The influence of gut-derived bacteria on systemic adaptive immune responses

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Erklärung

Diese Dissertation wurde im Sinne von § 4 der Promotionsordnung vom 7.1.2004 im Zeitraum von Oktober 2005 bis Juli 2010 von Herrn Prof. Dr. Percy A. Knolle betreut.

Eidesstattliche Erklärung

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

Bonn, den 19. Juli 2010

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Summary

Sepsis and sepsis-associated multi-organ failure are major challenges for clinicians and scientists and are characterized by high patient morbidity and mortality. One factor thought to be important in the etiology of systemic inflammatory response syndrome (SIRS) is the breakdown of the intestinal barrier resulting in bacterial translocation and subsequent bacteremia causing sepsis. Whereas the influence of sepsis on the innate immune system is well described, the counter regulatory mechanisms and the impact on adaptive immunity are still largely unknown. In this thesis, the influences of gut-derived bacteria on the induction of systemic adaptive immune responses were investigated.

The results presented demonstrate that the loss of gut barrier function led to release of vast amounts of intestinal bacteria, which rapidly disseminated in the organism. Bacteremia caused suppression of adaptive cytotoxic immune responses against subsequent infections with viral and bacterial pathogens. This suppression was characterized by impaired expansion of antigen-specific cytotoxic T cells and a lack of antigen-specific cytotoxicity. Interestingly, bacterial translocation from the gut did not necessarily result in suppression of T cell responses. If only low amounts of bacteria translocated, bacteria were retained within the liver which prevented dissemination of gut-derived bacteria, bacteremia, and subsequent immunosuppression. Importantly, the induction of local immunity at peripheral sites was not affected by bacteremia, contradicting the current opinion of a general, systemic immunosuppression following sepsis. Moreover, bacteremia exclusively inhibited the generation of subsequent adaptive immune responses, whereas already initiated antigen-specific CTL responses were further stimulated by systemic bacteria. Although *E.coli,* which was used in this study as a model organism for translocating bacteria, stimulates a broad variety of Toll-like receptors (TLRs), suppression was solely dependent on TLR4 activation. Interestingly, neither TLR4 downstream signaling via MyD88 nor expression of the potent suppressive cytokine IL-10 contributed to the observed immunosuppression after bacteremia. Instead signaling via TRIF and subsequent expression of type I interferons (IFNs) were critically involved in *E.coli*-mediated CTL suppression.

We could demonstrate that adaptive immune responses towards systemic pathogens were generated in the spleen and furthermore, that suppression of systemic CTL responses strictly depended on the presence of bacteria in the spleen. Splenic macrophages and DCs, which are crucially involved in the induction of T cell responses as metallophilic marginal

zone macrophages (MMMs) efficiently phagocytose blood borne antigens and transfer them to cross-presenting DCs (Backer et al., 2010). These cells were impaired in their ability to induce adaptive immunity to subsequent adenoviral infection after exposure to *E.coli*. These findings underline a central role of splenic macrophages and DCs in immunosuppression after sepsis.

The observed suppression of adaptive immunity was surprising as pathogens are known to be potent activators of the innate immune system by stimulation of pattern-recognition receptors, such as TLRs. Activation of the innate immune system is an essential prerequesite for the initiation of effective adaptive immune response (Akira et al., 2006; Iwasaki and Medzhitov, 2004). However, in this study we showed that activation of innate immunity indeed strictly depended on TLR signaling, whereas TLR signaling was dispensable for the generation of effective adaptive immune responses. Activation of TLRs led to effective induction of innate immunity but also exerts mechanisms, namely secretion of type I IFNs, which regulated adaptive immune responses in a paracrine manner.

Taken together, the findings presented in this study, demonstrate a dual role of TLR signaling. Depending on the anatomical site, dose, and time point of TLR ligand application systemic adaptive immune response are either stimulated or suppressed. Moreover, these data may provide further insights for the development of new therapeutic approaches to circumvent suppression of systemic adaptive immune response in septic patients.

Zusammenfassung

Sepsis und durch Sepsis ausgelöstes multiples Organ Versagen sind eine große Herausforderung für Mediziner und Wissenschaftler und zeichnen sich durch eine hohe Morbidität und Sterblichkeit aus. Ein Faktor, der seit langem mit der Ätiologie des systemischen inflammtorischen Response Syndrom (SIRS) assoziiert wird, ist der Verlust der Darmbarriere, in dessen Folge eine Translokation von Darmbakterien zu einer Sepsis führen kann. Obwohl der Einfluss der Sepsis auf das angeborene (innate) Immunsystem in der Literatur hinreichend beschrieben ist, sind die regulierenden Mechanismen des Organismus und die Auswirkungen der Sepsis auf das erworbene (adaptive) Immunsystem weitgehend unbekannt. In der vorliegenden Arbeit wurde der Einfluss von aus dem Darm translozierten Bakterien auf die Induktion von adaptiven Immunantworten untersucht.

Die präsentierten Ergebnisse zeigen, dass es durch den Verlust der Darmbarriere zum Übertritt von Bakterien kommt, die sich schnell im Organismus verteilen. In Folge dieser Bakteriämie wurde die Induktion von adaptiven Immunantworten auf eine nachfolgende virale oder bakterielle Infektion unterdrückt. Diese Suppression war gekennzeichnet durch eine beeinträchtigte Proliferation Antigen-spezifischer zytotoxischer T Lymphocyten (ZTL), so wie durch einen Verlust der Antigen-spezifischen Zytotoxizität der CD8 T Zellen. Jedoch führte eine bakterielle Translokation aus dem Darm nicht zwingend zu einer Suppression der T Zellen, da die Leber in der Lage war Bakterien zurückzuhalten und damit sowohl eine systemische Verteilung der Bakterien, als auch ein nachfolgende Immunsuppression zu verhindern. Die Induktion adaptiver, lokaler Immunantworten durch systemisch vorhandene Bakterien war nicht beeinträchtigt. Dieser Befund widerspricht der allgemeinen Meinung einer generellen Immunparalyse in Folge von Sepsis. Zudem verhindert eine Bakteriämie ausschließlich die Induktion einer adaptiven Immunantwort gegen nachfolgende Infektionen, hingegen wurden bereits bestehende ZTL Immunantworten durch systemisch vorhandene Bakterien stimuliert. Obwohl *E.coli*, welches in dieser Studie als Modell für translozierende Bakterien verwendet wurde, eine Vielzahl von Toll-like Rezeptoren (TLR) stimuliert, war die Suppression ausschließlich von der Aktivierung von TLR4 abhängig. Interessanterweise war weder die Signalkaskade über MyD88 noch die nachfolgende Expression des immunsuppressiven Zytokins IL-10 an der beobachteten Suppression beteiligt. Stattdessen spielten das Adaptormolekül TRIF, sowie die nachfol-

gende Expression von Typ I Interferonen (IFN) in der durch *E.coli* hervorgerufenen Immunsuppression eine wichtige Rolle.

Wir konnten zeigen, dass die Induktion adaptiver Immunantworten gegen systemische Infektionen in der Milz erfolgte und dass die Suppression systemischer ZTL Antworten nur ausgelöst wurde, wenn die Bakterien die Milz erreichten. Metallophile Makrophagen (MMM) und dendritische Zellen (DZ) der Milz, sind ursächlich an der Generierung von adaptiven T Zell Antworten beteiligt, da die MMM höchst effizient Antigene aus dem Blut aufnehmen und diese an kreuzpräsentierende DZ weiterreichen (Backer et al., 2010). Diese Zellen waren nicht mehr in der Lage eine adaptive Immunantwort auf eine nachfolgende Infektion zu generieren, wenn diese *E.coli* ausgesetzt wurden. Diese Befunde verdeutlichen die zentrale Rolle von DZ und MMM in der Induktion der Immunsuppression nach Sepsis.

