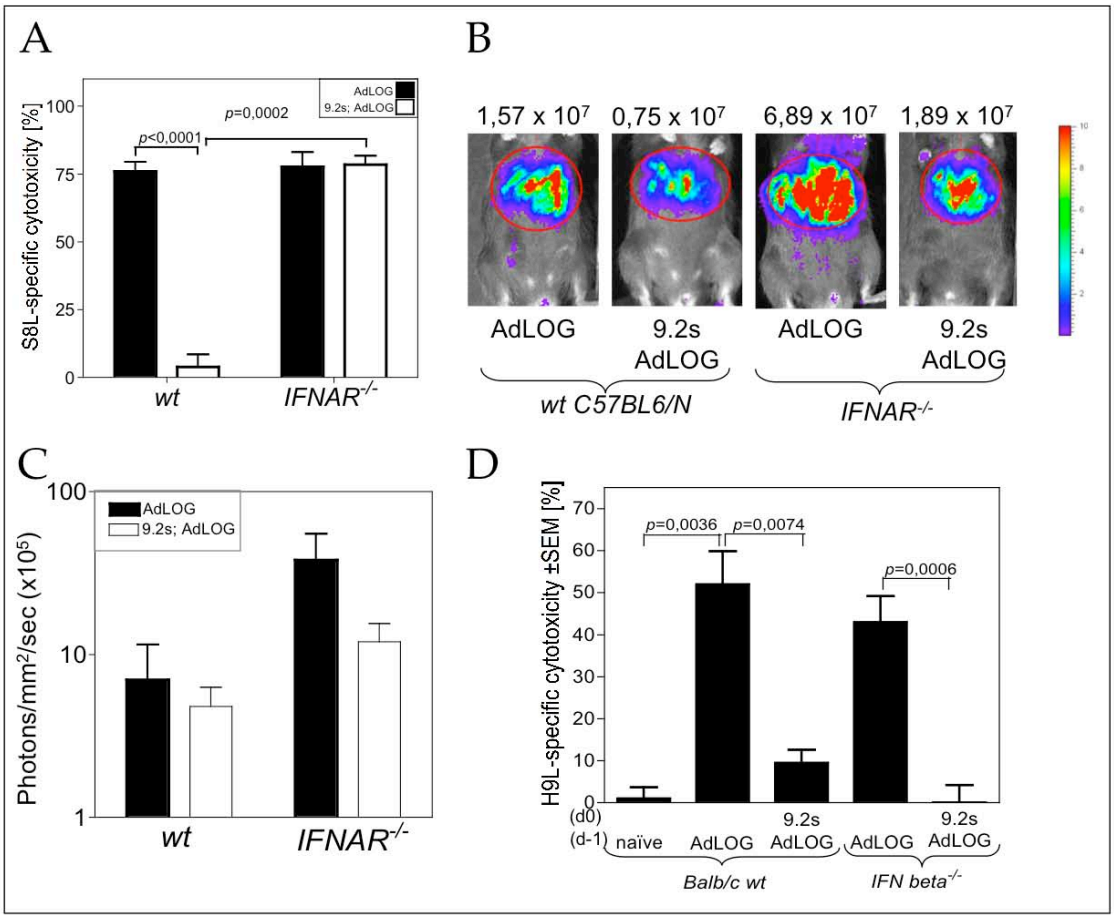

Figure 34 siRNA induced CTL inhibition required type I interferon.

(a) Determination of S8L-specific cytotoxicity in C57BL/6 wild type or IFNAR$^{-/-}$ mice after infection with recombinant AdLOG with or without systemic 9.2s-n pre-treatment. (b, c) *In vivo* Imaging of C57BL/6 WT or IFNAR$^{-/-}$ mice 24 hours after AdLOG infection; mice were injected with 10µg 9.2s siRNA complexed with 30µl DOTAP 24 hours before AdLOG infection or not. Numbers indicate photons per mm2 per second x10e7 detected in the regions of interest. Representative animals are shown. (c) Bioluminescence was quantified by photons per mm2 per second, n=3 per group. (d) H9L-specific CTL response was determined in Balb/c WT and IFN beta$^{-/-}$ mice 5 days after infection with 1x10e7 recombinant AdLOG i.v. Indicated groups of mice were injected with 10µg 9.2s siRNA i.v. one day before AdLOG infection. Each bar represents the mean ±SEM; n=3 per group.

7.3.5 Summary of chapter 7.3

Systemic injection of ligands for TLR4 or TLR7 also inhibited CTL responses towards infection of mice with recombinant AdOVA, as it was previously observed for CpG. Importantly, siRNA-induced CTL suppression, which was dependent on TLR7. The observation that TLR7 ligand siRNA suppressed CTL responses raised the hypothesis that therapeutic application of siRNA might inhibit the generation of CTL responses towards subsequent infections. Indeed, experimental therapeutic application of siRNA inhibited CTL responses directed against subsequent infections of mice with recombinant AdOVA in two different tumor models, although siRNA treatment positively influenced the anti-tumor response. Most importantly, siRNA-induced inhibition of immune responses could be attributed to negative regulatory effects of type I IFN.

8 Discussion

The aim of this thesis was to investigate how Toll-like receptor (TLR) ligands regulate effector immune responses towards viral or parasitic infections. TLR recognize conserved molecular patterns of microorganisms and are essential for the activation of host immunity. Whereas the immune-stimulatory effects of TLR ligands are well described, the circumstances of TLR-mediated inhibition of immune responses are still largely unknown. The results within this thesis point out a key function of TLRs in negative regulation of inflammatory immune responses. In contrast to subcutaneous application of TLRs that stimulate immune responses, systemic application of TLR ligands in high doses prior to infection suppressed innate and adaptive immune responses. The results demonstrate a dual role of TLRs as that the anatomical site, dose and time-point of TLR ligand application determined if an immune response was stimulated or suppressed. TLR-ligand mediated suppression of adaptive immune responses – i.e. antigen-specific CTL responses, was mediated by inhibition of $CD4^+$ T cell help. Physiological relevance of TLR mediated suppression of spleen dependent-immune responses was demonstrated in two different mouse models of infectious diseases. TLR-dependent negative regulation suppressed CTL responses in mice following infection with recombinant AdOVA and protected *Plasmodium berghei* infected mice against lethal inflammatory processes. However, the data emphasize the risk of TLR ligand mediated bystander suppression of unrelated immune responses targeted against subsequent infections, which may have important implications for future therapeutic application of TLR ligands.

Immune activation is a double-edged sword for the host. The destructive potential of the effectors has to be strictly controlled because the armory used against pathogens may also exert detrimental effects to the host. Self-inflicted damage is inevitable during a protective response, either by direct lysis of infected cells by CTLs or indirectly by the release of antimicrobial factors (cytokines, free radicals) that are toxic to the host. Thus, excessive inflammation causing immune damage must be prevented. Therefore, we addressed the question how TLR ligand-mediated inflammatory immune responses are regulated and in particular, how TLR-L influenced the generation of antigen-specific $CD8^+$ T cell cytotoxicity in response to various infections, such as adenoviral vectors, *Listeria monocytogenes* or *Plasmodium ssp*. All these infections required the spleen for the induction of $CD8^+$ T cell responses against antigens expressed by these pathogens (Figure 4,Figure 15).

8.1 TLR ligand-mediated inhibition of adaptive immune responses

8.1.1 TLR7-ligand and TLR9 ligand-mediated inhibition of T cell activation by interfering DC-T cell interaction

Interestingly, we observed that TLR-L differently affected CTL responses if given locally or systemically. While subcutaneous injection of the TLR9 ligand CpG enhanced specific CTL responses towards co-administered antigen (Figure 2A), intravenous injection of TLR ligands in high doses inhibited the generation of adaptive immune responses (Figure 2, Figure 26, Figure 27). The stimulatory effect of local administration of TLR-L together with antigen is in line with many reports where TLR-L are used as adjuvans in the induction of adaptive immunity (Krieg, 2006).

Largly unexpected though was the finding that intravenous injection of TLR ligands in high doses inhibited the generation of adaptive immune responses (Figure 2). TLR ligand-mediated immune suppression was characterized by strongly reduced numbers of antigen-specific T cells and a lack of antigen-specific cytotoxicity (Figure 2B, C). Importantly, this effect was strongly dependent on TLRs, as TLR deficient mice generated normal CTL response even upon injection of the ligand (Figure 3, Figure 26, Figure 28).

It is assumed that cellular responses to TLR ligands are influenced by an individual expression pattern of single TLRs in respect of the cell type, localization and tissue (Kawai and Akira, 2005). The striking finding demonstrating that subcutaneous application of CpG enhanced CTL responses whereas intravenous application of CpG inhibited CTL responses suggests that the circumstances of immune activation determine whether a CTL response was induced or not.

In particular, we observed that the time point of TLR ligation in respect to immunization/application of antigen and the dosage of TLR-L injected determined whether CTL induction was successful or not. With respect to the time point, systemic injection of TLR-L prior to immunization inhibited the CTL responses whereas injection of TLR-L one day after infection enhanced the CTL response (Figure 26). In this line, low doses of systemic TLR-L were stimulatory, while doses above a certain threshold inhibited CTL responses.

To determine the mechanisms underlying the suppressive effect of TLR-L on the generation of CTL responses, one has to carefully look at the factors known to be essential for the activation of CD8 T cells: Firstly, DC have to take up antigen and present it to T cells (signal1). Secondly, DC have to be licensed by TLR stimulation and/ or CD40-CD40-Ligand interaction provided by $CD4^+$ T cells. Thirdly, activated DC have to provide co-stimulation in form of receptors (signal 2; CD80/86) or cytokines (signal 3; IL-12, type I interferon). Finally, activated CD4 T cells have to provide help to CD8 T cells either by licensing DC, i.e. by maximizing costimulation or by providing IL-2 (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

We first investigated whether TLR-L impeded antigen presentation. As it is known that TLR triggering enhances the antiviral activity of innate cells (Blander and Medzhitov, 2004), TLR ligands could have diminished the viral load in a way that CD8 T cell activation was inhibited. We can exclude this possibility, as our results indicate that although CpG decreased the amount of antigen, the strength of the CTL response did not correlate with viral load. In particular, both high and low doses of CpG reduced the viral load equally strong (Figure 2F). But while the systemic application of CpG in high doses suppressed the CTL responses directed against a subsequent AdOVA infection, low doses of CpG even stimulated the CTL response compared to control-infected mice (Figure 2E). A recent study by the group of Villadangos demonstrated impaired phagocytosis and strongly reduced cross-presentation of OVA class I antigen by splenic $CD8a^+$ DCs upon systemic injection of CpG (Wilson et al., 2006). In agreement with Wilson et al., we also detected decreased cross-presentation of OVA class I antigen after systemic application of CpG (data not shown). However, our results demonstrated that reduced cross-presentation did not affect CD8 T cell activation and therefore was not responsible for CpG induced CTL suppression.

We could further exclude an inhibitory effect of TLR-L on antigen presentation based on transfer experiments. Adoptive transfer of exogenously antigen-loaded and matured APCs did not overcome the CTL suppression in TLR ligand treated mice, although untreated control mice generated a strong CTL response (Figure 11). These results rather supported the theory that TLR-L induced an inhibitory milieu, which was responsible for the observed block of T cell activation. Although TLR ligand-induced impairment of IL-12 and other pro-inflammatory cytokine production was a major characteristic of immune paralysis (Figure 7, Figure 8), the data suggest that although direct negative effects of TLR ligands on APCs can not be excluded, these effects can not exclusively be responsible for inhibition of endogenous T cell responses.

$CD8^+$ T cell activation greatly depends on the activation of DC ("licensing"), which either occurs by provision of $CD4^+$ help via CD40 or TLR stimulation. As $CD4^+$ T cells are important factors in the induction of CD8 T cell responses, we investigated whether systemic TLR-L blocked the generation of $CD8^+$ T cell cytotoxicity via inhibition of $CD4^+$ help. The importance of $CD4^+$ help for the generation of cytotoxic $CD8^+$ T cells against adenoviral antigens was demonstrated by two experiments. First, lack of OVA-specific CTL response in CD40-deficient animals upon infection with recombinant AdOVA demonstrated that the generation of OVA-specific CTL responses required CD40 co-stimulation. Second, mice genetically deficient $CD4^+$ T cells or MHC class II did not generate OVA-specific CTL responses upon infection with recombinant AdOVA (Figure 5). These findings support current models postulating that CTL responses require $CD4^+$ T cell help (Behrens et al., 2004; Bevan, 2004)Castellino et al., 2006).

Indeed, data presented in this thesis provide evidence that systemic TLR ligands negatively affected CD4 help. This was concluded from the observation that an adoptive transfer of activated antigen-

specific CD4+ T helper cells restored antigen-specific CTL response in mice treated with ligands for TLR9 and TLR7 (Figure 13; Figure 33). This transfer experiment strongly indicated that systemic application of TLR ligands mainly affected CD4+ T cell help, whereas impairment of DCs seemed to be less important as otherwise activation of CD8 T cells by antigen presenting DC would not have been possible. Even if TLR ligands caused sub-optimal conditions of T cell priming by reducing the levels of class I presented antigen and lack of costimulatory cytokines such as IL-12, these deficits had been clearly overcome by the adoptively transferred exogenously activated CD4+ T cells.

In summary, the transfer experiments imply two major facts: (1) inhibition of adaptive immunity could not solely be attributed to a functional impairment of DCs; the amount of antigen that is presented on class I must be sufficient for T cell activation and (2) a possible inhibitory milieu can be bypassed by transferring the activated CD4+ T cells. Also here, a hyper-reactivity of the transgenic T cells cannot be completely excluded.

Future experiments are required to examine the role of CD40-CD40L triggered DC-T cell interaction during TLR ligand-induced suppression. To investigate this question, TLR ligand treated mice could be injected with a stimulatory anti-CD40 antibody or receive DCs that were *in vitro* transduced with a recombinant adenovirus expressing CD40L via an adoptive transfer. This may help to determine whether DC licensing via CD40-CD40L is affected during TLR ligand induced suppression.