Die beobachtete Suppression adaptiver Immunantworten war unerwartet, da Pathogene TLR aktivieren und somit das innate Immunsystem stimulieren. Ferner ist die Aktivierung des innaten Immunsystems essentiell für das Einleiten einer effektiven adaptiven Immunantwort (Akira et al., 2006; Iwasaki and Medzhitov, 2004). Hier konnten wir zeigen, dass zwar die Aktivierung des innaten Immunsystems tatsächlich vom TLR-Signal abhängig war, für die Generierung einer effektiven, adaptiven Immunantwort hingegen die Aktivierung der TLR nicht vonnöten war. Die Aktivierung von TLR führte auf der einen Seite zur Aktivierung des innaten Immunsystems, auf der anderen Seite wurden aber auch Mechanismen ausgelöst, die die adaptive Immunantwort durch Typ I IFN parakrin regulierten.

Zusammenfassend zeigen die hier präsentierten Ergebnisse eine duale Funktion der TLR-Signalwirkung. Abhängig von der anatomischen Verteilung, Dosis und des Zeitpunktes der Gabe von TLR Liganden, wurde eine systemische Immunantwort entweder stimuliert, oder unterdrückt. Darüber hinaus könnten diese Daten weitere Erkenntnisse für die Entwicklung neuer Therapien bieten, um in Zukunft die Suppression des systemisch, adaptiven Immunsystems zu verhindern.

1 Introduction

1.1 The immune system

The immune system (from Latin *immunis* literally *untouched, free*) allows organisms to fight efficiently against invading and life-threatening pathogens such as viruses, bacteria, fungi, and parasites as well as foreign or malignant cells. To defend the body against these pathogens multiple mechanisms of immune defense have evolved which are divided into two major subdivisions:

1) The innate immune system is the first line of defense and protects the host by recognition of pathogenic patterns that are non-self and common to many pathogens.

2) The acquired or adaptive immune system is composed of cells which recognize specific antigens and provide elimination of pathogens in the later phase of infection. Importantly, cells of the adaptive immune system form an immunological memory which allows a more rapid response to re-infection with the same pathogen.

1.1.1 The innate immune system

The elements of the innate immune system include anatomical barriers, secretory molecules, and cellular components. Mechanical anatomical barriers are formed by the skin and internal epithelial layers, the movement of the intestines, and the oscillation of bronchopulmonary cilia all of which prevents pathogens to enter the organism. Associated with these protective surfaces are chemical and biological agents. Lysozym and phospholipase which breakdown bacterial cell walls can be found in tears, nasal secretions, and salvia. Very small peptides called defensins bear strong antimicrobial activity and cover the surface of the skin, lung, and gastrointestinal tract. Even though these anatomical surface barriers are quiet efficient, they can be broken by mechanical stress thereby allowing pathogens to invade the organism. After passing the anatomical barrier, pathogens encounter a set of soluble factors and cells that efficiently eliminate invading microorganisms. The complement system lyses bacteria and mark them via opsonization for phagocytic cells like

macrophages. Cytokines and chemokines activate phagocyting cells, lead them to the site of infection and display antibacterial and antiviral effects as well.

Cells of the innate immune system recognize germs via a variety of highly conserved, germ line-encoded pattern recognition receptors (PPRs) that detect components of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs). PRRs are expressed on the cell surface or in intracellular compartments of many cell types including macrophages and dendritic cells (DCs). Different PRRs react with specific PAMPs, activate specific signaling pathways, and lead to distinct anti-pathogen responses. Most importantly, stimulation of PRRs leads to activation and maturation of professional antigen presenting cells (APCs) which in turn stimulate adaptive immune responses. The most prominent APCs are DCs, which upon activation by PAMPs, efficiently prime T cells responses and thus display the linkage between innate and adaptive immunity (Medzhitov and Janeway, 1997). Overall the innate immune system uses a variety of highly efficient immune effectors to combat pathogens. Nevertheless the efficacy of these effectors is limited due to the immunopathology they can cause.

1.1.2 The adaptive immune system

The adaptive immune system minimizes collateral damage by focusing immune defense in an antigen-specific manner. The most important effectors of the adaptive immune system are T cells, mediating cellular immunity, and B cells, mediating humoral immunity. In contrast to the strictly conserved PRRs of the innate immune system, the receptors of adaptive immunity, namely the B cell (BCR) and the T cell receptor (TCR), are not encoded in the germ line but instead develop during maturation by somatic recombination. Therefore every B and T cell carries a unique receptor with one distinct antigen-specificity which results in an enormous repertoire of antigens that can be recognized. In addition to cell-bound BCRs, B cells secrete antibodies, the soluble form of the BCR which bind to the respective antigen in its native conformation. Most toxins and extracellular pathogens are rapidly neutralized or tracked for phagocytosis by antibodies. In contrast, infected cells are recognized and eliminated by antigen-specific T cells. For activation of T cells, the respective antigenic peptide has to be presented on major histocompatibility molecules (MHC) expressed for example by APCs. Upon activation antigen-specific T and B cells undergo substantial proliferation which allows rapid and effective clearance of the infection.

In contrast to innate immune responses which offer immediate protection, the onset of adaptive immune reactions can take up to weeks. For this reason the adaptive immune system developed a unique immunological memory which allows immediate and effective antigen-specific immune responses upon re-exposure to the cognate pathogen.

During TCR and BCR development, an enormous variety of antigen-specificity of the adaptive immune system is created that is not able to unfailingly discriminate between "infectious non-self" and "non-infectious self". Importantly, T and B cells that recognize self are eliminated during development. However, some cells recognizing self might survive that can lead to detrimental consequences for the host such as autoimmune diseases. To prevent the host from damage of its own misguided immune system the adaptive immune system is precisely tuned by the innate immune system. This ensures that APCs trigger adaptive immunity only in the presence of PAMPs.

1.2 Pattern recognition receptors

To avoid detrimental activity of the immune system to the organism it is obligatory to discriminate between self, infectious non-self, and innocuous non-self which was proposed by Charles Janeway 20 years ago (Janeway, 1989, 1992; Janeway and Medzhitov, 2002; Medzhitov and Janeway, 2002). Cells of the innate immune system recognize germs via a variety of highly conserved, germ line-encoded PPRs that have evolved to detect conserved structures of pathogens referred to as PAMPs. To fulfill this function, PPRs have several important properties. First, they only recognize PAMPs which are unique to microbes. Second, they sense a broad variety of pathogens so that a limited number of germ line-encoded receptors detect nearly all infections. Third, PAMPs are essential for the pathogen to survive and therefore are not easily eliminated due to mutations (Akira et al., 2006; Medzhitov and Janeway, 2002; Palm and Medzhitov, 2009). Beside the secreted form of PRRs like the mannan-binding protein or the C-reactive protein, membrane-bound PRRs are divided into two main classes:

1) Transmembrane PRRs which recognize PAMPs in the extracellular space or in phagosomes and endosomes.
2) Cytosolic PRRs sensing PAMPs in the cytosol.