8.1.2 TLR7 ligand but not TLR9 ligand-mediated CTL suppression is dependent on type I interferons

In addition to TLR9 ligand CpG, also systemic application of TLR7 ligand siRNA prior to AdOVA infection suppressed antigen-specific CTL responses in mice (Figure 27, Figure 28). In agreement with the results obtained in TLR9 ligand experiments, also the transfer of activated antigen-specific CD4+ helper cells rescued the CTL response in TLR7 ligand treated mice. In contrast to TLR9 ligand CpG, induction of immune suppression by TLR7 ligand siRNA was mediated by type I IFNs (Figure 33). This finding contrasts the general association of type I IFNs with immune stimulation, as type I IFN can enhance maturation of DCs and provide signal 3 for T cell activation (Curtsinger et al., 2005). Besides the well described stimulatory effects of type I interferons, anti-inflammatory functions of type I IFN are observed in several models of autoimmunity (Hron and Peng, 2004; Katakura et al., 2005; Touil et al., 2006; Yarilina et al., 2007). Type I IFN were shown to protect neonates from acute inflammation by induction of IL-10 producing B cells (Zhang et al., 2007). Furthermore, a report by Lehner et al. demonstrated induction of apoptosis in monocyte-derived DCs by type I IFN (Lehner et al., 2001). The groups of Welsh and Reimann demonstrate negative regulation of CTL responses by type I IFNs (Bahl et al., 2006; Dikopoulos et al., 2005). IFNβ interferes with CD25 expression and is also described to induce CD95 (Noronha et al., 1993; Rep et al., 1999).

How type I interferon influences DC mediated T cell activation plays a similar role in the TLR-L induced suppression of CTL responses, as described here, needs to be analyzed in future studies. This involves investigation of the cellular source, which produces high levels of type I interferon. In a preliminary experiment depletion of plasmacytoid dendritic cells as a primary responder to TLR7 ligand and source of type I IFN was performed. Antibody mediated pDC depletion abrogated siRNA-induced CTL suppression significantly, but not completely (data not shown). Although pDC are considered to be the most important source of type I interferons following TLR7 stimulation, our experiment suggests that other cells might produce type I interferons following challenge with TLR7-L. Possible candidates are B cells as these cells express TLR7 and TLR9. Preliminary experiments that were performed in B cell deficient $\mu MT^{-/-}$ mice did neither indicate a role for B cells in TLR9 ligand nor in TLR7 ligand-mediated suppression (data not shown). However, $\mu MT^{-/-}$ mice still express pre-B cells (B1, B2) that may be involved in suppression. In addition, the use of genetically deficient animals may be limited to draw conclusions as these animals may compensate for the genetic lack of a certain molecule or cells by increased activation of other mechanisms.

8.1.3 Therapeutic application of TLR ligands harbors possible risks of immune suppression

Our data show that an intravenous injection of siRNA can suppress CTL responses in a TLR7 dependent manner if siRNA is complexed to a carrier that targets the endosome. Importantly, the experimental dose of siRNA that induced suppression of adaptive immune responses in our experiments, was previously reported to exert immune stimulatory effects (Hornung et al., 2005). Our data demonstrate a relevance of siRNA-mediated suppression of immune responses against a secondary adenoviral infection in two experimental models of tumor therapy (Figure 30, Figure 32). In addition to previously described immune-stimulatory consequences that are mainly attributed to induction of high levels of type I interferon (Hornung et al., 2005), our data point out a critical role for type I interferons in immune suppression.

Immune stimulatory properties of TLR ligands provide a promising tool for therapeutic application. Induction of pro-inflammatory cytokines facilitates the attraction of lymphocytes and a “productive” encounter with APCs. This is favored in circumstances of insufficient immunogenicity or actively induced “tolerance”, which may be induced by persistent infections or tumors in order to evade attack by the immune system. CpG and siRNA treatment currently represent a highly interesting approach of tumor therapy (Lu et al., 2005; Muller and Scherle, 2006; Xie et al., 2006). Certain RNA sequences are described to elicit immune stimulatory properties in a TLR7 dependent manner (Hornung et al., 2005). Data from this thesis provide evidence that siRNA can suppress adaptive immune responses towards subsequent infections if circumstances of the application meet the criteria of a potential “danger” signal. Here, we define “danger” as the risk to induce systemic overwhelming inflammation that may be dangerous for the host.

We recommend that therapeutic application of TLR ligands should be carefully considered in order to prevent "accidental" T cell suppression towards subsequent infections during therapeutic application of immune-stimulatory TLR ligands. Our data indicate an increased risk of systemic application whereas local application did not inhibit immune responses. Our data show that intracellular targeting of siRNA into the cytoplasm instead of the endosome by choosing the appropriate carrier may offer a possibility to prevent "accidental" immune suppression due to systemic application of TLR ligands (Figure 28B). These observations may be the basis of future research to improve success and safety of TLR ligands in therapeutic applications.

8.2 The role of TLRs in the induction of experimental cerebral malaria (ECM)

8.2.1 Induction of pro-inflammatory effector mechanisms during *Plasmodium* infection is dependent on TLRs

It is yet unresolved how immune effector mechanisms during *Plasmodium ssp*. infection are regulated that are necessary to efficiently combat parasites but may cause detrimental consequences due to excessive immune activation. Cerebral malaria (CM) is a major life-threatening complication due to *P. falciparum* infection in humans and is assumed to be a complex inflammatory syndrome. CM pathogenesis may be partially explained by a long-standing hypothesis postulating that immuno-pathology is caused by inflammatory responses, leading to the disruption of the blood-brain barrier. Vascular leakage facilitated infiltration of parasitized erythrocytes and immune cells into the brain that harbors the risk of brain damage due to sequestration of parasitized erythrocytes or due to immune damage caused by parasite-specific $CD8^+$ T cells. In humans, the examination of postmortem tissue or tissue biopsies exerts the only way of evaluating pathological processes. Thus, experimental studies in animal models are essential to identify biological processes during *Plasmodium* infection causing cerebral malaria. Among many different experimental malaria model systems, *Plasmodium berghei ANKA* (PbA) infection of C57BL/6 mice is the most accepted model to study the pathogenesis of experimental cerebral malaria processes *in vivo*, as most of the data generated in this system are considered to be similar to the human situation (Schofield and Grau, 2005).

In the PbA infection, IFNγ and T cells have been identified to be among those factors that are essential for the pathogenesis of ECM. This was concluded from experimental approaches performed in genetically deficient animals or by use of depleting antibodies (Yanez et al., 1999; Belnoue et al., 2002; Hermsen et al., 1998) and was confirmed in this study (Figure 15).

Until recently, the role of TLRs in the pathogenesis of ECM was unknown. First hints that *Plasmodium*-derived components are recognized through TLRs and induce immune activation are based on three recently published studies. TLR-mediated recognition of plasmodial components GPI occurs through TLR2, whereas TLR9 recognizes hemoglobin-degradation product hemozoin /parasite-

derived DNA (Coban et al., 2005; Krishnegowda et al., 2005; Parroche et al., 2007). However, the biological relevance for TLRs in ECM pathogenesis was not examined up to now.

We investigated the role of TLRs and their contribution to IFNγ dependent effector mechanisms in the generation of innate and immune responses following *Plasmodium berghei ANKA* (PbA) infection. Mice deficient in TLR2, TLR3 or TLR9 were significantly protected against ECM upon PbA infection, which suggested that detection of PbA through TLR2, TLR3 and TLR9 leads to induction of detrimental inflammatory responses (Figure 14). These data are in line with a recent study from Coban et al., who attributed TLR2, TLR9 and MyD88 to the induction of ECM following PbA infection (Coban et al., 2007). Contradictory results were published by two other groups, that either excluded any relevance of TLRs in PbA infection (Togbe et al., 2007) or limited it to MyD88 (Lepenies et al., 2007). A possible explanation for these discrepant observations may be that clones of PbA used in these studies differ in their ability to induce CM (Amani et al., 1998).

For a long time it has been speculated where pathogenic T cells and inflammatory cytokines causing ECM are generated. Among direct effects on the brain, the spleen has been considered to play a role in ECM induction (Engwerda, 2005; Hermsen et al., 1998). Therefore, we studied whether and how the spleen influenced ECM induction. Interestingly, splenectomized animals were completely protected against developing ECM after PbA infection (Figure 15). Similar observations were published before in an infection model utilizing the related strain *P. berghei K173* in mice (Hermsen et al., 1998; Kamiyama et al., 1987), suggesting that a requirement of the spleen represents a general mechanism for priming of T cell responses following infection by different *Plasmodium* species (Engwerda et al., 2005b). Additionally, our data indicate relevance for an intact micro-architecture of the spleen. Previous studies reported protection from ECM of RAG-1 deficient mice is based on the lack of effector B and T cells. The lack of these important lymphocyte populations in RAG deficient mice is likely to cause a disintegrated micro-architecture of the spleen (Mebius and Kraal, 2005). Transfer of $CD8^+$ effector cells isolated from wild type donors 5 days after PbA infection induced ECM in RAG-1 deficient recipients (Nitcheu et al., 2003). Here, we show that the generation of effector cells required an intact micro-architecture of the spleen as simple reconstitution of RAG-1 deficient mice with $CD8^+$ and $CD4^+$ splenocytes isolated from naïve wild type donors did not restore the ability to generate detrimental effector responses upon PbA infection (Figure 15). In addition, re-transfer of single-splenocyte suspension after splenectomy of PbA-infected mice did not cause ECM development (data not shown).

Our findings that depletion of dendritic cells resulted in protection of PbA infected mice from ECM pathology (Figure 16), is in line with a recent study (deWalick et al., 2007) and emphasizes the importance of DC in the induction of PbA-specific T cell responses as well as the production of inflammatory cytokines IL-12 and INFγ. Depletion of $CD4^+$ and $CD8^+$ T lymphocytes protected PbA infected mice and demonstrated that T cells were critical effectors in ECM (Belnoue et al., 2002; Hermsen et al., 1998). However, as parasite-specific CTL responses cannot be determined in the

murine PbA infection model, we investigated the generation of CTL responses towards *P. falciparum* infection *in vivo* in HLA-A2 transgenic mice, which represent a humanized mouse model for *P. falciparum* infection (Ureta-Vidal et al., 1999). Importantly, the data from this thesis provide the first evidence that *P. falciparum*-specific T cell responses can be induced in a mouse model. In the HLA-A2 transgenic model, the generation of *P. falciparum*-specific CTL was dependent on the spleen, as shown before for mouse model of PbA infection (Figure 22).

Induction of IFNγ depends on IL-12 and represents one of the most important biological activities of IL-12 that is linked to induction of a T_H1 response (Hsieh et al., 1993; Kobayashi et al., 1989; Macatonia et al., 1995; Stern et al., 1990). Major producers of IFNγ are activated T cells, but also NK cells and DCs. To investigate whether IL-12 was indeed involved in ECM induction, we performed experiments in mice deficient for IL-12p35. Interestingly, IL-12p35 deficient mice were protected against development of ECM following PbA infection (Figure 17), which indicated that the detection of parasites triggered effector mechanisms in an IL-12p35 dependent manner that finally led to immuno-pathological complications.

Although the mechanism how inflammatory cytokines promote ECM is currently unknown, it is speculated that the induction of IFNγ triggers effector mechanisms destabilizing the integrity of the blood-brain barrier. The resulting imbalance between neuroprotective and neurotoxic mediators is considered to be the cause of the subsequent brain damage (Medana et al., 2003; Medana et al., 2002). Catabolites of the tryptophan metabolism, kynurenic acid and quinolinic acid, are among those neurotoxic catabolites (Clark et al., 2005; Hunt et al., 2006; Sanni et al., 1998). The kynurenine pathway is activated by IFNγ and generates neurotoxic and neuroprotective metabolites (Alberati-Giani et al., 1996). Indoleamine 2,3 dioxygenase (IDO) is a key enzyme mediating tryptophan degradation, which can be blocked with 1-methyl tryptophan (1-MT). Preliminary data from our laboratory and work from others indicate that blockage of IDO by oral application of 1-MT can protect PbA infected mice against ECM (data not shown and (Clark et al., 2005). The authors observed that a shift of the ratio between the neurotoxic metabolite quinolinic acid and the neuroprotective metabolite kynurenic acid correlated with the protection from ECM induction (Clark et al., 2005).

Taken together, our data suggest that during *Plasmodium* infection TLRs trigger a cascade of innate and adaptive immune mechanisms culminating in immune-mediated tissue damage.

8.2.2 *Plasmodium* itself inhibits detrimental effector mechanisms upon infection

A key fining of this thesis was the observation that PbA itself was able to prevent detrimental effector responses. Whereas in other infections an increased dose of pathogens accelerates disease progess, here, wild type (WT) mice, that received higher doses of PbA (PbA^{high}), survived PbA infection almost completely, whereas all mice died that were infected with low dose PbA (Figure 23). Our results show that TLR2, TLR3 and TLR9 were involved in mediating the protection from ECM and that induction of this protection was completely dependent on IL-12/IL-23p40 (Figure 23, Figure 24). In addition, mice deficient in IL-10 or iNOS were significantly less well protected against ECM upon infection with PbA^{high} (Figure 25). A possible protective role for IL-10 and iNOS in prevention of ECM pathogenesis was reported by others (Kossodo et al., 1997); (Gramaglia et al., 2006). Both IL-10 and iNOS can be induced by IL-12 due to induction of a negative feedback mechanism. Protection against ECM is assumed to result from antagonizing pro-inflammatory mediators and inhibition of neurotoxic mediators (IDO) supporting a stabilized integrity of the blood brain barrier.

Our data suggest that a subsequent induction of anti-inflammatory regulatory mediators are likely to be responsible for protection as seen in a recent study from Mitchell et al. (Mitchell et al., 2005). There, co-infection of C57BL/6 mice with *P. berghei ANKA* and *P. berghei K173* prevented ECM–related death. The authors determined increased levels of pro-inflammatory cytokines including IL-12 and IFNγ one day after co-infection and attributed this to the increased survival of the mice (Mitchell et al., 2005). Excessive production of pro-inflammatory mediators can activate negative feed-back mechanisms such as IL-10 to dampen inflammation. IL-10 is a potent inhibitor of IL-12 production by blocking transcription of both encoding genes (Aste-Amezaga et al., 1998). The important role of IL-10 in immune regulation has been shown by uncontrolled, lethal systemic inflammation to various microorganisms in IL-10 deficient mice (Gazzinelli et al., 1996).