1.2.1 Transmembrane PRR

The best characterized members of the transmembrane PRR family are the family of Toll-like receptors (TLRs). Originally the protein "Toll" was discovered by the group of Christiane Nüsslein-Volhard in 1985 and was shown to be essential in the embryogenesis of *Drosophila melanogaster* (Anderson et al., 1985). Later, TLRs where shown to play a crucial role in the induction of immune responses in Drosophila as well as in mammals (Lemaitre et al., 1996). TLRs sense infection trough the recognition of PAMPs and induce innate and adaptive immune responses (Iwasaki and Medzhitov, 2004; Pasare and Medzhitov, 2004). Furthermore, TLRs have been shown to be sufficient to control adaptive immunity leading to an adequate adaptive immune response. Another member of the transmembrane PRRs is Dectin-1 which recognizes β-glucans from fungal pathogens such as *Candida albicans* (Brown and Gordon, 2001). Analogous to TLRs, Dectin-1 stimulation alone has been shown to be sufficient to induce adaptive immune responses (LeibundGut-Landmann et al., 2007). In contrast to TLRs Dectin-1, is reported to enhance phagocytosis and influence phagosome trafficking (Herre et al., 2004).

1.2.2 Cytosolic PRR

The cytosolic PRRs can be divided into two subclasses based on their mechanism of activation. PRR as the nucleotide-binding oligomerization domain (Nod) containing Nod1 and Nod2, the retinoic acid-inducible gene I (RIG-I)-like helicases and the interferon stimulator DNA (ISD) sensor can be activated directly by their respective ligands (Meylan et al., 2006; Takeuchi and Akira, 2007). Nod1 and Nod2 sense bacterial infections by detection of peptidoglycan fragments in the cytosol. In contrast, RIG-I recognizes RNA viruses in most cell types and activates an antiviral innate immune response which is characterized by type I interferon (IFN) production. The ISD sensor is activated by cytosolic DNA derived from DNA viruses and retroviruses. Both, RIG-I as well as ISD activation, has been shown to be sufficient for the induction of T cell and antibody responses. The second class contains members of the (Nod)-like receptors (NLR) family that is involved in the formation and activation of multimeric protein complexes, namely the inflammasomes. They control the activation of caspase-1 and furthermore the secretion of caspase-1-dependent cytokines like IL-1β upon stimulation.

1.2.3 Toll-like receptors

The expression pattern of TLRs is quiet conserved among the mammals (Rast et al., 2006). Currently, 13 mammalian TLRs , expressed on the cell surface or in the endoplasmatic reticulum and endosomal compartments have been identified (Baccala et al., 2007). Cell surface TLRs 1, 2, 4, 5, 6, and 11 sense lipids, lipoproteins, or peptidoglycans from bacteria, fungi, or protozoa. Intracellular TLRs 3, 7, 8 and 9 detect bacterial and viral nucleic acids. The ligands for TLRs 10, 12, and 13 have not yet been identified. TLR10 is expressed in humans but not mice, TLR8 is not functional in mice, and TLRs 11, 12, and 13 are exclusively expressed in mice but not humans.

TLRs are type I membrane glycoproteins with a trimodular structure. They consist of an extracellular domain containing leucine-rich-repeat motifs that promote the detection of PAMPs and a cytoplasmic domain which allows downstream signaling and subsequent expression of proinflammatory cytokines and chemokines. TLRs are expressed on a broad range of immune cells such as DCs, macrophages, and B cells. These TLRs serve to sense bacteria, funghi, protozoa, and viruses and therefore play an important role in initiation of appropriate immune responses.

1.2.4 Toll-like receptor ligands

TLRs recognize a broad variety of PAMPs, also referred to as TLR ligands (TLR-L) (Figure 1.1). The cell wall of gram positive and gram negative bacteria are sensed by TLR1, TLR2, TLR4, and TLR6. The most potent immunostimulant is a lipid portion of lipopolysaccharide (LPS) mainly released from gram negative bacteria such as *Escherichia coli*. LPS associates with LPS binding protein (LBP) and binds to CD14. LPS is then transferred to MD-2 which associates with the extracellular portion of TLR4. The cell wall of gram positive bacteria such as *Staphylococcus aureus* contains only very low amounts of LPS. Instead TLRs are stimulated by lipoteichoic acid (LTA), lipoproteins, and pepdidoglycan (PG) derived from the cell wall of gram positive bacteria. TLR2 plays a major role in detecting gram positive bacteria and interacts physically with TLR1 and TLR6. Another component of many bacteria, falgellin is sensed by TLR5. Flagellin is a constituent of bacterial flagella, the motility apparatus used by many microbial pathogens. Furthermore bacterial DNA can be recognized by TLR9 present in the endosome. TLR9 senses the unmethylated CpG oligonucleotide which is abundant in bacterial genomes.

Viruses are recognized by endosomal TLRs, namely TLR3, TLR7, TLR8, and TLR9. DNA viruses such as herpes simplex virus 1 (HSV-1) or murine cytomegalovirus (MCMV) contain genomes that are rich in CpG-DNA motives and therefore activate TLR9. TLR7 and TLR8 are highly homologous, are both expressed in the endosomal membrane and sense single-stranded RNA (ssRNA) of both viral and host origin. Double stranded RNA (dsRNA) is generated during viral replication of ssRNA viruses and is recognized by TLR3. Mostly viral stimulation of TLRs leads to profound expression of type I interferons and proinflammatory cytokines.

1.2.5 Toll-like receptor signaling

Stimulation of TLRs by their respective ligands triggers potent activation of signaling cascades leading to the induction of genes involved in host defense. TLRs signal via the cytoplasmic domain which is homologous to the cytoplasmic region of the IL-1 receptor, known as the Toll/IL-1 receptor (TIR) domain. Upon stimulation, TLRs dimerize and undergo conformational changes which arrange recruitment of TIR-domain-containing adaptor molecules to the TIR domain of the TLR. Five TIR-domain containing adaptors are known to be involved in TLR signaling namely MyD88, MyD88-adaptor-like (MAL, also known as TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF, also known as TICAM1), TRIF-related adaptor molecule (TRAM) and sterile α-and armadillo-motif-containing protein (SARM) (O'Neill and Bowie, 2007; Oshiumi et al., 2003a). The diversity of responses to distinct TLR ligands partially refers to selective usage of these adaptor molecules. In most TLRs except TLR3 downstream signaling is mediated by the TIR domain-containing adaptor molecule MyD88 (Akira et al., 2006). Upon binding of a ligand to its respective TLR, MyD88 is recruited and in turn recruits a variety of IL-1 receptor-associated kinases (IRAKs) which leads to robust activation of NF-κB and subsequent expression of inflammatory cytokines such as Interleukin-6 (IL-6), IL-12 or tumor necrosis factor (TNF). The second TLR adaptor molecule is the TIR-domain-containing adapter-inducing interferon-β (TRIF) which initiates downstream signaling of TLR3 and TLR4. TRIF is known to control the TLR4-induced MyD88-independent pathway and is the only adaptor associated with TLR3. Activation of TRIF is associated with profound expression of interferons (IFN) through the activation of interferon regulatory factor 3 (IRF3) (Fitzgerald et al., 2003b; McWhirter et al., 2004). Furthermore, in contrast to

MyD88, TRIF can directly activate NF-κB and IRF3 which activates the interferon-β transcription. TLR4 is unique among the TLRs because it is the only TLR that is able to recruit both adaptor molecules, MyD88 and TRIF. MAL is required for signaling by TLR2 and TLR4, serving as a bridge to recruit MyD88. TRAM is the most restricted adaptor in TLR signaling as it exclusively interacts with TRIF in the TLR4 pathway(Fitzgerald et al., 2003b; Oshiumi et al., 2003b; Yamamoto et al., 2003b). TRAM is membrane-associated and acts analogous to MAL in MyD88 signaling as a bridging adaptor for TRIF (Hornung et al., 2006). SRAM, in contrast to the other four adaptor molecules, is a negative regulator of NF-κB and IRF activation (Carty et al., 2006). SRAM expression was shown to specifically inhibit TRIF-dependent gene induction without affecting the MyD88-dependent pathway of TLR signaling.