8.2.3 TLR9 ligand CpG prevented effector mechanisms in *Plasmodium* infection through induction of DC paralysis and T cell suppression

Protection from ECM induction following high dose PbA infection was partially absent in TLR9 deficient mice (Figure 23), which emphasizes the impact of TLR-mediated regulation of detrimental inflammatory responses. The requirements for the induction of CTL responses were similar in *Plasmodium ssp*. infection and AdOVA infection with respect to the spleen. In both infection models, the induction of CTL responses required the spleen and DCs. As in the AdOVA infection model adaptive effector responses i.e. antigen-specific CTLs -were inhibited upon systemic application with synthetic TLR9 ligand (in a TLR9 dependent manner), we initially hypothesized that the generation of detrimental effector immune responses - i.e. inflammatory cytokines and CTLs - culminating in ECM following PbA infection, could be inhibited by systemic application of TLR9 ligand CpG.

Indeed, PbA-infected mice were protected against ECM if CpG was injected one day prior to or one day after PbA infection (Figure 18, Figure 20A). DCs are primary targets of TLR ligation (Reis e Sousa, 2006). Our data indicate a key role for these antigen-presenting cells in TLR ligand-induced inhibition of immune responses. A single systemic application of CpG led to the maturation of splenic DCs, which was characterized by an immediate production of IL-12 and increased expression of co-stimulatory molecules (Figure 6). In contrast, repeated intravenous injections of mice with CpG induced a refractory state in splenic DCs as these cells were unable to produce IL-12, TNFα and IFNγ (Figure 7). Reduced levels of IL-12 in the serum reflected a systemic down-regulation of inflammation (Figure 8D). Similar findings were described in 1999 by Reis e Sousa and coworkers in a murine model of *Toxoplasma gondii* infection. (Reis e Sousa et al., 1999). The inability of DCs to sustain IL-12 production after repeated microbial stimulation *in vivo* was termed "immune paralysis" of DCs and was shown to confer protection from IFNγ dependent immune damage. In our experiments the DC paralysis protected PbA infected mice from immune damage (ECM) and lasted for up to seven days and was again in line with the results from Reis e Sousa (Reis e Sousa et al., 1999). Pathogen-induced counter-regulation of IL-12 expression is not restricted to malaria or toxoplasmosis, as many reports exist, which demonstrate that control of IL-12 production can prevent tissue damage caused by excessive inflammation. In addition to *Toxoplasma gondii,* active induction of antigen presenting cell paralysis and IL-12 suppression mediated via viral pathogens is described for cytomegalovirus, Influenza virus or Measles virus (Andrews et al., 2001; Karp et al., 1996; Noone et al., 2005; Oldstone et al., 1999; Schneider-Schaulies et al., 2001; Slifka et al., 2003).

Taken together, as DC-depleted as well as IL-12p35 deficient mice were protected against ECM upon PbA infection, we hypothesize that CpG-mediated protection resulted from the inability of DCs to produce IL-12 during PbA infection. TLR9 ligand CpG therefore induced not only a refractory state in DCs but inhibited PbA-specific cytotoxic T cell responses. This was strongly supported by the finding that TLR9 ligand CpG treatment caused a suppression of *Plasmodium*-specific CTL responses in HLA-A2 transgenic mice, a humanized model of *P. falciparum* infection (Figure 22). This is the first description of TLR ligand mediated inhibition of *P. falciparum*-specific CTL responses and emphasizes the relevance of TLRs in immune regulation of adaptive immune responses to *Plasmodium* infections.

Overall, our results provide strong evidence for prevention of *Plasmodium*-induced inflammatory processes by systemic application of synthetic TLR ligands. Future therapeutic application of TLR ligands during Plasmodium infection requires a decisive staging of disease course and clinical progress of *Plasmodium* infection in humans. Our data show that TLR application in mice proximately to ECM onset harbors the risk of acceleration of inflammatory responses, thus therapeutic intervention aiming at stabilizing the blood-brain-barrier by regulation the balance between neurotoxic and neuroprotective factors may prevent progress in infection. The induction of an anti-inflammatory status in the infected host might offer a promising strategy for future therapeutic interventions.

8.3 Cellular refractoriness caused by TLR ligands

The TLR ligand mediated immune suppression to virally expressed antigens was observed in a mouse model and is similar to a well-known phenomenon in patients that survived septic shock. These patients often suffer from insufficient protection against subsequent infections due to induction of immune paralysis characterized by lack of immune responses (Volk et al., 1991). This form of 'immunological paralysis' is manifested by suppressed monocyte function, including low levels of MHC class II expression, reduced capacity for antigen presentation, and suppressed cytokine production (Munoz et al., 1991; Volk et al., 1996; Volk et al., 1991).

Our observation regarding TLR-ligand-mediated immune suppression raise the question whether this negative effect of TLRs can be compared to the so-called "LPS tolerance" that is observed during sepsis (Karp et al.; 1998). Suppression of IL-12, loss of DCs and macrophage hypo-responsiveness are well-described symptoms of experimental endotoxin tolerance (Karp et al., 1998; Wysocka et al., 2001). Some studies attribute hypo-responsiveness following repeated LPS challenge to down-regulated expression and trapping of the receptor TLR4 to the Golgi apparatus (Hornef et al., 2002).

As TLR 7 and TLR9 are intracellular receptors, an altered receptor expression upon repeated stimulation or retaining the receptors in different stages of the endosome/lysosome remain possibilities that would require extensive analysis. Recent studies suggest induction of several negative regulators to disturb/inhibit cellular responses to TLRs (Liew et al., 2005). These include soluble decoy receptors, but also downstream of the receptors several intracellular negative regulators may be involved. It remains to be determined whether specific negative regulators are activated by ligands for TLR9 or TLR7.

8.4 Conclusion and outlook

Our findings support the importance of TLRs in the regulation of immune responses towards infections. The data from this thesis provide strong evidence that suppression of T cell responses by systemic application of TLR ligands and pathogens is the result of interference in DC-T cell interaction. In particular, all TLR ligands examined in this thesis were able to suppress CTL responses if they were applied systemically in high doses prior to antigen application. We suggest that the induction of immune suppression represents a type of "emergency shutdown" that is induced when circumstances of TLR activation or infection represent a serious danger by excessive inflammation.

We conclude that TLR mediated CTL suppression cannot be attributed to a simple molecular switch but rather is the result of multiple factors that interfere with CTL priming.

Our data suggest that pathogens such as *Plasmodium ssp*. may benefit from the mechanisms of immune suppression - evolved and dedicated for the host as mechanism of protection - as a possibility

of immune evasion or silencing. Prevention of detrimental host responses is favorable for *Plasmodium*, as this parasite has to complete its life cycle.

We hypothesize that TLR dependent induction of immune suppression by recognition of endogenous ligands represents an ongoing physiological mechanism to prevent potentially undesired or even harmful immune response.

9 References

Ahmed, R., Butler, L. D., and Bhatti, L. (1988). T4+ T helper cell function in vivo: differential requirement for induction of antiviral cytotoxic T-cell and antibody responses. J Virol *62*, 2102-2106.

Airoldi, I., Gri, G., Marshall, J. D., Corcione, A., Facchetti, P., Guglielmino, R., Trinchieri, G., and Pistoia, V. (2000). Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells. J Immunol *165*, 6880-6888.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat Rev Immunol *4*, 499-511.

Akira, S., Takeda, K., and Kaisho, T. (2001). Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol *2*, 675-680.

Alberati-Giani, D., Ricciardi-Castagnoli, P., Kohler, C., and Cesura, A. M. (1996). Regulation of the kynurenine metabolic pathway by interferon-gamma in murine cloned macrophages and microglial cells. J Neurochem *66*, 996-1004.

Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature *413*, 732-738.

Amani, V., Boubou, M. I., Pied, S., Marussig, M., Walliker, D., Mazier, D., and Renia, L. (1998). Cloned lines of Plasmodium berghei ANKA differ in their abilities to induce experimental cerebral malaria. Infect Immun *66*, 4093-4099.

Anderson, K. V., Bokla, L., and Nusslein-Volhard, C. (1985a). Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. Cell *42*, 791-798.

Anderson, K. V., Jurgens, G., and Nusslein-Volhard, C. (1985b). Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. Cell *42*, 779-789.

Andrews, D. M., Andoniou, C. E., Granucci, F., Ricciardi-Castagnoli, P., and Degli-Esposti, M. A. (2001). Infection of dendritic cells by murine cytomegalovirus induces functional paralysis. Nat Immunol *2*, 1077-1084.

Aste-Amezaga, M., Ma, X., Sartori, A., and Trinchieri, G. (1998). Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. J Immunol *160*, 5936-5944.

Bachmann, M. F., and Kopf, M. (2002). Balancing protective immunity and immunopathology. Curr Opin Immunol *14*, 413-419.

Badovinac, V. P., Porter, B. B., and Harty, J. T. (2002). Programmed contraction of CD8(+) T cells after infection. Nat Immunol *3*, 619-626.

Bahl, K., Kim, S. K., Calcagno, C., Ghersi, D., Puzone, R., Celada, F., Selin, L. K., and Welsh, R. M. (2006). IFN-induced attrition of CD8 T cells in the presence or absence of cognate antigen during the early stages of viral infections. J Immunol *176*, 4284-4295.

Barnden, M. J., Allison, J., Heath, W. R., and Carbone, F. R. (1998). Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol *76*, 34-40.

Behrens, G., Li, M., Smith, C. M., Belz, G. T., Mintern, J., Carbone, F. R., and Heath, W. R. (2004). Helper T cells, dendritic cells and CTL Immunity. Immunol Cell Biol *82*, 84-90.

Belnoue, E., Kayibanda, M., Vigario, A. M., Deschemin, J. C., van Rooijen, N., Viguier, M., Snounou, G., and Renia, L. (2002). On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria. J Immunol *169*, 6369-6375.

Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and Heath, W. R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*, 478-480.

Bevan, M. J. (1976). Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med *143*, 1283-1288.

Bevan, M. J. (2004). Helping the CD8(+) T-cell response. Nat Rev Immunol *4*, 595-602.

Billiau, A. (2006). Anti-inflammatory properties of Type I interferons. Antiviral Res *71*, 108-116.

Blander, J. M., and Medzhitov, R. (2004). Regulation of phagosome maturation by signals from toll-like receptors. Science *304*, 1014-1018.

Boubou, M. I., Collette, A., Voegtle, D., Mazier, D., Cazenave, P. A., and Pied, S. (1999). T cell response in malaria pathogenesis: selective increase in T cells carrying the TCR V(beta)8 during experimental cerebral malaria. Int Immunol *11*, 1553-1562.

Bowie, A., and O'Neill, L. A. (2000). The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. J Leukoc Biol *67*, 508-514.

Cardin, R. D., Brooks, J. W., Sarawar, S. R., and Doherty, P. C. (1996). Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. J Exp Med *184*, 863-871.

Castellino, F., and Germain, R. N. (2006). Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu Rev Immunol *24*, 519-540.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med *184*, 747-752.

Clark, C. J., Mackay, G. M., Smythe, G. A., Bustamante, S., Stone, T. W., and Phillips, R. S. (2005). Prolonged survival of a murine model of cerebral malaria by kynurenine pathway inhibition. Infect Immun *73*, 5249-5251.

Coban, C., Ishii, K. J., Kawai, T., Hemmi, H., Sato, S., Uematsu, S., Yamamoto, M., Takeuchi, O., Itagaki, S., Kumar, N*., et al.* (2005). Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med *201*, 19-25.

Coban, C., Ishii, K. J., Uematsu, S., Arisue, N., Sato, S., Yamamoto, M., Kawai, T., Takeuchi, O., Hisaeda, H., Horii, T., and Akira, S. (2007). Pathological role of Toll-like receptor signaling in cerebral malaria. Int Immunol *19*, 67-79.

Cooper, A. M., and Khader, S. A. (2007). IL-12p40: an inherently agonistic cytokine. Trends Immunol *28*, 33-38.

Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C., and Mathis, D. (1991). Mice lacking MHC class II molecules. Cell *66*, 1051-1066.

Cousens, L. P., Peterson, R., Hsu, S., Dorner, A., Altman, J. D., Ahmed, R., and Biron, C. A. (1999). Two roads diverged: interferon alpha/beta- and interleukin 12-mediated pathways in promoting T cell interferon gamma responses during viral infection. J Exp Med *189*, 1315-1328.

Curtsinger, J. M., Lins, D. C., and Mescher, M. F. (2003). Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J Exp Med *197*, 1141-1151.

Curtsinger, J. M., Valenzuela, J. O., Agarwal, P., Lins, D., and Mescher, M. F. (2005). Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol *174*, 4465-4469.

D'Andrea, A., Rengaraju, M., Valiante, N. M., Chehimi, J., Kubin, M., Aste, M., Chan, S. H., Kobayashi, M., Young, D., Nickbarg, E., and et al. (1992). Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J Exp Med *176*, 1387-1398.

deWalick, S., Amante, F. H., McSweeney, K. A., Randall, L. M., Stanley, A. C., Haque, A., Kuns, R. D., MacDonald, K. P., Hill, G. R., and Engwerda, C. R. (2007). Cutting Edge: conventional dendritic cells are the critical APC required for the induction of experimental cerebral malaria. J Immunol *178*, 6033-6037.

Diebold, S. S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science *303*, 1529-1531.

Dikopoulos, N., Bertoletti, A., Kroger, A., Hauser, H., Schirmbeck, R., and Reimann, J. (2005). Type I IFN negatively regulates CD8+ T cell responses through IL-10-producing CD4+ T regulatory 1 cells. J Immunol *174*, 99-109.

Dondorp, A. M., Kager, P. A., Vreeken, J., and White, N. J. (2000). Abnormal blood flow and red blood cell deformability in severe malaria. Parasitol Today *16*, 228-232.

Doyle, S. L., and O'Neill, L. A. (2006). Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. Biochem Pharmacol *72*, 1102-1113.

Dutton, R. W. (1967). In vitro studies of immunological responses of lymphoid cells. Adv Immunol *6*, 253-336.

Engwerda, C., Belnoue, E., Gruner, A. C., and Renia, L. (2005a). Experimental models of cerebral malaria. Curr Top Microbiol Immunol *297*, 103-143.

Engwerda, C. R., Beattie, L., and Amante, F. H. (2005b). The importance of the spleen in malaria. Trends Parasitol *21*, 75-80.

Finley, R. W., Mackey, L. J., and Lambert, P. H. (1982). Virulent P. berghei malaria: prolonged survival and decreased cerebral pathology in cell-dependent nude mice. J Immunol *129*, 2213-2218.

Flandin, J. F., Chano, F., and Descoteaux, A. (2006). RNA interference reveals a role for TLR2 and TLR3 in the recognition of Leishmania donovani promastigotes by interferon-gamma-primed macrophages. Eur J Immunol *36*, 411-420.