In general TLR signaling is mediated by selective use of different adaptor molecule combinations by different TLRs. Activation of TLRs by their respective ligands leads to distinct and characteristic intracellular signaling and expression of proinflammatory cytokines by induction of NF-κB. Additionally, several TLR signaling pathways lead to the expression of type I interferons by activation of TRIF.

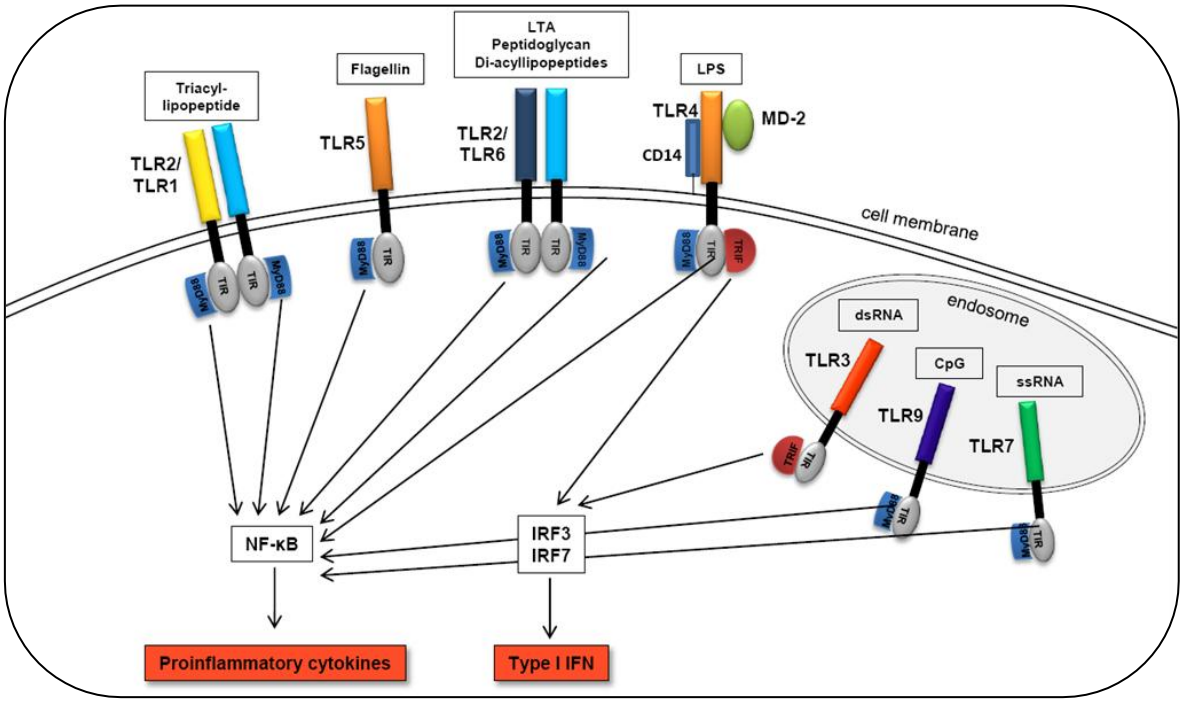

Figure 1.1 Toll-like **receptors and their ligands.**
Toll-like receptors (TLRs) are expressed on the cell surface or in intracellular compartments and recognize distinct conserved pathogen-associated molecular patterns (PAMPs), which are also known as TLR ligands. Upon TLR stimulation, intracellular adaptor molecules are recruited which initiate intracellular signaling pathways that activate NFκB- and IRF-dependent target genes resulting in the expression of proinflammatory cytokines and type I interferons (IFN).

1.3 Activation of T cells by APCs

Naïve T cells constantly circulate within the lymphatic tissue where they encounter their specific antigen presented by APCs on MHC molecules. Two classes of MHC molecules exist, namely MHC class I and MHC class II (Germain, 1994). MHC class I molecules are expressed on all nucleated cells and predominantly present endogenous peptides to antigen-specific T cells expressing the CD8 co-receptor. Therefore presentation of cytosolic peptides on MHC class I is a crucial mechanism in viral defense as activated cytotoxic CD8 T cells are able to sense and lyse infected cells. In contrast, MHC class II molecules are almost exclusively expressed on APCs such as DCs, macrophages, and B cells. For presentation, exogenous antigen is internalized by APCs and upon processing in lysosomes antigen-derived peptides are loaded on MHC class II. After transfer of the MHC class II molecule to the cell surface the antigenic peptide is presented to antigen-specific CD4-expressing T cells (Wolf and Ploegh, 1995).

Beside the recognition of the specific antigen presented on MHC molecules, which is also known as signal 1, T cell activation requires additional signals. Signal 2 involves co-stimulation by molecules such as CD80/86 provided on APC side which bind to CD28 on the T cell and trigger survival of T cells as well as secretion of cytokines (Boise et al., 1995; Carreno and Collins, 2002) like IL-2, a cytokine supporting the proliferation of T cells (Cantrell and Smith, 1984). Signal 3 is provided by cytokines such as IL-12 or type I IFNs which are secreted by matured APCs upon TLR stimulation or CD40 ligation (Curtsinger et al., 1999; Trinchieri et al., 2003). CD40 expressed by matured DCs interacts with CD40L, which is expressed on activated CD4 T helper cells. In that way, CD4 T cell help licenses the DC to initiate an efficient cytotoxic CD8 T cell response and stimulate generation of functional CD8 T cell memory.

Differentiation of naïve T cells into effector T cells strictly dependents on theses 3 signals (Curtsinger et al., 2003a; Curtsinger et al., 2003b). Moreover CD4 T cells develop into T helper cells (T_H), which are subdivided into T_H1, T_H2 and T_H17 cells (Moser and Murphy, 2000). T_H1 cells are crucial for the protection against intracellular pathogens as they produce IFNγ upon stimulation. T_H2 are directed against extracellular pathogens and induce the production of IL-4 (Moser and Murphy, 2000). TH17 cells are characterized by the

secretion of IL-17 and are involved in the clearance of extracellular pathogens (Bettelli et al., 2008).

Antigen-specific naïve T cells exist at very low frequencies in the host. However, upon activation they undergo massive expansion. This expansion phase lasts for five to eight days and CD8 T cell numbers may increase by > 10,000-fold (Badovinac and Harty, 2002; Butz and Bevan, 1998; Doherty, 1998). When cytotoxic T cells recognize infected cells they eliminate these cells by the release of cytotoxic agents such as granzymes, perforins and granulysin. A second way to kill target cells is the induction of apoptosis by stimulation of the FAS receptor by FAS ligand on the T cell. In the contraction phase after clearance of pathogens and infected cells, 90%-95% of the effector T cells undergo elimination (Sprent and Tough, 2001).

1.4 The immunopathogenesis of sepsis

The term sepsis describes a complex clinical syndrome that results from a harmful or damaging host response to infection. In Germany about 150,000 cases of sepsis are reported each year with an overall mortality of 30-50% (Brunkhorst, 2006).