Gays, F., Unnikrishnan, M., Shrestha, S., Fraser, K. P., Brown, A. R., Tristram, C. M., Chrzanowska-Lightowlers, Z. M., and Brooks, C. G. (2000). The mouse tumor cell lines EL4 and RMA display mosaic expression of NK-related and certain other surface molecules and appear to have a common origin. J Immunol *164*, 5094-5102.

Gazzinelli, R. T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G., and Sher, A. (1996). In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol *157*, 798-805.

Gerosa, F., Paganin, C., Peritt, D., Paiola, F., Scupoli, M. T., Aste-Amezaga, M., Frank, I., and Trinchieri, G. (1996). Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. J Exp Med *183*, 2559-2569.

Glenister, F. K., Coppel, R. L., Cowman, A. F., Mohandas, N., and Cooke, B. M. (2002). Contribution of parasite proteins to altered mechanical properties of malaria-infected red blood cells. Blood *99*, 1060-1063.

Gramaglia, I., Sobolewski, P., Meays, D., Contreras, R., Nolan, J. P., Frangos, J. A., Intaglietta, M., and van der Heyde, H. C. (2006). Low nitric oxide bioavailability contributes to the genesis of experimental cerebral malaria. Nat Med *12*, 1417-1422.

Grau, G. E., Piguet, P. F., Engers, H. D., Louis, J. A., Vassalli, P., and Lambert, P. H. (1986). L3T4+ T lymphocytes play a major role in the pathogenesis of murine cerebral malaria. J Immunol *137*, 2348-2354.

Greenberg, J., and Kendrick, L. P. (1957a). Parasitemia and survival in inbred strains of mice infected with Plasmodium berghei. J Parasitol *43*, 413-419.

Greenberg, J., and Kendrick, L. P. (1957b). Some characteristics of Plasmodium berghei passed within inbred strains of mice. J Parasitol *43*, 420-427.

Grohmann, U., Belladonna, M. L., Bianchi, R., Orabona, C., Ayroldi, E., Fioretti, M. C., and Puccetti, P. (1998). IL-12 acts directly on DC to promote nuclear localization of NF-kappaB and primes DC for IL-12 production. Immunity *9*, 315-323.

Grusby, M. J., Johnson, R. S., Papaioannou, V. E., and Glimcher, L. H. (1991). Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. Science *253*, 1417-1420.

Harui, A., Roth, M. D., Kiertscher, S. M., Mitani, K., and Basak, S. K. (2004). Vaccination with helper-dependent adenovirus enhances the generation of transgene-specific CTL. Gene Ther *11*, 1617-1626.

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature *410*, 1099-1103.

Heil, F., Ahmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., Dietrich, H., Lipford, G., Takeda, K., Akira, S*., et al.* (2003). The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur J Immunol *33*, 2987-2997.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science *303*, 1526-1529.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., and Akira, S. (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol *3*, 196-200.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740-745.

Hermsen, C., van de Wiel, T., Mommers, E., Sauerwein, R., and Eling, W. (1997). Depletion of CD4+ or CD8+ T-cells prevents Plasmodium berghei induced cerebral malaria in end-stage disease. Parasitology *114 (Pt 1)*, 7-12.

Hermsen, C. C., Mommers, E., van de Wiel, T., Sauerwein, R. W., and Eling, W. M. (1998). Convulsions due to increased permeability of the blood-brain barrier in experimental cerebral malaria can be prevented by splenectomy or anti-T cell treatment. J Infect Dis *178*, 1225-1227.

Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J., and Carbone, F. R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17-27.

Hornef, M. W., Frisan, T., Vandewalle, A., Normark, S., and Richter-Dahlfors, A. (2002). Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. J Exp Med *195*, 559-570.

Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A.*, et al.* (2005). Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med *11*, 263-270.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol *162*, 3749-3752.

Hron, J. D., and Peng, S. L. (2004). Type I IFN protects against murine lupus. J Immunol *173*, 2134-2142.

Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., and Murphy, K. M. (1993). Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. Science *260*, 547-549.

Huang, F. P., Feng, G. J., Lindop, G., Stott, D. I., and Liew, F. Y. (1996). The role of interleukin 12 and nitric oxide in the development of spontaneous autoimmune disease in MRL/MP-lpr/lpr mice. J Exp Med *183*, 1447-1459.

Hunt, N. H., Golenser, J., Chan-Ling, T., Parekh, S., Rae, C., Potter, S., Medana, I. M., Miu, J., and Ball, H. J. (2006). Immunopathogenesis of cerebral malaria. Int J Parasitol *36*, 569-582.

Hunt, N. H., and Grau, G. E. (2003). Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends Immunol *24*, 491-499.

Janeway, C. A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol *54 Pt 1*, 1-13.

Janeway, C. A., Jr., and Bottomly, K. (1994). Signals and signs for lymphocyte responses. Cell *76*, 275-285.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F.*, et al.* (2002). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F., and Liu, Y. J. (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J Exp Med *194*, 863-869.

Kaech, S. M., Hemby, S., Kersh, E., and Ahmed, R. (2002). Molecular and functional profiling of memory CD8 T cell differentiation. Cell *111*, 837-851.

Kamiyama, T., Tatsumi, M., Matsubara, J., Yamamoto, K., Rubio, Z., Cortes, G., and Fujii, H. (1987). Manifestation of cerebral malaria-like symptoms in the WM/Ms rat infected with Plasmodium berghei strain NK65. J Parasitol *73*, 1138-1145.

Karp, C. L., Wysocka, M., Ma, X., Marovich, M., Factor, R. E., Nutman, T., Armant, M., Wahl, L., Cuomo, P., and Trinchieri, G. (1998). Potent suppression of IL-12 production from monocytes and dendritic cells during endotoxin tolerance. Eur J Immunol *28*, 3128-3136.

Karp, C. L., Wysocka, M., Wahl, L. M., Ahearn, J. M., Cuomo, P. J., Sherry, B., Trinchieri, G., and Griffin, D. E. (1996). Mechanism of suppression of cell-mediated immunity by measles virus. Science *273*, 228-231.

Kast, W. M., Bronkhorst, A. M., de Waal, L. P., and Melief, C. J. (1986). Cooperation between cytotoxic and helper T lymphocytes in protection against lethal Sendai virus infection. Protection by T cells is MHC-restricted and MHC-regulated; a model for MHC-disease associations. J Exp Med *164*, 723-738.

Katakura, K., Lee, J., Rachmilewitz, D., Li, G., Eckmann, L., and Raz, E. (2005). Toll-like receptor 9-induced type I IFN protects mice from experimental colitis. J Clin Invest *115*, 695-702.

Kawai, T., and Akira, S. (2005). Pathogen recognition with Toll-like receptors. Curr Opin Immunol *17*, 338-344.

Kichler, A., Remy, J. S., Boussif, O., Frisch, B., Boeckler, C., Behr, J. P., and Schuber, F. (1995). Efficient gene delivery with neutral complexes of lipospermine and thiol-reactive phospholipids. Biochem Biophys Res Commun *209*, 444-450.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B., and Trinchieri, G. (1989). Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med *170*, 827-845.

Koblish, H. K., Hunter, C. A., Wysocka, M., Trinchieri, G., and Lee, W. M. (1998). Immune suppression by recombinant interleukin (rIL)-12 involves interferon gamma induction of nitric oxide synthase 2 (iNOS) activity: inhibitors of NO generation reveal the extent of rIL-12 vaccine adjuvant effect. J Exp Med *188*, 1603-1610.

Kossodo, S., Monso, C., Juillard, P., Velu, T., Goldman, M., and Grau, G. E. (1997). Interleukin-10 modulates susceptibility in experimental cerebral malaria. Immunology *91*, 536-540.

Krieg, A. M. (2006). Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov *5*, 471-484.

Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature *374*, 546-549.

Krishnegowda, G., Hajjar, A. M., Zhu, J., Douglass, E. J., Uematsu, S., Akira, S., Woods, A. S., and Gowda, D. C. (2005). Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. J Biol Chem *280*, 8606-8616.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263-274.

Lehner, M., Felzmann, T., Clodi, K., and Holter, W. (2001). Type I interferons in combination with bacterial stimuli induce apoptosis of monocyte-derived dendritic cells. Blood *98*, 736-742.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973-983.

Lepenies, B., Cramer, J. P., Burchard, G. D., Wagner, H., Kirschning, C. J., and Jacobs, T. (2007). Induction of experimental cerebral malaria is independent of TLR2/4/9. Med Microbiol Immunol.

Li, K., Chen, Z., Kato, N., Gale, M., Jr., and Lemon, S. M. (2005). Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J Biol Chem *280*, 16739-16747.

Lien, E., Means, T. K., Heine, H., Yoshimura, A., Kusumoto, S., Fukase, K., Fenton, M. J., Oikawa, M., Qureshi, N., Monks, B.*, et al.* (2000). Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J Clin Invest *105*, 497-504.

Liew, F. Y., Xu, D., Brint, E. K., and O'Neill, L. A. (2005). Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol *5*, 446-458.

Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1990). Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. Oncogene *5*, 1095-1097.

Lu, P. Y., Xie, F., and Woodle, M. C. (2005). In vivo application of RNA interference: from functional genomics to therapeutics. Adv Genet *54*, 117-142.

Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P., Hsieh, C. S., Culpepper, J. A., Wysocka, M., Trinchieri, G., Murphy, K. M., and O'Garra, A. (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol *154*, 5071-5079.

Marrack, P., Kappler, J., and Mitchell, T. (1999). Type I interferons keep activated T cells alive. J Exp Med *189*, 521-530.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu Rev Immunol *12*, 991-1045.

Matzinger, P. (2002). The danger model: a renewed sense of self. Science *296*, 301-305.

Mebius, R. E., and Kraal, G. (2005). Structure and function of the spleen. Nat Rev Immunol *5*, 606-616.

Medana, I. M., Day, N. P., Salahifar-Sabet, H., Stocker, R., Smythe, G., Bwanaisa, L., Njobvu, A., Kayira, K., Turner, G. D., Taylor, T. E., and Hunt, N. H. (2003). Metabolites of the kynurenine pathway of tryptophan metabolism in the cerebrospinal fluid of Malawian children with malaria. J Infect Dis *188*, 844-849.

Medana, I. M., Hien, T. T., Day, N. P., Phu, N. H., Mai, N. T., Chu'ong, L. V., Chau, T. T., Taylor, A., Salahifar, H., Stocker, R.*, et al.* (2002). The clinical significance of cerebrospinal fluid levels of kynurenine pathway metabolites and lactate in severe malaria. J Infect Dis *185*, 650-656.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. Nat Rev Immunol *1*, 135-145.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell *2*, 253-258.

Mescher, M. F., Agarwal, P., Casey, K. A., Hammerbeck, C. D., Xiao, Z., and Curtsinger, J. M. (2007). Molecular basis for checkpoints in the CD8 T cell response: tolerance versus activation. Semin Immunol *19*, 153-161.

Meyvisch, C., and Mareel, M. (1982). Influence of implantation site of MO4 cell aggregates on the formation of metastases. Invasion Metastasis *2*, 51-60.

Mitchell, A. J., Hansen, A. M., Hee, L., Ball, H. J., Potter, S. M., Walker, J. C., and Hunt, N. H. (2005). Early cytokine production is associated with protection from murine cerebral malaria. Infect Immun *73*, 5645-5653.

Muller, A. J., and Scherle, P. A. (2006). Targeting the mechanisms of tumoral immune tolerance with small-molecule inhibitors. Nat Rev Cancer *6*, 613-625.

Munoz, C., Carlet, J., Fitting, C., Misset, B., Bleriot, J. P., and Cavaillon, J. M. (1991). Dysregulation of in vitro cytokine production by monocytes during sepsis. J Clin Invest *88*, 1747-1754.

Muzio, M., Ni, J., Feng, P., and Dixit, V. M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science *278*, 1612-1615.

Nitcheu, J., Bonduelle, O., Combadiere, C., Tefit, M., Seilhean, D., Mazier, D., and Combadiere, B. (2003). Perforin-dependent brain-infiltrating cytotoxic CD8+ T lymphocytes mediate experimental cerebral malaria pathogenesis. J Immunol *170*, 2221-2228.

Noone, C. M., Lewis, E. A., Frawely, A. B., Newman, R. W., Mahon, B. P., Mills, K. H., and Johnson, P. A. (2005). Novel mechanism of immunosuppression by influenza virus haemagglutinin: selective suppression of interleukin 12 p35 transcription in murine bone marrow-derived dendritic cells. J Gen Virol *86*, 1885-1890.

Noronha, A., Toscas, A., and Jensen, M. A. (1993). Interferon beta decreases T cell activation and interferon gamma production in multiple sclerosis. J Neuroimmunol *46*, 145-153.

O'Neill, L. A., and Bowie, A. G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol *7*, 353-364.

Ohashi, K., Burkart, V., Flohe, S., and Kolb, H. (2000). Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. J Immunol *164*, 558-561.

Okamura, Y., Watari, M., Jerud, E. S., Young, D. W., Ishizaka, S. T., Rose, J., Chow, J. C., and Strauss, J. F., 3rd (2001). The extra domain A of fibronectin activates Toll-like receptor 4. J Biol Chem *276*, 10229-10233.

Oldstone, M. B., Lewicki, H., Thomas, D., Tishon, A., Dales, S., Patterson, J., Manchester, M., Homann, D., Naniche, D., and Holz, A. (1999). Measles virus infection in a transgenic model: virus-induced immunosuppression and central nervous system disease. Cell *98*, 629-640.

Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K.*, et al.* (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity *13*, 715-725.

Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc Natl Acad Sci U S A *97*, 13766-13771.

Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., Chora, A., Rodrigues, C. D., Gregoire, I. P., Cunha-Rodrigues, M., *et al.* (2007). Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat Med.

Parham, C., Chirica, M., Timans, J., Vaisberg, E., Travis, M., Cheung, J., Pflanz, S., Zhang, R., Singh, K. P., Vega, F., *et al.* (2002). A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. J Immunol *168*, 5699-5708.

Parroche, P., Lauw, F. N., Goutagny, N., Latz, E., Monks, B. G., Visintin, A., Halmen, K. A., Lamphier, M., Olivier, M., Bartholomeu, D. C., *et al.* (2007). Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. Proc Natl Acad Sci U S A *104*, 1919-1924.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., *et al.* (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science *282*, 2085-2088.