The host immune response during sepsis can be separated into distinct but overlapping phases. The initial response in sepsis, referred to as the systemic inflammatory response syndrome (SIRS), is characterized by release of a number of inflammatory mediators including early response cytokines such as TNF, IL-1β, IL-6, IL-12, leukocyte-active chemokines, adhesion molecules, and leukotrienes (Dinarello, 2000; Levy et al., 2003). This initiation of substantial cytokine release is due to a systemic activation of immune responses by high levels of PAMPS and/or damaged host tissue, which in turn leads to overstimulation of immune cells. This so-called “cytokine storm” is accompanied by a dysregulation of the complement system characterized by excessive production of the complement factor C5a. The effects of C5a contribute to immunoparalysis, multi-organ failure, and imbalances in the coagulation system (Huber-Lang et al., 2001; Huber-Lang et al., 2002; Laudes et al., 2002). This cytokine storm, the massive release of complement, as well as the loss of coagulation can end up in multi organ dysfunction syndrome (MODS) and death (Rittirsch et al., 2008). The initial hyper-inflammatory phase is followed by counterregulation of the immune system characterized by the profound release of inhibitory molecules including anti-inflammatory cytokines such as IL-10, transforming growth factor-beta

(TGF-β), suppressors of pathogen recognition signaling cascades, immunomodulatory eicosanoids and hormones (van der Poll and van Deventer, 1999). This counter-regulatory phase is referred to as the compensatory anti-inflammatory response syndrome (CARS) (Bone, 1996a, b). SIRS and CARS overlap considerably and the overall immune status of patients depends on which response is dominant (Lyn-Kew and Standiford, 2008).

Moreover multiple immune cells are affected in sepsis and thus contribute to CARS. Changes in macrophages and monocyte function in sepsis includes the down-regulation of inflammatory cytokines such as IL-6 and TNF as well as the incapacity to release nitric oxide which illustrates the lack of respiratory burst (Goya et al., 1992; Munoz et al., 1991; Reddy et al., 2001). Splenic DCs undergo rapid apoptosis upon systemic endotoxin administration in mice. Similarly, there is a prolonged loss of DCs up to 15 days post induction of abdominal sepsis in both lung and spleen (Hotchkiss et al., 2002; Wen et al., 2008). Like macrophages and DCs, various lymphocyte populations are influenced by sepsis and likely contribute to the immunosuppressive effect of sepsis. DCs not undergoing apoptosis produce less IL-12 after exposure to high doses of endotoxin. Sepsis results in a substantial drop in the number of circulating B cells and CD4 T cells whereas no alterations in the CD8 T cell population could be observed (Hotchkiss et al., 2001). The late phase of sepsis is characterized by the expression of anti-inflammatory cytokines. IL-10 is one of the most potent anti-inflammatory cytokines which mediates leukocyte deactivation during sepsis. Upon stimulation with PAMPs, it is produced by many cells of the adaptive immune system such as T cells and B cells as well by cells of the innate immune system, for instance DCs, macrophages, mast cells, NK cells, eosinophiles and neutrophils (Siewe et al., 2006; Zhang et al., 2009). During infection, IL-10 inhibits the activity of Th1 cells, NK cells, and macrophages, which all contribute to an effective clearance of pathogens but tissue damage as well (Couper et al., 2008). When exposed to IL-10, DCs fail to induce a potent Th1 immune response. Furthermore, IL-10 inhibits the production of cytokines such as IL-2, TNF, and IL-5 as well as the proliferation of CD4 T cells and activation of T cells in the presence of IL-10 is described to result in anergy. In contrast, IL-10 has stimulatory effects on CD8 T cells and induces their recruitment, cytotoxic activity, and proliferation. (Couper et al., 2008; Moore et al., 2001; Saraiva and O'Garra, 2010).

Overall, the pathomechanisms of sepsis are very complex, dynamic, and not fully understood. Due to the fact that not a single key mediator causes sepsis, trials to neutralize such a

factor mostly failed and during the past years the mortality and rates of sepsis have not decreased (Dombrovskiy et al., 2007a, b).

1.5 Bacterial translocation from the gut

The human gut harbors approximately $1x10^{14}$ bacteria; tenfold more than the number of eukaryotic cells in an adult's body (Gill et al., 2006). Therefore, the gut represents a critical barrier between this huge reservoir of endotoxins and the entire body. Breakdown or overwhelming of this barrier results in the egress of bacteria and endotoxins with subsequent development of sepsis, initiation of a cytokine-mediated SIRS, MODS, and death. This process was termed "bacterial translocation" and describes the so-called "gut origin of sepsis hypothesis" (Berg and Garlington, 1979; Fraenkel, 1891; Nieuwenhuijzen et al., 1996a; Pastores et al., 1996). Therefore the gut may be an important factor of multiple organs failure and elucidates the absence of a discreet focus of infection in most patients with delayed SIRS and MODS.

Although there is growing evidence that bacterial translocation from the intestine is a normal phenomenon occurring in homeostasis, detrimental breakdown of the gut barrier function is described in several circumstances (Garside et al., 2004; Spahn and Kucharzik, 2004). Factors that influence bacterial translocation are believed to act on the homeostatic equilibrium between luminal organisms and the gut barrier (Wells, 1990) such as intestinal obstruction (Deitch, 1989; Sedman et al., 1994), jaundice (Deitch et al., 1990b; Sakrak et al., 2003), inflammatory bowel disease (MacFie et al., 2006; Takesue et al., 2002), ischaemia-reperfusion injury (Deitch, 2002), and several traumas including burn (Magnotti and Deitch, 2005) and stroke (Caso et al., 2009).

Even though bacterial translocation from the gastrointestinal tract is a well described phenomenon, the predominant route of translocating bacteria is controversially discussed (Berg, 1985, 1990, 1992, 1995; Deitch and Berg, 1987a; Deitch et al., 1990a). With respect to the anatomy of the gut two distinct routes are involved in dissemination of gut-derived bacteria: first, translocation via the mesenteric lymph nodes (mLNs) and/or second, vascular dissemination via the gut draining blood vessels. During their passage to the mLNs, most bacteria may be killed by the host´s defense system such as macrophages. The spread of bacteria beyond the mLN is supposed to go along the cysterna chilli and via the thoracic

duct draining in the left subclavin vein. Draining of bacteria via the vascular route allocates them along the vena mesenterica superior or vena mesenterica inferior and the portal vein into the liver. The route of bacterial dissemination is supposed to depend on the mechanism that promotes bacterial translocation. According to this, penetration of the intestinal epithelium and subsequent intracellular passage leads to translocation via the lymphatic vessels, whereas disruption of the tight junctions would enable the extracellular passage of gut-derived bacteria resulting in their vascular distribution (Berg, 1995).

1.6 Role of the liver and Kupffer cells in host defense

The liver is the largest organ in the abdomen, weighing approximately 1,5 kg in healthy adult humans. It serves a variety of functions such as synthesis and turnover of plasma proteins, the storage and metabolism of carbohydrates, the production of bile, and the detoxification of potentially dangerous chemicals. In addition, the liver plays a major role in clearing systemic bacterial infections (North, 1974). The clearance of bacteria as well as endotoxins is generally ascribed to fixed tissue macrophages namely Kupffer cells, which line the liver sinusoids and constitute the largest compartment of tissue macrophages present in the body (Klein et al., 1994). Nutrient rich venous blood is drained from the intestine via the portal vein which terminates in the liver and about 75% of hepatic blood is derived from this vein (Parikh et al., 2010). Kupffer cells are predominantly found in the periportal area and represent the first macrophage population to get in contact with bacteria and endotoxin derived from the gastrointestinal tract (Fox et al., 1987).