Potter, S., Chaudhri, G., Hansen, A., and Hunt, N. H. (1999). Fas and perforin contribute to the pathogenesis of murine cerebral malaria. Redox Rep *4*, 333-335.

Presky, D. H., Yang, H., Minetti, L. J., Chua, A. O., Nabavi, N., Wu, C. Y., Gately, M. K., and Gubler, U. (1996). A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. Proc Natl Acad Sci U S A *93*, 14002-14007.

Rahemtulla, A., Fung-Leung, W. P., Schilham, M. W., Kundig, T. M., Sambhara, S. R., Narendran, A., Arabian, A., Wakeham, A., Paige, C. J., Zinkernagel, R. M., and et al. (1991). Normal development and function of CD8+ cells but markedly decreased helper cell activity in mice lacking CD4. Nature *353*, 180-184.

Reis e Sousa, C. (2006). Dendritic cells in a mature age. Nat Rev Immunol *6*, 476-483.

Reis e Sousa, C., Yap, G., Schulz, O., Rogers, N., Schito, M., Aliberti, J., Hieny, S., and Sher, A. (1999). Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology. Immunity *11*, 637-647.

Remy, J. S., Kichler, A., Mordvinov, V., Schuber, F., and Behr, J. P. (1995). Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: a stage toward artificial viruses. Proc Natl Acad Sci U S A *92*, 1744-1748.

Rep, M. H., Schrijver, H. M., van Lopik, T., Hintzen, R. Q., Roos, M. T., Ader, H. J., Polman, C. H., and van Lier, R. A. (1999). Interferon (IFN)-beta treatment enhances CD95 and interleukin 10 expression but reduces interferon-gamma producing T cells in MS patients. J Neuroimmunol *96*, 92-100.

Ridge, J. P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature *393*, 474-478.

Rogge, L., Barberis-Maino, L., Biffi, M., Passini, N., Presky, D. H., Gubler, U., and Sinigaglia, F. (1997). Selective expression of an interleukin-12 receptor component by human T helper 1 cells. J Exp Med *185*, 825-831.

Sanni, L. A., Thomas, S. R., Tattam, B. N., Moore, D. E., Chaudhri, G., Stocker, R., and Hunt, N. H. (1998). Dramatic changes in oxidative tryptophan metabolism along the kynurenine pathway in experimental cerebral and noncerebral malaria. Am J Pathol *152*, 611-619.

Schneider-Schaulies, S., Niewiesk, S., Schneider-Schaulies, J., and ter Meulen, V. (2001). Measles virus induced immunosuppression: targets and effector mechanisms. Curr Mol Med *1*, 163-181.

Schoenberger, S. P., Toes, R. E., van der Voort, E. I., Offringa, R., and Melief, C. J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480-483.

Schofield, L., and Grau, G. E. (2005). Immunological processes in malaria pathogenesis. Nat Rev Immunol *5*, 722-735.

Schweichel, D., Steitz, J., Tormo, D., Gaffal, E., Ferrer, A., Buchs, S., Speuser, P., Limmer, A., and Tuting, T. (2006). Evaluation of DNA vaccination with recombinant adenoviruses using bioluminescence imaging of antigen expression: impact of application routes and delivery with dendritic cells. J Gene Med *8*, 1243-1250.

Slifka, M. K., Homann, D., Tishon, A., Pagarigan, R., and Oldstone, M. B. (2003). Measles virus infection results in suppression of both innate and adaptive immune responses to secondary bacterial infection. J Clin Invest *111*, 805-810.

Sprent, J., and Tough, D. F. (2001). T cell death and memory. Science *293*, 245-248.

Stern, A. S., Podlaski, F. J., Hulmes, J. D., Pan, Y. C., Quinn, P. M., Wolitzky, A. G., Familletti, P. C., Stremlo, D. L., Truitt, T., Chizzonite, R., and et al. (1990). Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc Natl Acad Sci U S A *87*, 6808-6812.

Storme, G., Mareel, M., and De Bruyne, G. (1981). Influence of cells number on directional migration of MO4 cells in vitro. Arch Geschwulstforsch *51*, 45-50.

Szabo, S. J., Dighe, A. S., Gubler, U., and Murphy, K. M. (1997). Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J Exp Med *185*, 817-824.

Takaoka, A., and Yanai, H. (2006). Interferon signalling network in innate defence. Cell Microbiol *8*, 907-922.

Takeda, K., Kaisho, T., and Akira, S. (2003). Toll-like receptors. Annu Rev Immunol *21*, 335-376.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity *11*, 443-451.

Togbe, D., Schofield, L., Grau, G. E., Schnyder, B., Boissay, V., Charron, S., Rose, S., Beutler, B., Quesniaux, V. F., and Ryffel, B. (2007). Murine cerebral malaria development is independent of toll-like receptor signaling. Am J Pathol *170*, 1640-1648.

Touil, T., Fitzgerald, D., Zhang, G. X., Rostami, A., and Gran, B. (2006). Cutting Edge: TLR3 stimulation suppresses experimental autoimmune encephalomyelitis by inducing endogenous IFN-beta. J Immunol *177*, 7505-7509.

Trinchieri, G. (1998). Interleukin-12: a cytokine at the interface of inflammation and immunity. Adv Immunol *70*, 83-243.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol *3*, 133-146.

Unanue, E. R., and Askonas, B. A. (1968). The immune response of mice to antigen in macrophages. Immunology *15*, 287-296.

Ureta-Vidal, A., Firat, H., Perarnau, B., and Lemonnier, F. A. (1999). Phenotypical and functional characterization of the CD8+ T cell repertoire of HLA-A2.1 transgenic, H-2KbnullDbnull double knockout mice. J Immunol *163*, 2555-2560.

Vabulas, R. M., Braedel, S., Hilf, N., Singh-Jasuja, H., Herter, S., Ahmad-Nejad, P., Kirschning, C. J., Da Costa, C., Rammensee, H. G., Wagner, H., and Schild, H. (2002). The endoplasmic reticulum-resident heat shock protein Gp96 activates dendritic cells via the Toll-like receptor 2/4 pathway. J Biol Chem *277*, 20847-20853.

Van Bleek, G. M., and Nathenson, S. G. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. Nature *348*, 213-216.

van den Broek, M. F., Muller, U., Huang, S., Zinkernagel, R. M., and Aguet, M. (1995). Immune defence in mice lacking type I and/or type II interferon receptors. Immunol Rev *148*, 5-18.

van der Heyde, H. C., Nolan, J., Combes, V., Gramaglia, I., and Grau, G. E. (2006). A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction. Trends Parasitol *22*, 503-508.

Vincke, I. H., Bafort, J., and Scheepers-Biva, M. (1966). [Recent observations on the cyclic transmission of Plasmodium berghei]. Ann Soc Belg Med Trop *46*, 327-336.

Volk, H. D., Reinke, P., Krausch, D., Zuckermann, H., Asadullah, K., Muller, J. M., Docke, W. D., and Kox, W. J. (1996). Monocyte deactivation--rationale for a new therapeutic strategy in sepsis. Intensive Care Med *22 Suppl 4*, S474-481.

Volk, H. D., Thieme, M., Heym, S., Docke, W. D., Ruppe, U., Tausch, W., Manger, D., Zuckermann, S., Golosubow, A., Nieter, B., and et al. (1991). Alterations in function and phenotype of monocytes from patients with septic disease--predictive value and new therapeutic strategies. Behring Inst Mitt, 208-215.

Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997). MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity *7*, 837-847.

Wilson, N. S., Behrens, G. M., Lundie, R. J., Smith, C. M., Waithman, J., Young, L., Forehan, S. P., Mount, A., Steptoe, R. J., Shortman, K. D*., et al.* (2006). Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. Nat Immunol *7*, 165-172.

Wysocka, M., Robertson, S., Riemann, H., Caamano, J., Hunter, C., Mackiewicz, A., Montaner, L. J., Trinchieri, G., and Karp, C. L. (2001). IL-12 suppression during experimental endotoxin tolerance: dendritic cell loss and macrophage hyporesponsiveness. J Immunol *166*, 7504-7513.

Xie, F. Y., Woodle, M. C., and Lu, P. Y. (2006). Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. Drug Discov Today *11*, 67-73.

Yanez, D. M., Manning, D. D., Cooley, A. J., Weidanz, W. P., and van der Heyde, H. C. (1996). Participation of lymphocyte subpopulations in the pathogenesis of experimental murine cerebral malaria. J Immunol *157*, 1620-1624.

Yarilina, A., DiCarlo, E., and Ivashkiv, L. B. (2007). Suppression of the effector phase of inflammatory arthritis by double-stranded RNA is mediated by type I IFNs. J Immunol *178*, 2204-2211.

Zhang, X., Deriaud, E., Jiao, X., Braun, D., Leclerc, C., and Lo-Man, R. (2007). Type I interferons protect neonates from acute inflammation through interleukin 10-producing B cells. J Exp Med *204*, 1107-1118.

Zhang, Y., Chirmule, N., Gao, G. P., Qian, R., Croyle, M., Joshi, B., Tazelaar, J., and Wilson, J. M. (2001). Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. Mol Ther *3*, 697-707.

10 Appendices

10.1 Publications and contributions to scientific conferences

Parts of this thesis are published

- G. Wingender*, N. Garbi*, **B. Schumak***, F. Jüngerkes*, E. Endl, D. von Bubnoff, J. Steitz, J. Striegler, G. Moldenhauer, T. Tüting, A. Heit, K. M. Huster, O. Takikawa, S. Akira, D. H. Busch, H. Wagner, G. J. Hämmerling, P. A. Knolle and A. Limmer (2006).
  Systemic application of CpG-rich DNA suppresses adaptive T cell immunity via induction of IDO. *Eur J Immunol* 36, 12-20.

*These authors contributed equally to this work

Contribution to other publications:

- Wingender, G., **B. Schumak**, A. Schurich, J. E. Gessner, E. Endl, A. Limmer & P. A. Knolle (2006).
  Rapid and preferential distribution of blood-borne alphaCD3epsilonAb to the liver is followed by local stimulation of T cells and natural killer T cells. *Immunology* 117 (1); 117-26.

- Limmer, A, J. Ohl, G. Wingender, M. Berg, F. Jüngerkes, **B. Schumak**, D. Djandji, K. Scholz, A. Klevenz, S. Hegenbarth, F. Momburg, G.J. Hämmerling, B. Arnold & P. A. Knolle (2005)
  Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur J Immunol* 35 (10): 2970-81.

Scientific contribution to conferences

Oral and/or poster presentation of data at national and international congresses

2003 Meeting of the German Society of Immunology, Berlin, Germany
2004 12th International Congress of Immunology, Montreal, Canada
2004 Meeting of the Dutch and the German Societies of Immunology, Maastricht, The Netherlands
2005 Meeting of the Scandinavian and the German Societies of Immunology, Kiel
2006 1st Meeting of the European Societies of Immunology, Paris, France
2007 1st World Immune Regulation Meeting in Davos, Switzerland
2007 37th Meeting of the German Society of Immunology, Heidelberg

10.2 Prizes and Awards

2004 Poster prize of the 8th Semester-Meeting of the Bonner Forum Biomedizin Walberberg, Germany

2004 Travel Award of the 12th International Congress of Immunology, Montreal, Canada

2007 Poster Prize of the 1st World Immune Regulation Meeting, Davos, Switzerland

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Highlights

FRONTLINE:

Systemic application of CpG-rich DNA suppresses adaptive T cell immunity via induction of IDO

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CpG-rich oligonucleotides (CpG-ODN) bind to Toll-like receptor 9 (TLR9) and are used as powerful adjuvants for vaccination. Here we report that CpG-ODN not only act as immune stimulatory agents but can also induce strong immune suppression depending on the anatomical location of application. In agreement with the adjuvant effect, subcutaneous application of antigen plus CpG-ODN resulted in antigen-specific T cell activation in local lymph nodes. In contrast, systemic application of CpG-ODN resulted in suppression of T cell expansion and CTL activity in the spleen. The suppressive effect was mediated by indoleamine 2,3-dioxygenase (IDO) as indicated by the observation that CpG-ODN induced IDO in the spleen and that T cell suppression could be abrogated by 1-methyl-tryptophan (1-MT), an inhibitor of IDO. No expression of IDO was observed in lymph nodes after injection of CpG-ODN, explaining why suppression was restricted to the spleen. Studies with a set of knockout mice demonstrated that the CpG-ODN-induced immune suppression is dependent on TLR9 stimulation and independent of type I and type II interferons. The present study shows that for the use of CpG-ODN as an adjuvant in vaccines, the route of application is crucial and needs to be considered. In addition, the results indicate that down-modulation of immune responses by CpG-ODN may be possible in certain pathological conditions.

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Abbreviations: **1-MT:** 1-methyl-tryptophan · **AdLucGFP:** recombinant adenovirus expressing luciferase and GFP · **AdOVA:** recombinant adenovirus expressing ovalbumin · **CpG-ODN:** CpG-rich ODN · **IDO:** indoleamine 2,3-dioxygenase · **LmOVA:** recombinant *Listeria monocytogenes* secreting ovalbumin · **ODN:** oligonucleotides

Introduction

The immune system has developed mechanisms for recognition of conserved structures on pathogens [1, 2]. Toll-like receptors (TLR), expressed on various cells of the innate immune system, are well-known examples for such pattern recognition receptors. Activation of cells via TLR ligands leads to the rapid production of proinflammatory mediators, which control infections locally and attract leukocytes to the site of infection [1, 3]. TLR9 is expressed on various cells including B cells, macrophages and DC, and binding of its ligand, CpG-rich oligonucleotides (CpG-ODN), induces strong production of proinflammatory cytokines (*e.g.* IL-1, IL-6, IL-12, type I interferons), chemokines (*e.g.* MCP-1, RANTES, IP10) and costimulatory molecules (*e.g.* CD40, CD80, CD86) [1, 4, 5]. As a result, direct activation of DC by TLR9 signaling is a prerequisite for induction of full T cell function [6]. Activation of DC via TLR thus provides a link between innate and adaptive immunity [4, 5, 7–10]. Therefore, CpG-ODN applied locally has been successfully used as an adjuvant for vaccination in animal models and clinical trials [11–15].

Here we show that in addition to its immune stimulatory effect, CpG-ODN can also be immunosuppressive. Namely, when applied systemically CpG-ODN inhibited T cell responses in the spleen. The suppressive effect was mediated by induction of indoleamine 2,3-dioxygenase (IDO), an enzyme known to suppress T cell expansion. These results indicate that the route of CpG-ODN application is critical for regulating T cell immunity.