The rapid elimination of bacteria taken up by the liver is dependent on the complex interaction of Kupffer cells and microbicidal neutrophils, which immigrate rapidly in response to infection (Gregory et al., 2002; Gregory and Wing, 2002). Beside their phagocytic capacity, Kupffer cells are shown to bind bacteria extracellular by interaction of lectins on Kupffer cells and carbohydrate residues expressed by bacteria (Ofek and Sharon, 1988; Perry and Ofek, 1984). Bacteria bound to Kupffer cells are eliminated by immigrating neutrophils. Additionally, platelets have been shown to contribute to the inflammatory processes and participate on the fight against infection (Klinger and Jelkmann, 2002). Activation of platelets by TLR4 ligands leads to their binding to adherent neutrophils in the sinusoids which in turn leads to robust neutrophil activation and rapid formation of “neutrophils extracellular traps” (NETs) which are web-like structures of DNA that trap and kill bacteria (Brinkmann et al., 2004; Clark et al., 2007). Overall the majority of bacteria and

endotoxin coming from the gut or the circulation is cleared within the liver by Kupffer cells with the aid of neutrophils and platelets.

1.7 The spleen

The spleen is a secondary lymphoid organ that harbors about one fourth of the body´s lymphocytes and half of body´s monocytes. It fulfills two important functions in the body. First, the spleen has an important filter function for the blood and second, initiates immune responses to blood-borne antigens (Balogh et al., 2004; Cesta, 2006; Nolte et al., 2002). Splenectomized patients are more susceptible to infections with encapsulated pathogens including streptococci, pneumococci and meningococci (Cadili and de Gara, 2008).

The unique architecture of the spleen consists of two functionally and morphologically distinct compartments: the white pulp and the red pulp which are separated in space by the marginal zone (Figure 1.2 **A**).

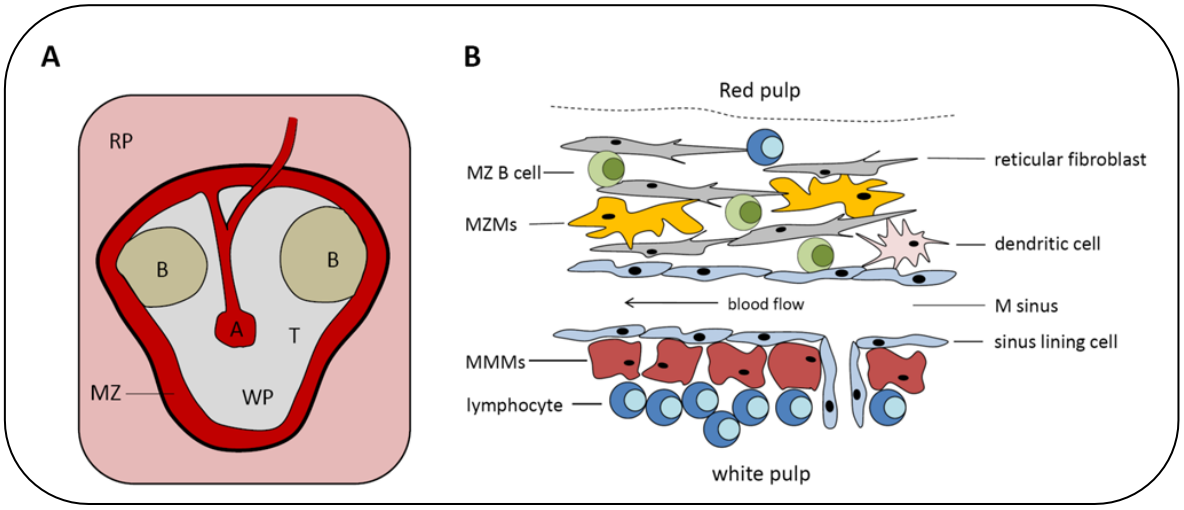

Figure 1.2 Structure of the white pulp of the spleen
(**A**) The central ateriol [A] is sheathed by the white pulp (WP). The WP consists of T cell areas (T) and B cell zones [B] both of which are separated from the red pulp (RP) by the marginal zone (MZ).
(**B**) The outer ring of the marginal zone adjacent to the red pulp consists of marginal zone macrophages (MZMs), reticular fibroblasts, dendritic cells, and marginal zone B cells (MZ B cells). The inner ring mainly consists of marginal zone metallophilic macrophages (MMMs) and lymphocytes. The marginal sinus, that separates the outer and the inner ring, is lined by endothelial cells. Adapted from (Mebius and Kraal, 2005).

1.7.1 The red pulp

The red pulp represents the non-lymphoid compartment of the spleen and functions as a blood filter that removes cellular debris and senescent red blood cells. Furthermore the red pulp stores erythrocytes, platelets, and iron. Arterial blood arrives into cords in the red pulp, a three dimensional meshwork that is build up by fibroblasts, reticular fibers, and associated macrophages. It forms an open blood system without an endothelial lining (Groom et al., 1991). Due to their stiffened membranes, senescent erythrocytes are unable to pass the splenic cords into the venous sinuses (Bratosin et al., 1998; Mebius and Kraal, 2005) and are actively phagocytosed by macrophages in the red pulp. These macrophages then recycle the iron of aging erythrocytes (Knutson and Wessling-Resnick, 2003).

1.7.2 The white pulp

The white pulp represents the lymphoid compartment and closely resembles the structure of a lymph node (Nolte et al., 2000). It is organized by lymphoid sheaths with T and B cell zones. In the T-cell zone, also known as periarteriolar lympohoid sheaths (PALS), T cells interact with DCs and passing B cells. In the B-cell zone activated B cells undergo clonal expansion which leads to isotype switching and somatic hypermutation. The integrity of the T- and B-cell zone is maintained by selective recruitment of T and B cells to their respective site by chemokines. CC chemokine ligand 19 (CCL19) and CCL21 attracts T cells and DCs to the T-cell zone, whereas CXCL13 is required for B cell recruitment to the B-cell zone (Ansel et al., 2000; Forster et al., 1999; Gunn et al., 1999).

1.7.3 The marginal zone

The marginal zone surrounds the white pulp and is involved in the induction of innate as well as adaptive immune responses. All cells that migrate from the red pulp into the white pulp have to pass the marginal zone. This is believed to be an active process in which G-protein coupled receptors, chemokines, and cell-cell interaction are essentially involved (Cyster and Goodnow, 1995; Johnston and Butcher, 2002; Kraal and Mebius, 2006). Besides being a transit area, the marginal zone contains a large number of resident cells that not only have unique properties but also seem to depend on each other for their localization

thereby establishing and maintaining the integrity of the marginal zone (Mebius and Kraal, 2005). Adjacent to the red pulp, the marginal zone forms a thicker outer ring which is composed of sinus lining endothelial cells, marginal zone macrophages (MZMs), reticular fibroblasts, DCs, and marginal zone B cells. The thinner, inner ring is build up by sinus lining endothelial cells separating the marginal sinus from the MZMs and metallophilic marginal zone macrophages (MMMs). MZMs and MMMs differ in their expression of surface molecules. MZMs express SIGNR1, a C-type lectin, and a type I scavenger receptor, namely macrophage receptor with collagenous structure (MARCO). MMMs are characterized by the expression of the sialic acid-binding immunoglobulin-like lectin-1 (Siglec-1, CD169). These surface molecules are closely associated with recognition and binding of pathogens. SIGNR1 on MZMs mediates binding to polysaccharides on pathogens such as *Streptococcus pneumoniae* or *Mycobacterium tuberculosis* and Siglec-1 on MMMs has been shown to serve as a phagocytic receptor for sialylated pathogens such as *Neisseria meningitides* (Jones et al., 2003; Kang et al., 2004; Lanoue et al., 2004). The other important receptor MARCO, present at the surface of MZMs and in less extend on MMMs, can recognize many pathogens including *Escherichia coli* and *Staphylococcus aureus* (Elomaa et al., 1995). These receptors enable macrophages in the marginal zone so sense, trap, and eliminate blood-borne bacteria. Importantly, our group recently could show that MMMs efficiently capture adenoviruses and exclusively transfer antigen to CD8+ splenic DCs, thereby essentially contributing to initiation of adaptive immune responses in the spleen (Backer et al., 2010).

1.7.4 Dendritic cells in the spleen

DCs are professional APCs that most efficiently prime T cell responses. Spleen-resident DCs can be divided into different subsets based on their localization, expression of phenotypic markers, and function (Heath et al., 2004). DCs positive for CD8 are predominantly found in the T cell zone and outer marginal zone of the spleen and are further characterized by the expression of DEC205 (CD205) (Idoyaga et al., 2009). The subpopulation of DEC205+ DCs present in the marginal zone is specialized in cross-presentation of antigens and activation as well as tolerization of CD8+, cytotoxic T lymphocytes (Belz et al., 2005; den Haan et al., 2000; Schulz and Reis e Sousa, 2002). CD8- DCIR2+ DCs are mainly localized in the marginal zone and are specialized in the activation of CD4+ T lymphocytes by presentation of antigens on MHCII molecules (Dudziak et al., 2007). Upon activation

by microbial products, all DC subsets present in the marginal zone migrate to the T cell areas of the spleen where they interact with antigen-specific T cells (De Smedt et al., 1996)

1.8 Type I interferons

Type I interferon (IFN) was discovered by Isaacs and Lindenmann in 1957 and originally described as an "activity" that was secreted by virus-infected cells that protects other cells from infection (Isaacs and Lindenmann, 1957). The name IFNs describes their ability to stimulate the synthesis of a large set of antiviral gene products which "interfere" with viral replication and spread (Katze et al., 2002). In mammals the type I IFN genes form a large multigene family comprising the species α, β, κ, ω, and τ (Pestka et al., 2004b). During viral and bacterial infection, IFN-α and IFN-β are the main synthesized type I IFNs. A single IFN-β gene is found in most mammals, but the IFN-α family is formed by 14 functional genes in mice. However, the biological significance of having many IFN-α genes is not yet clear. The production of type I IFN is triggered upon stimulation with viral and bacterial PAMPs in almost all cell types including macrophages, DC subsets, natural killer cells (NK cells), and T cells (Bogdan et al., 2004). PPR stimulation results in a secretion of immediate-early type I IFNs. This fast expression of type I IFNs is promoted by interferon regulatory factor 3 (IRF3) which is expressed in probably all cells (Fitzgerald et al., 2003a). IRF3-induced type I IFN is rapid and results in low amounts of IFN-α/β that initiates an amplification loop by autocrine and paracrine stimulation of the type I IFN receptor (IFNAR). Both, IFN-α and IFN-β, signal via the IFNAR complex which comprises two subunits, IFNAR1 and IFNAR2 (Stark et al., 1998). These two chains are permanently associated with members of the Janus protein tyrosine kinase family, specifically with the tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1). Upon IFN binding to IFNAR, janus kinases phosphorylate the receptor and Signal Transducers and Activator of Transcription (STATs). Phosphorylation initiates signal transduction in association with IRF9 which binds to the IFN-stimulated response element (ISRE) and leads to subsequent expression of interferon-regulated genes like iNOS, CD80, CD86, or IRF 7 (Noppert et al., 2007). In turn, IRF7 binds to the IRF binding site (IRFBDS) and induces delayed but profound production of INFα (Figure 1.3). Besides their well known antiviral properties, type I IFNs enhance DC maturation and promote the activation as well as differentiation of CD8 T

cells by providing signal 3 (Curtsinger et al., 2005; Luft et al., 1998; Montoya et al., 2002). Thus, type I IFNs are crucially involved in linking innate and adaptive immune responses (Uematsu and Akira, 2007).

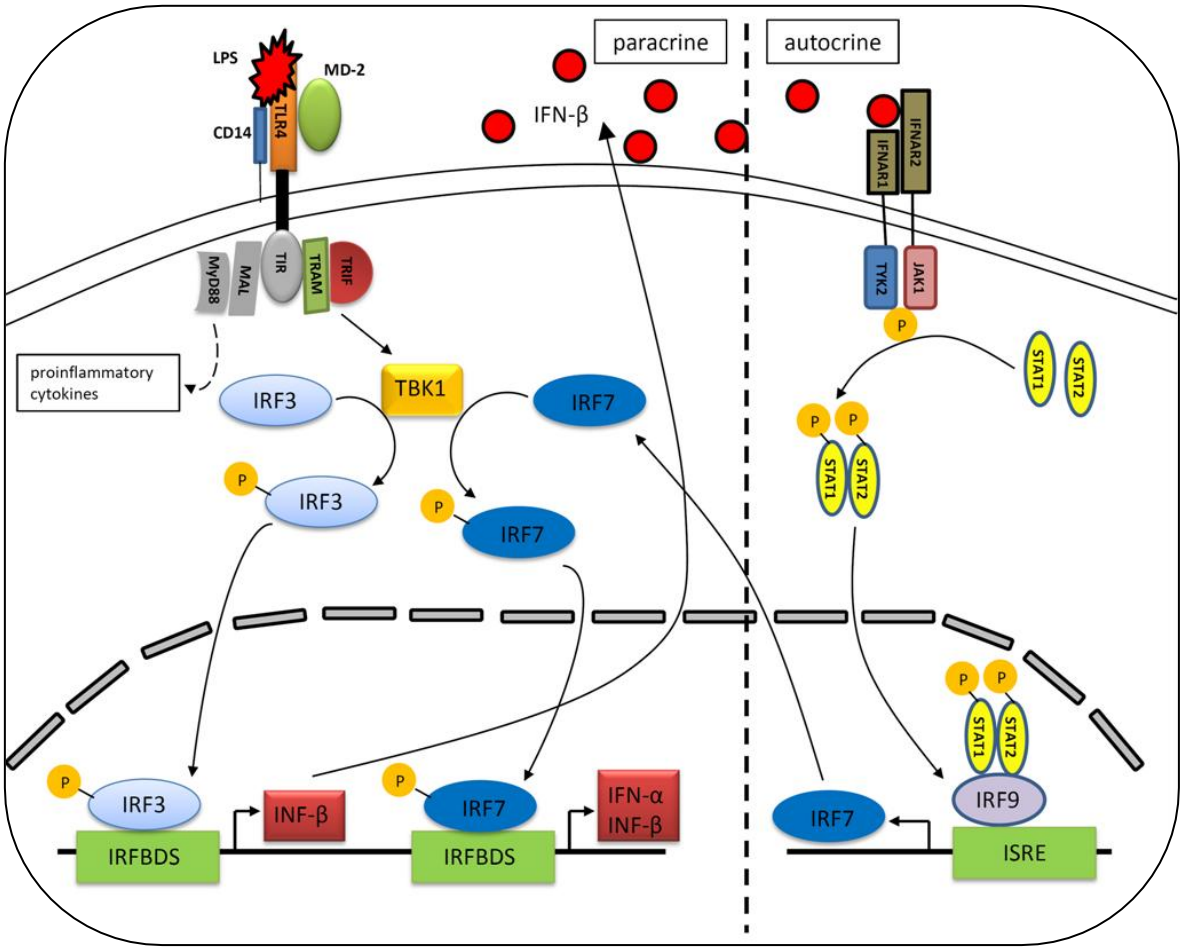

Figure 1.3 LPS-induced production of type I IFNs
In cells that not constitutively express little or no IRF7, amounts of the protein are increased by an amplification loop. Rapid signal transduction through TLR4 causes IRF3 activation, IFN-β synthesis, and signal transduction through the IFNAR. IFNAR signal transduction includes phosphorylation of IRF9 and subsequent IRF7 expression because the protein is encoded by an IFN-inducible gene (ISRE). Activation of newly synthesized IRF7 by tank-binding kinase (TBK1) leads to increased expression of IFN-β and many IFN-α genes.
Adapted from (Decker et al., 2005) and (Mahieu and Libert, 2007)