Results

Systemic CpG-ODN administration suppresses CTL activity *in vivo*

The synthetic TLR9 ligand CpG-ODN, a model for microbial DNA, is an established adjuvant that augments immune reactions [7, 9, 16]. Accordingly, we observed increased ovalbumin (OVA)-specific CTL activity in the draining lymph node following co-administration of OVA and CpG-ODN s.c. (Fig. 1A). Injected target cells loaded with the OVA-derived peptide SIINFEKL were lysed in the draining lymph node about ten times more efficiently in mice immunized s.c. with OVA plus CpG-ODN compared with mice immunized s.c. with OVA alone. In the spleens of mice immunized s.c. with OVA plus CpG-ODN, we also observed about 40% specific cytotoxicity, suggesting that a number of CTL had migrated from the lymph node to the spleen (data not shown). It has been previously reported that i.v. application of soluble (LPS-depleted) OVA induces CTL activity [17, 18]. Consistent with this, we observed about 30–40% *in vivo* CTL activity in mice injected i.v. with OVA (Fig. 1A). Surprisingly, co-administration of OVA and CpG-ODN i.v. strongly reduced the specific CTL response *in vivo* compared with mice injected i.v. with OVA alone (Fig. 1A).

The contrasting effects of CpG-ODN applied locally or systemically on CTL activity may be explained by the different routes of administration. Therefore, we next investigated the effect of CpG-ODN on the generation of CTL activity in isolated cells from peripheral lymph nodes and spleen, because these represent the main lymphoid organs targeted by s.c. and i.v. application, respectively. For this, we incubated lymph node cells and splenocytes

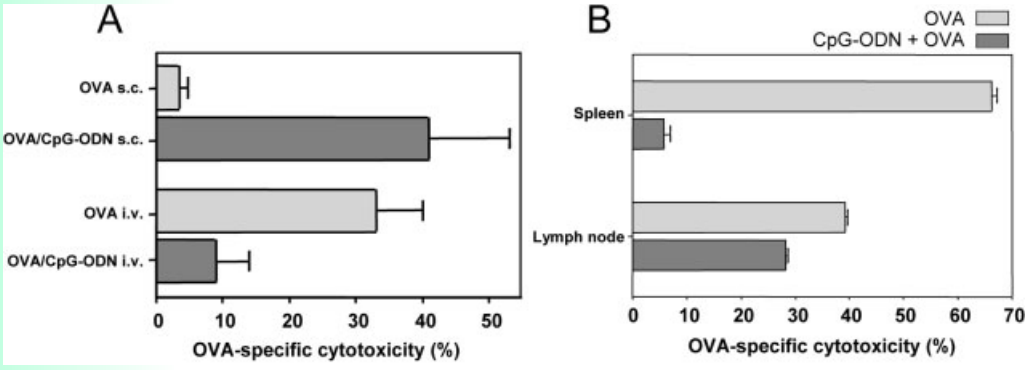

Figure 1. Generation of cytotoxicity is inhibited by systemic application of CpG-ODN. (A) C57BL/6 mice were injected with 100 μg OVA alone or in combination with 50 μg CpG-ODN (either s.c. or i.v.). *In vivo* cytotoxicity was determined 5 days later by quantifying the lysis of target cells loaded with the OVA-derived, H2-K^b-restricted SIINFEKL in the draining lymph node (s.c. immunization) or in the spleen (i.v. immunization) 16 h after target cell transfer into the effector mice. (B) Spleen or lymph node cells from OT-I mice were incubated *in vitro* with 500 μg/mL OVA alone (light grey) or in the presence of 5 μg/mL CpG-ODN (dark grey), and 4 days later *in vitro* cytotoxicity was determined by a standard $^{[51]}$Cr-release assay. Shown is specific cytotoxicity at an E:T ratio of 25:1. Results are expressed as mean ± standard error of the mean (SEM). Representative data are shown.

from OT-I mice, which harbor transgenic OVA-specific $CD8^+$ T cells, with OVA alone or together with CpG-ODN. Development of CTL activity in the spleen was strongly suppressed by CpG-ODN (about 10-fold) (Fig. 1B), in agreement with the reduced CTL activity observed in the spleen after i.v. immunization (Fig. 1A). In contrast, CpG-ODN had only a minor effect on isolated lymph node cells with respect to CTL activity (Fig. 1B), suggesting that organ-resident cells in lymph node and spleen respond differentially to CpG-ODN. Thus, these results indicate that the suppressive effect of systemic CpG-ODN application on CTL generation is mainly due to a negative effect of CpG-ODN in the spleen.

Systemic CpG-ODN application suppresses anti-viral T cell responses in a dose-dependent fashion

Next, we studied whether the inhibitory activity of CpG-ODN on CTL generation also affects virus-specific T cell responses. CpG-ODN were injected i.v. into mice, followed 1 day later by i.v. infection with adenovirus expressing OVA (AdOVA). The generation of CTL specific

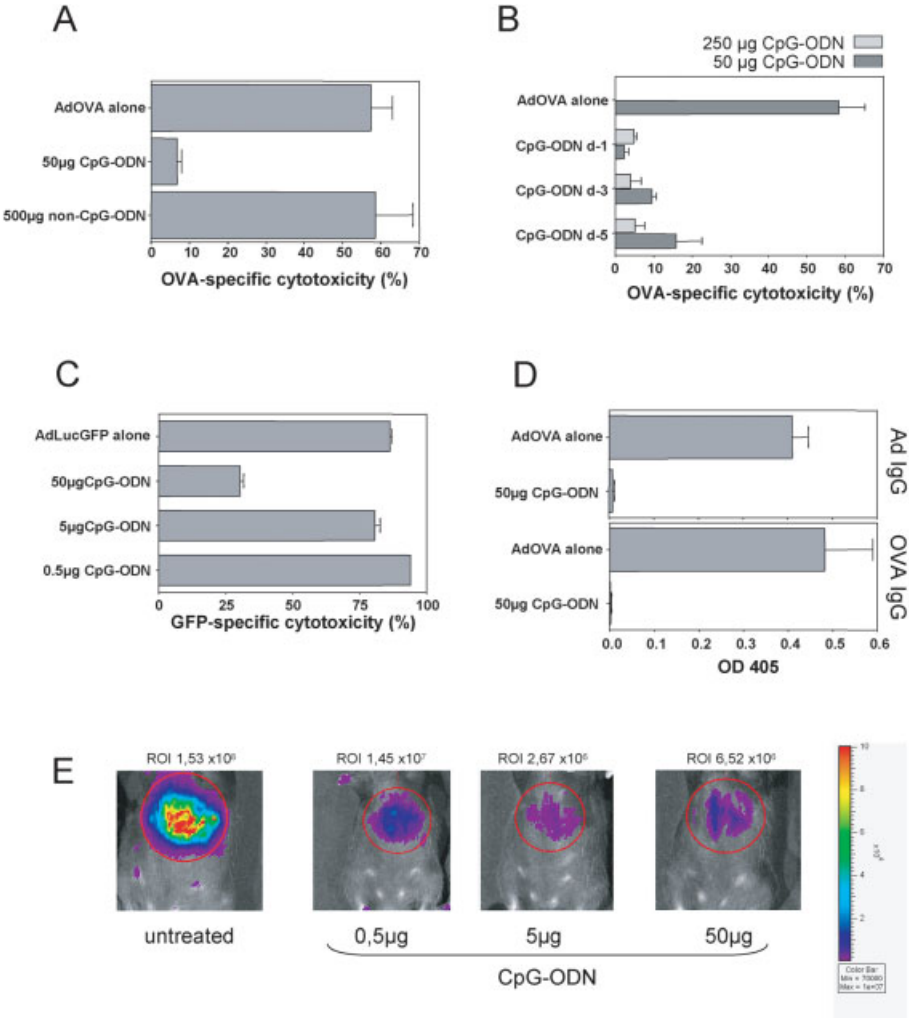

Figure 2. Systemic CpG-ODN inhibits generation of anti-viral T cell responses. (A) CpG-ODN or non-CpG-ODN were injected i.v. into C57BL/6 mice 1 day before AdOVA immunization (1×10^8 pfu i.v.). *In vivo* cytotoxicity in the spleen was quantified 5 days later in a 4-h assay as described in the legend to Fig. 1A. (B) CpG-ODN were injected i.v. into C57BL/6 mice 1 to 5 days before immunizing mice with AdOVA (1×10^8 pfu i.v.). *In vivo* cytotoxicity was analyzed as described in (A). (C) Different doses of CpG-ODN were injected i.v. into C57BL/6 × BALB/c F1 1 day before infection with AdLucGFP (1×10^8 pfu i.v.). *In vivo* cytotoxicity was analyzed 5 days later by quantifying lysis of target cells loaded with GFP-derived, H2-K^d-restricted HYLSTQSAL. (D) IgG titers specific for adenovirus (αAd IgG, top) or OVA (αOVA IgG, bottom) in the plasma of C57BL/6 mice pretreated or not with CpG-ODN i.v. and immunized 1 day later with AdOVA were quantified by ELISA. Shown are serum IgG levels obtained 21 days after immunization. (E) C57BL/6 × BALB/c F1 mice were injected i.v. with the indicated doses of CpG-ODN 1 day before immunization with AdLucGFP. Adenovirus-related bioluminescence was quantified 3 days after immunization to determine viral load in the host. ROI is the region of interest; ROI numbers indicate light intensity in the ROI. Results are expressed as mean ± standard error of the mean (SEM). Representative data are shown.

for OVA (H2-K^b/SIINFEKL) was again strongly suppressed by systemic CpG-ODN application (Fig. 2A). This suppressive effect was specific to CpG motifs, because non-CpG-ODN had no effect on CTL activity (Fig. 2A), and long-lasting, since a single CpG-ODN application at least 5 days before infection with AdOVA inhibited generation of specific CTL activity (Fig. 2B).

We then investigated the dose dependency of the CpG-ODN-induced T cell suppression. For this purpose, adenovirus expressing GFP and luciferase (AdLucGFP) was used for infection of mice so that we could quantify both CTL responses against GFP (K^d/HYLSTQSAL) as well as the adenoviral load by bioluminescence imaging. The suppressive effect of CpG-ODN on CTL generation was dose-dependent, since a clear inhibition was observed when a relatively high dose of 50 μg was injected i.v., but injection of 5 or 0.5 μg CpG-ODN had no effect (Fig. 2C). Because all doses of CpG-ODN resulted in reduced adenoviral load in the host (Fig. 2E), the suppressive effect on CTL generation cannot be solely attributed to decreased antigen expression.

To investigate whether or not systemic CpG-ODN administration also suppresses CD4$^+$ T helper cell function, we quantified anti-adenovirus and anti-OVA IgG serum titers in mice treated with CpG-ODN i.v. and infected with AdOVA i.v. 1 day later, since T helper cell function is required for optimal Ig isotype class switch. Induction of serum IgM antibodies specific for adenovirus or OVA was not negatively affected by systemic CpG-ODN application (data not shown). However, systemic application of CpG-ODN dramatically reduced serum titers of anti-adenovirus and anti-OVA IgG in mice infected with AdOVA (Fig. 2D).

CpG-ODN treatment impairs antigen-specific clonal T cell expansion

The reduction in CTL activity caused by systemic CpG-ODN treatment may have been the result of impaired T cell expansion. To investigate whether T cell expansion is inhibited by CpG-ODN treatment, CFSE-labeled spleen cell suspensions from OT-I (OVA-specific) TCR-transgenic mice were incubated *in vitro* with OVA alone or in combination with CpG-ODN. In agreement with the decreased cytotoxicity described earlier (Fig. 1B), CD8$^+$ T cell proliferation was strongly inhibited by CpG-ODN treatment (Fig. 3A). Likewise, OVA-specific CD4$^+$ T cell proliferation was reduced, although to a lesser extent (Fig. 3A). These results indicate that one mechanism by which CpG-ODN treatment inhibits T cell function is the limitation of antigen-specific expansion. Furthermore, the suppression of cell proliferation was T cell-specific, because B cells proliferated extensively in response to CpG-ODN (Fig. 3A), as previously reported [7]. In agreement with these data on *in vitro* proliferation, CD8$^+$ T cell expansion *in vivo* was also inhibited, because the percentage of K^b/SIINFEKL tetramer-positive cells was strongly reduced in wild-type mice injected i.v. with CpG-ODN followed by AdOVA 1 day later (Fig. 3B).

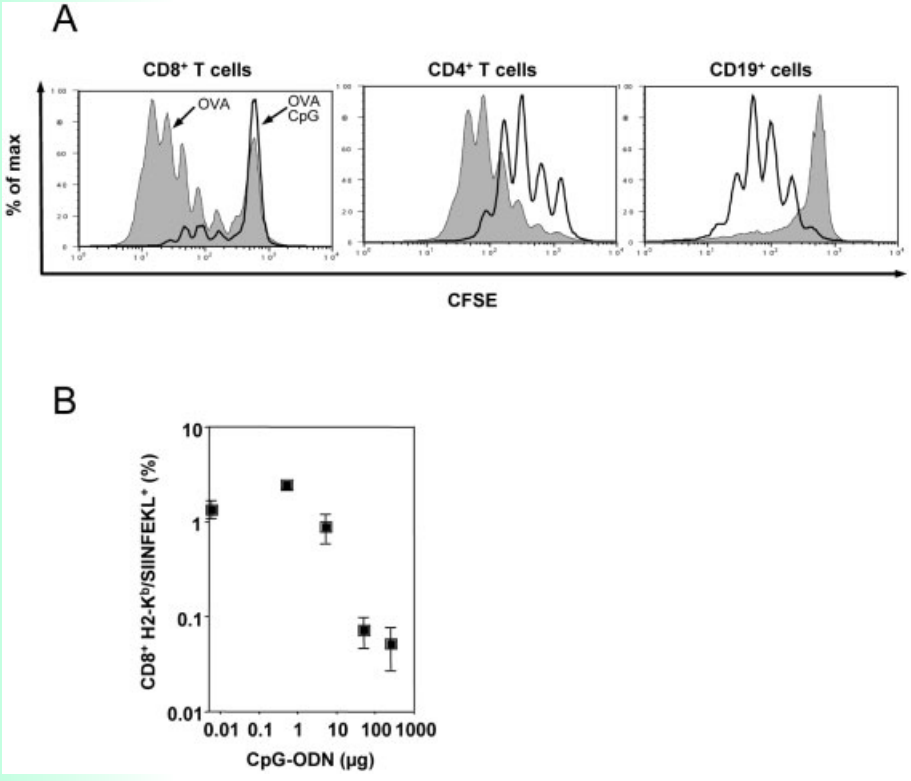

Figure 3. Suppression by CpG-ODN is due to impaired antigen-specific T cell proliferation. (A) CFSE-labeled splenocytes from OT-I mice (left and right) or OT-II mice (middle) were incubated *in vitro* with 500 μg/mL OVA alone (filled histogram) or in the presence of 5 μg/mL CpG-ODN (bold line). The indicated cell populations were analyzed for proliferation by flow cytometry 4 days later. (B) Indicated amounts of CpG-ODN were injected i.v. into C57BL/6 mice 1 day before challenge with AdOVA (1×10^8 pfu i.v.). The percentage of H2-K^b/SIINFEKL-specific CD8$^+$ T cells in the spleen was determined 5 days later by flow cytometry using specific tetramers. Results are expressed as mean ± standard error of the mean (SEM). Representative data are shown.

Systemic CpG-ODN administration induces IDO expression in the spleen

It has been reported that activation of the enzyme IDO can inhibit T cell proliferation via depletion of tryptophan and/or via generation of suppressive tryptophan cleavage products [19, 20]. We investigated whether the suppressive effect of systemic CpG-ODN application on T cell expansion is linked to IDO activity. First, the serum of C57BL/6 mice treated with CpG-ODN i.v. was analyzed for the presence of tryptophan metabolites. The ratio of the tryptophan cleavage product kynurenine to tryptophan increased in the serum shortly after CpG-ODN injection (Fig. 4A), indicating that IDO activity was induced *in vivo* by systemic CpG-ODN challenge.

Next we investigated by immunohistology whether IDO is expressed in the spleen after systemic application of CpG-ODN. A dose of 0.5 μg CpG-ODN failed to induce IDO (data not shown), although it resulted in protective innate immunity against adenovirus (Fig. 2E). However, 24 h after i.v. injection of 50 μg CpG-ODN, expression of IDO in the spleen was observed (Fig. 4B). In contrast to the spleen, no IDO expression was found in peripheral lymph nodes after i.v. treatment with CpG-ODN (Fig. 4B). Systemic administration of a single dose of CpG-ODN did not caused structural changes in the splenic architecture, as has been reported when multiple doses of CpG-ODN were applied [21].

T cell suppression by systemic CpG-ODN treatment is mediated by IDO activity

For investigation of the functional relevance of IDO expression, the tryptophan analogue 1-methyl-tryptophan (1-MT), a well-established competitive inhibitor of IDO [19, 20, 22], was used. OVA-induced proliferation of CFSE-labeled OT-I cells was blocked by the presence of CpG-ODN in *in vitro* culture (Fig. 5A). Addition of 1-MT interfered with this suppression, resulting in proliferation of the OT-I cells. Likewise, the *in vitro* generation of OVA-specific CTL was inhibited by CpG-ODN, but not when IDO was blocked with 1-MT (Fig. 5B). Importantly, by giving mice 1-MT in the drinking water, antigen-specific $CD8^+$ T cell proliferation and cytotoxicity to soluble antigen (OVA) was restored (Fig. 5C, left). As expected, the restoration of cytotoxicity was accompanied by an increase in the number of tetramer-positive, OVA-specific $CD8^+$ T cells (Fig. 5C, right).

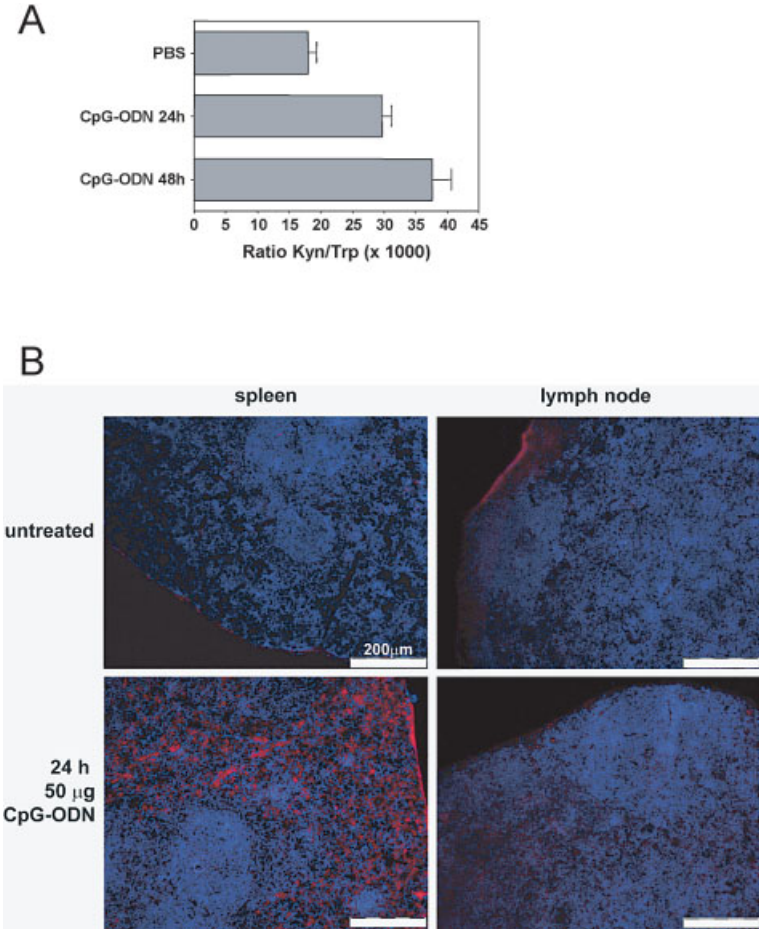

Figure 4. Systemic CpG-ODN induce IDO expression in the spleen. (A) C57BL/6 mice were treated i.v. with 50 μg CpG-ODN, and after 24 or 48 h, the amounts of tryptophan and its cleavage product kynurenine in serum were determined. Results are expressed as mean ± standard error of the mean (SEM). (B) C57BL/6 mice were treated i.v. with 50 μg CpG-ODN. After 24 h, spleen cryosections were stained for IDO (red) and nuclei counter-stained with Hoechst 33258 (blue) (size bar =200 μm). Representative data are shown. No induction of IDO was obtained with a control, non-CpG-ODN (not shown).

CpG-ODN-mediated immune suppression is TLR9-dependent but independent of IFNα and IFNγ

Immunostimulatory CpG-ODN are known to bind to TLR9 [23]. We used TLR9$^{-/-}$ mice to investigate whether the CpG-ODN-induced suppression is also dependent on TLR9. No suppression of OVA-specific cytotoxicity by CpG-ODN was found in TLR9-deficient mice after immunization with AdOVA (Fig. 6A), indicating a primary role for TLR9 in the suppressive effect. Comparable results were obtained in mice infected with *Listeria monocytogenes* expressing OVA as a transgene (LmOVA). Again, CpG-ODN prevented the formation of OVA-specific CD8$^+$ T cells, whereas no such suppression occurred in TLR9$^{-/-}$ mice (Fig. 6B).

Tumors and infections have been reported to induce IDO in an IFNγ-dependent way [24, 25]. Therefore we investigated whether type I and II interferons are responsible for the CpG-ODN-induced suppression described here. Strong suppression by systemic CpG-ODN application was still obtained in IFNαR$^{-/-}$ and IFNγ$^{-/-}$ mice immunized with AdOVA virus (Fig. 6C, D).

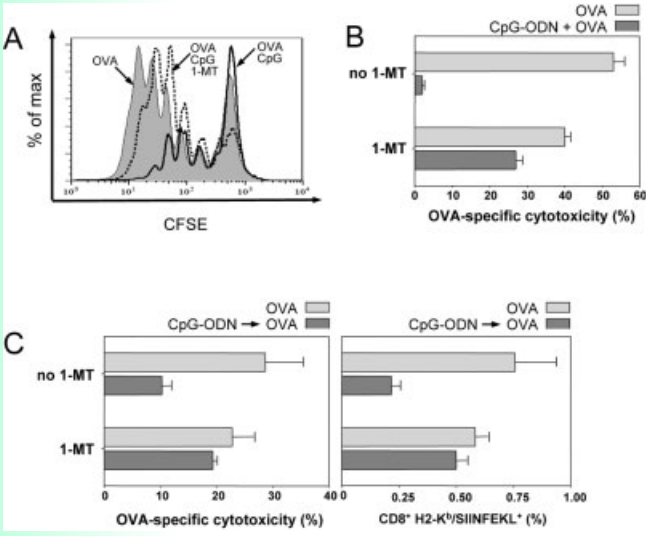

Figure 5. CpG-ODN-induced suppression is mediated by IDO activity. (A) CFSE-labeled splenocytes from OT-I mice were incubated *in vitro* with 500 μg/mL OVA alone (filled histogram), together with 5 μg/mL CpG-ODN (dotted line) or together with 5 μg/mL CpG-ODN and 1 mM 1-MT (bold line), and CD8$^+$ T cells were analyzed for proliferation 3 days later. (B) OT-I splenocytes were incubated *in vitro* with 500 μg/mL OVA alone or in combination with 5 μg/mL CpG-ODN and 1 mM 1-MT, and 4 days later cytotoxicity *in vitro* was determined by $^{[51]}$Cr-release assay (E:T =100:1). (C) C57BL/6 mice were treated i.v. with 100 μg OVA alone or in combination with 50 μg CpG-ODN and 1-MT (5 mg/mL in the drinking water) as indicated, and 5 days later *in vivo* cytotoxicity (left panel) and the percentage of H2-K^b/SIINFEKL-specific CD8$^+$ T cells (right panel) were determined. Results are expressed as mean ± standard error of the mean (SEM). Representative data are shown.

These findings argue against a decisive role of type I and II interferons in CpG-ODN induced suppression.

Discussion

TLR ligands such as CpG-ODN are potent stimulators of innate and adaptive immune responses and are, therefore, used as adjuvants for immunization. The present study demonstrates that CpG-ODN can also have adverse effects and suppress T cell responses. The anatomical site of application appears to be critical in determining the immunosuppressive *versus* stimulatory effects of CpG-ODN. Stimulation and suppression of immunity are induced in distinct lymphoid organs. Thus, subcutaneous injection of antigen plus CpG-ODN, as performed for many types of vaccinations, results in a strong T cell response in the local lymph node. In contrast, systemically injected CpG-ODN induced suppression of T cell proliferation and the generation of effector cells in the spleen. CpG-ODN did not negatively affect antigen-presenting DC and effector T cells directly (data not shown). Rather, this suppression was found to be mediated by induction of IDO, an enzyme known to degrade tryptophan, an essential amino acid for

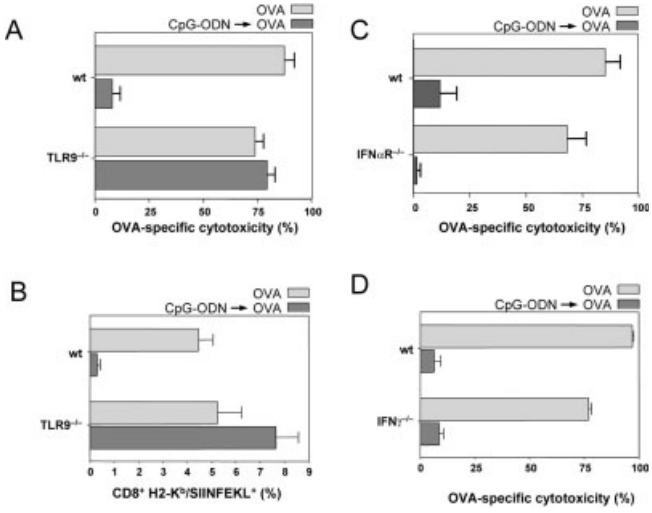

Figure 6. CpG-ODN-mediated immunosuppression is TLR9-dependent but independent of IFNα and IFNγ. TLR9-deficient mice (TLR9$^{-/-}$) or wild-type C57BL/6 littermates (wt) were injected i.v. with 50 μg CpG-ODN 1 day before AdOVA (1×10^8 pfu i.v.) (A) or OVA-expressing *L. monocytogenes* (2×10^3 LmOVA i.v.) (B) challenge. OVA-specific cytotoxicity *in vivo* was quantified in the spleen 5 days after infection (A). OVA-specific CD8$^+$ T cells were quantified in the spleen using specific tetramers 7 days after infection (B). IFNγ$^{-/-}$ (C) or IFNαR$^{-/-}$ (D) mice were injected i.v. with 50 μg CpG-ODN 1 day before AdOVA (1×10^8 pfu i.v.), and 5 days later OVA-specific *in vivo* cytotoxicity was determined in the spleen. Results are expressed as mean ± standard error of the mean (SEM). Representative data are shown.

proliferation of $CD4^+$ and $CD8^+$ T lymphocytes [26, 27]. IDO can be produced by various cell types, including macrophages, immature DC, plasmacytoid DC (pDC) and non-hematopoietic cells such as trophoblasts and lung epithelial cells [25, 27]. The nature of the IDO-producing cell types in the spleen is not yet clear. Their identification may be complicated, because it is known that cells can express IDO in a functionally inactive form [20, 25]. Preliminary experiments indicate that pDC are not involved (or solely responsible), as *in vivo* depletion with a pDC-specific antibody had no effect on suppression (own unpublished data).

Induction of IDO and T cell suppression in the spleen was dose-dependent and observed only at a dose of 50 μg CpG-ODN per mouse. It is interesting to note that smaller doses of CpG-ODN (0.5 and 5 μg) appear to be sufficient for induction of innate immunity, as indicated by the finding that these doses drastically reduced the load of adenovirus in the host (see Fig. 2E). These observations suggest that induction of IDO requires much higher concentrations of CpG-ODN than induction of mediators of innate immunity, such as IL-12 and type I and II interferons, which are likely to be involved in the protection against virus. Work with various knockout mice demonstrated that interaction of CpG-ODN with TLR9 is required for T cell suppression in the spleen. However, it is not clear if TLR9-positive cells directly produce IDO upon TLR9 engagement or if IDO induction is an indirect effect mediated by soluble factors that are released upon TLR9 stimulation. We could exclude type I and type II interferons as mediators of suppression, because CpG-ODN-induced inhibition was still observed in $IFN\alpha R^{-/-}$ and $IFN\gamma^{-/-}$ mice. It is known that tumors and infections can induce IDO in various cells types in a IFNγ-dependent manner [24, 25, 27]. The experiments described here failed to reveal a role for type I and II interferons in CpG-ODN-induced and IDO-mediated suppression and therefore argue for a different mode of IDO induction. Further studies are required for elucidation of the mechanism of IDO induction by CpG-ODN.