2 Aim of the thesis

Sepsis is currently the 10th leading cause of death and accounts for significant healthcare expenditures in the developed world. As the gut represents a large reservoir for endotoxins, it is assumed that the loss of gut barrier function results in bacterial translocation and subsequent sepsis. The pathophysiology of sepsis is inherently complex and research efforts in the field of sepsis have largely focused on the innate immune system, whereas the influence on adaptive immunity remains largely unknown.

The aim of this thesis was to analyze the influence of gut-derived bacteria on the induction of systemic immune responses. We were particularly interested in the following questions:

- Does bacterial translocation influence the induction of adaptive immunity?
- What is the liver's role in preventing the dissemination of gut-derived bacteria?
- Which role does the spleen play in the suppression of adaptive immunity? Which splenic cell types mediate immunity? Are they affected in their function to generate immunity by exposure to *E.coli*?
- Is activation of TLR and its downstream signaling involved in mediating immune suppression?

3 Material and Methods

3.1 Materials

3.1.1 General laboratory equipment

Autoclave	Belimed, Köln
Balances	U4100-OD2.2 and MC BA 100 (Sartorius, Göttingen)
Beakers	5 ml, 10 ml, 25 ml, 50 ml, 100 ml, 150 ml, 250 ml, 500 ml (Schott, Mainz)
Centrifuges	Multifuge3 S-R, Biofuge fresco, Biofuge pico (Heraeus, Braunschweig)
Counting Chamber	Neubauer (Brand, Wertheim)
Digital camera	CCD-1300 (Vosskuehler, Osnabrück)
Erlenmeyer flasks	50 ml, 100 ml, 250 ml, 500 ml (Schott, Mainz)
Flow cytometers	FACSCalibur, CantoII, LSRII (BD Biosciences, Heidelberg)
Freezers (-20°C)	Liebherr, Biberach
Freezers (-80°C)	Hera freeze (Heraeus, Braunschweig)
Heating block	ThermoStat plus (Eppendorf, Hamburg)
Ice machine	Icematic (Scotsman®, Frimont Bettolinc, Pogliano, Italy)
Incubators	HERAcell (Heraeus, Braunschweig)
IVIS®200	Xenogen, San Francisco, USA
Magnet stirrer	IKA® Laboratory Equipment, Staufen
Measuring cylinders	50 ml, 100 ml, 250 ml, 500 ml, 1l (Schott, Mainz)

Microscopes	IX71 and CKX31 (Olympus, Hamburg)
	Epifluorescence microscope Edipse TE 2000 ($Ni^{-/-}$n, Düsseldorf)
Microwave	Panasonic, Wiesbaden
pH-meter	pH 523 (Wissenschaftlich-Technische Werkstätten, Weilheim)
Pipette-Boy	Pipetus (Hirschmann Labortechnik, Eberstadt)
Pipettes	Gilson, Heidelberg and Eppendorf, Hamburg
Preparation Instruments	Labotec, Göttingen
Refrigerators (+4°C)	Bosch, Stuttgart and Liebherr, Biberach
Sieves, steel	University Bonn, Department "Feinmechanik"
Sonificator	UW2070/Sonoplus (Bandeln electronic, Berlin)
Spectrophotometers	Ultrospec 3100 UV/VIS (Amersham Pharmacia, Freiburg);
	NanoDrop™ ND 1000 (NanoDrop Products, Wilmington, USA)
Threaded bottles	100 ml, 250 ml, 500 ml, 1l, 2l (Schott, Mainz)
Ultra-pure water system	NANOpure Diamond, Barnstead (Werner, Reinstwassersysteme, Leverkusen)
Waterbath	TW8 (Julabo, Seelbach)
Workbench, sterile	HERAsafe (Heraeus, Hanau)

3.1.2 Software

Endnote X1	Thomson ISI ResearchSoft, USA
FACS Diva V6.1.1	BD Biosciences, Heidelberg
Flowjo V8.8.4	Tree star, Inc., USA
Illustrator CS4	Adobe, USA

Living Image 2.50 (IVIS)	Xenogen, USA
Microsoft Office 2008	Microsoft, USA
Photoshop CS4	Adobe, USA
Prism 4 for Macintosh	GraphPad Software, USA

3.1.3 Consumables

Cover slides	21x46mm (Marienfeld, Lauda-Königshofen)
Cryo vials	VWR International, Darmstadt
Cryomolds	VWR International, Darmstadt
FACS tubes	polystyrene, 12/75 mm (Sarstedt, Nümbrecht)
Injection needles	27G (grey), 25G (orange), 20G (yellow) (BD Microlance, Heidelberg)
	33G (Fine science tools, Heidelberg)
Inoculation loops	10µl (Greiner bio-one, Solingen)
Gloves DermaClean™	Ansell, Red Bank New Jersey, USA
Microtiter plates	96-well, round and flat bottom (Greiner bio-one, Solingen)
Parafilm	Parafilm "M"® (American National Can TM, Greenwich, USA)
Pasteur pipettes	150 mm and 230 mm (Roth, Karlsruhe)
Petri dishes	10 cm (Greiner bio-one, Solingen)
Pipette tips	10 µl, 200 µl, 1000 µl (Greiner bio-one, Solingen)
Plastic Pipettes	5 ml, 10 ml, 25 ml (Sarstedt, Nümbrecht)
Polyamide tissue	"Gaze" (Labomedic, Bonn)
Polypropylene tubes	sterile, 15 ml and 50 ml (Greiner bio-one, Solingen)
Reaction tubes	0.5 ml, 1.5 ml, 2 ml (Eppendorf, Hamburg)
Scalpel	Feather (Osaka, Japan)

Sterile filter	0.2 µm (Schleicher & Schuell)
Syringes	2 ml, 5 ml, 10 ml, 20 ml BD Discardit™ (BD Bioscience, Heidelberg)
Tissue culture plates	6-, 12-, 24-, 48-, 96-well (TPP, St. Louis, USA or Sarstedt, Nümbrecht)
Tuberculin syringes	Omnifix®-F (Braun, Melsungen)

3.1.4 Chemicals and reagents

Ampicillin sodium salt	Sigma Aldrich, München
Bovine serum albumin (BSA)	Roth, Karlsruhe
Brain Heart Infusion Broth	Roth, Karlsruhe
CFSE	Molecular Probes, Leiden, Netherlandes
Columbia Blood Agar Base	Oxoid, Wesel
Dimethylsulfoxide (DMSO)	Merck, Darmstadt
Disodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt
DOTAP®	Roth, Karsruhe
Ethanol, absolute	Merck, Darmstadt
Ethylene diamine tetraacetic acid (EDTA)	Sigma Aldrich, München
Fetal calf serum (FCS)	PAA, Cölbe
Glycerol ($C_3H_8O_3$)	Roth, Karlsruhe
Heparin	Ratiopharm, Ulm
Hoechst 33258	