In conclusion, we have shown that CpG-ODN can be either immunostimulatory or suppressive, depending upon the anatomical site of application. These observations are important with regard to the use of CpG-ODN in vaccines, and they may explain why in clinical trials, s.c. immunization was superior to i.v. immunization in terms of the protection provided [12]. However, suppression by systemic CpG-ODN application may also be beneficial in some situations. For example, i.v. application of CpG-ODN in a mouse model of experimental asthma resulted in induction of IDO in the lung and amelioration of asthma, but the precise mechanism of action is not clear [26]. Our results indicate that T cell immunity can be regulated depending on the route of CpG-ODN administration, thus opening up the possibility to reduce (suppress) unwanted T cell responses.

Materials and methods

Mice

Mice were maintained under specific pathogen-free conditions at the animal facilities of the ZMBH and DKFZ (Heidelberg, Germany) and the IMMEI (Bonn, Germany). C57BL/6 and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) or Harlan Winkelmann (Borchen, Germany). OVA-specific $CD8^+$ T cell-transgenic OT-I mice [28], OVA-specific $CD4^+$ T cell-transgenic OT-II mice [29] and mice deficient for TLR9, IFNαR or IFNγ have been described elsewhere [30] and were all on a C57BL/6 background.

Reagents and antibodies

OVA grade II and VII was purchased from Sigma (Deisenhofen, Germany). Polymyxin-B columns for the removal of LPS contamination from soluble OVA were obtained from Pierce (Bonn, Germany). The peptides SIINFEKL ($OVA_{257–264}$, H2-K^b-binding) and HYLSTQSAL ($GFP_{200–208}$, H2-K^d-binding) were synthesized by Pineda (Berlin, Germany). CFSE and 7-aminoactinomycin D (7-AAD) were from Molecular Probes (Leiden, The Netherlands). LPS-free phosphorothioate-stabilized CpG-ODN 1668 (referred to as CpG-ODN) and mammalian non-CpG-ODN AP-1 [9] were synthesized by TIB MolBiol (Berlin, Germany). Recombinant adenovirus type 5 expressing OVA (AdOVA) or AdLucGFP were constructed as previously described [31]. Fluorochrome-labeled and biotinylated antibodies used for flow cytometry and immunohistology were from BD Biosciences (Heidelberg, Germany).

Immunizations

For immunizations with soluble OVA, C57BL/6 mice were injected i.v. or s.c. (intra ear pinna) with 100 μg OVA alone or in combination with 50 μg CpG-ODN. For adenoviral infections, the indicated doses of CpG-ODN or mammalian non-CpG ODN were applied i.v. 1 day before i.v. immunization with 1×10^8 pfu AdOVA . The dose dependency of CpG-ODN immunosuppression was investigated by administering CpG-ODN i.v. 1 day before infecting C57BL/6 × BALB/c F1 mice i.v. with 1×10^8 pfu AdLucGFP. K^b/SIINFEKL- or K^d/HYLSTQSAL-specific cytotoxicity *in vivo* was quantified in the spleen (i.v. immunization) or draining lymph nodes (s.c. immunization) as described below 5 days after immunization. LmOVA were grown in brain heart infusion broth. For infection with LmOVA, a sublethal dose of $2x10^3$ bacteria was injected into the tail veins of 8-week-old C57BL/6 mice. OVA-specific T cell counts (by K^b/SIINFEKL tetramers) were determined 7 days later in spleens of infected mice.

In vitro cell culture, cell proliferation and cytotoxicity

Spleen cells from C57BL/6, OT-I or OT-II mice were incubated at 1×10^6 cells/mL with 500 μg/mL OVA alone or in

combination with 5 μg/mL CpG-ODN. For proliferation studies *in vitro*, spleen cells were labeled with 5 μM CFSE as described previously [17, 18] before addition of OVA and CpG-ODN. Proliferation of $CD8^+$ T cells, $CD4^+$ T cells and B cells was analyzed by flow cytometry 3 or 4 days later using a FACScalibur or LSR II (BD Biosciences). The effect of CpG-ODN on OVA-specific $CD8^+$ T cell expansion was investigated in C57BL/6 mice immunized with AdOVA with and without CpG-ODN as described above. The percentage of H2-K^b/SIINFEKL-specific $CD8^+$ T cells in the spleen was determined by flow cytometry using specific tetramers as previously described [32].

CTL cytotoxicity was measured *in vivo* as previously described [17, 18]. Briefly, target spleen cells from C57BL/6 or C57BL/6 × BALB/c F1 mice were pulsed with 1 μg/mL SIINFEKL or HYLSTQSAL for 45 min at 37°C and subsequently labeled with 1 μM CFSE for 15 min at 37°C ($CFSE^{high}$, specific target cells) or were not pulsed with peptide and labeled with 0.1 μM CFSE ($CFSE^{low}$, non-specific target cells). The two target cell populations were mixed in equal numbers and injected i.v. into effector mice. Unless otherwise indicated, mice were killed 4 h later and the ratio of recovered specific *versus* non-specific target cells in spleen or draining lymph nodes quantified by flow cytometry. The percentage of specific lysis was calculated using the following equation:

$$100 - [100 \times (CFSE^{high} / CFSE^{low})_{immunized} / (CFSE^{high} / CFSE^{low})_{non\text{-}immunized}]$$

In vitro cytotoxicity was assessed with a standard 5-h $^{[51]}Cr$-release assay as previously described [18].

Antibody responses

C57BL/6 mice were immunized with AdOVA alone or in combination with CpG-ODN as described above. IgG serum titers specific for adenovirus or for OVA were quantified by ELISA 21 days after immunization using plates coated with adenoviral particles or OVA, respectively and using POX-conjugated goat-anti-mouse-IgG and goat-anti-mouse-IgM monoclonal antibodies (BD-Biosciences, Heidelberg, Germany)

IDO expression, activity and inhibition

IDO expression was assessed by fluorescent immunohistology on cryosections of mouse spleens fixed in acetone and stained with rabbit anti-IDO serum [24] followed by AlexaFluor 647-conjugated anti-rabbit mAb (Molecular Probes). Nuclei were counterstained with Hoechst 33258. Sections were analyzed on an Olympus IX71 inverted microscope (Olympus, Hamburg, Germany). The ratio of L-kynurenine/L-tryptophan in serum, an indicator of IDO activity, was quantified by HPLC (Shimadzu, Duisburg, Germany) as previously described [33]. IDO activity was blocked using the competitive inhibitor D-isomer of 1-methyl-tryptophan (1-MT; Sigma). For blocking IDO *in vitro*, 1 mM 1-MT was added to the cell cultures simultaneously with OVA and CpG-ODN. For inhibition of IDO activity *in vivo*, 5 mg/mL 1-MT was supplied in the drinking water 1 day before CpG-ODN administration and continued for 5 days[22].

Bioluminescence imaging

The AdLucGFP load in C57BL/6 × BALB/c F1 mice was quantified by *in vivo* bioluminescence using the real-time IVIS Imaging System 200 (Xenogen Corp., Alameda, CA, USA), as described elsewhere [34]. Briefly, mice were infected i.v. with 1×10^8 pfu AdLucGFP and analyzed for bioluminescence measurement 24 h later. Mice were injected with 2.5 mg luciferin (S039; Synchem, Kassel, Germany) and anesthetized with isofluran 5 min before quantification of bioluminescence. Data were analyzed using Living Image 2.50 software (Xenogen Corp.).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Comparisons were drawn using a two-tailed Student t-test (Excel, Microsoft Corporation, Redmond, USA) or a two-way ANOVA test (Prism, GraphPad Software Inc., San Diego, USA).

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References

1 **Takeda, K., Kaisho, T. and Akira, S.,** Toll-like receptors. *Annu. Rev. Immunol.* 2003. **21:** 335–376.

2 **Beutler, B.,** Innate immune sensing of microbial infection: the mechanism and the therapeutic challenge. *Wien. Med. Wochenschr.* 2002. **152:** 547–551.

3 **Davis, H. L., Suparto, II, Weeratna, R. R., Jumintarto, Iskandriati, D. D., Chamzah, S. S., Ma'ruf, A. A. *et al.*,** CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine* 2000. **18:** 1920–1924.

4 **Iwasaki, A. and Medzhitov, R.,** Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 2004. **5:** 987–995.

5 **Reis e Sousa, C.,** Toll-like receptors and dendritic cells: for whom the bug tolls. *Semin. Immunol.* 2004. **16:** 27–34.

6 **Sporri, R. and Reis e Sousa, C.,** Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of $CD4^+$ T cell populations lacking helper function. *Nat. Immunol.* 2005. **6:** 163–170.

7 **Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. and Klinman, D. M.,** CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995. **374:** 546–549.

8 **Banchereau, J. and Steinman, R. M.,** Dendritic cells and the control of immunity. *Nature* 1998. **392:** 245–252.

9 **Sparwasser, T., Koch, E. S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W. and Wagner, H.,** Bacterial DNA and immunostimulatory CpG

oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* 1998. **28:** 2045–2054.

10 **Krieg, A. M. and Wagner, H.,** Causing a commotion in the blood: immunotherapy progresses from bacteria to bacterial DNA. *Immunol. Today* 2000. **21:** 521–526.

11 **Horner, A. A. and Raz, E.,** Immunostimulatory sequence oligodeoxynucleotide-based vaccination and immunomodulation: two unique but complementary strategies for the treatment of allergic diseases. *J. Allergy Clin. Immunol.* 2002. **110:** 706–712.

12 **Krieg, A. M., Efler, S. M., Wittpoth, M., Al Adhami, M. J. and Davis, H. L.,** Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist. *J. Immunother.* 2004. **27:** 460–471.

13 **Rothenfusser, S., Tuma, E., Wagner, M., Endres, S. and Hartmann, G.,** Recent advances in immunostimulatory CpG oligonucleotides. *Curr. Opin. Mol. Ther.* 2003. **5:** 98–106.

14 **Ishii, K. J., Gursel, I., Gursel, M. and Klinman, D. M.,** Immunotherapeutic utility of stimulatory and suppressive oligodeoxynucleotides. *Curr. Opin. Mol. Ther.* 2004. **6:** 166–174.

15 **Klinman, D. M.,** Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* 2004. **4:** 249–258.

16 **Krieg, A. M., Hartmann, G. and Yi, A. K.,** Mechanism of action of CpG DNA. *Curr. Top. Microbiol. Immunol.* 2000. **247:** 1–21.

17 **Feuerer, M., Beckhove, P., Garbi, N., Mahnke, Y., Limmer, A., Hommel, M., Hammerling, G. J. *et al.*,** Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat. Med.* 2003. **9:** 1151–1157.

18 **Limmer, A., Ohl, J., Wingender, G., Berg, M., Jungerkes, F., Schumak, B., Djandji, D. *et al.*,** Cross-presentation of oral antigens by liver sinusoidal endothelial cells leads to CD8 T cell tolerance. *Eur. J. Immunol.* 2005. **35:** 2970–2981.

19 **Grohmann, U., Fallarino, F., Bianchi, R., Vacca, C., Orabona, C., Belladonna, M. L., Fioretti, M. C. and Puccetti, P.,** Tryptophan catabolism in nonobese diabetic mice. *Adv. Exp. Med. Biol.* 2003. **527:** 47–54.

20 **Mellor, A. L., Chandler, P., Baban, B., Hansen, A. M., Marshall, B., Pihkala, J., Waldmann, H. *et al.*,** Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase. *Int. Immunol.* 2004. **16:** 1391–1401.

21 **Heikenwalder, M., Polymenidou, M., Junt, T., Sigurdson, C., Wagner, H., Akira, S., Zinkernagel, R. and Aguzzi, A.,** Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat. Med.* 2004. **10:** 187–192.

22 **Uyttenhove, C., Pilotte, L., Theate, I., Stroobant, V., Colau, D., Parmentier, N., Boon, T. and Van den Eynde, B. J.,** Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat. Med.* 2003. **9:** 1269–1274.

23 **Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M. *et al.*,** A Toll-like receptor recognizes bacterial DNA. *Nature* 2000. **408:** 740–745.

24 **Takikawa, O., Habara-Ohkubo, A. and Yoshida, R.,** IFN-gamma is the inducer of indoleamine 2,3-dioxygenase in allografted tumor cells undergoing rejection. *J. Immunol.* 1990. **145:** 1246–1250.

25 **Moffett, J. R. and Namboodiri, M. A.,** Tryptophan and the immune response. *Immunol. Cell Biol.* 2003. **81:** 247–265.

26 **Hayashi, T., Beck, L., Rossetto, C., Gong, X., Takikawa, O., Takabayashi, K., Broide, D. H. *et al.*,** Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J. Clin. Invest.* 2004. **114:** 270–279.

27 **Mellor, A. L. and Munn, D. H.,** IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* 2004. **4:** 762–774.

28 **Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J. and Carbone, F. R.,** T cell receptor antagonist peptides induce positive selection. *Cell* 1994. **76:** 17–27.

29 **Barnden, M. J., Allison, J., Heath, W. R. and Carbone, F. R.,** Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 1998. **76:** 34–40.

30 **Krug, A., French, A. R., Barchet, W., Fischer, J. A., Dzionek, A., Pingel, J. T., Orihuela, M. M. *et al.*,** TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* 2004. **21:** 107–119.

31 **He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. and Vogelstein, B.,** A simplified system for generating recombinant adenouiruses. *Proc Natl Acad Sci USA.* 1998. **95:** 2509–2514.

32 **Haanen, J. B., Toebes, M., Cordaro, T. A., Wolkers, M. C., Kruisbeek, A. M. and Schumacher, T. N.,** Systemic T cell expansion during localized viral infection. *Eur. J. Immunol.* 1999. **29:** 1168–1174.

33 **von Bubnoff, D., Matz, H., Frahnert, C., Rao, M. L., Hanau, D., de la Salle, H. and Bieber, T.,** FcepsilonRI induces the tryptophan degradation pathway involved in regulating T cell responses. *J. Immunol.* 2002. **169:** 1810–1816.

34 **Doyle, T. C., Burns, S. M. and Contag, C. H.,** *In vivo* bioluminescence imaging for integrated studies of infection. *Cell. Microbiol.* 2004. **6:** 303–317.

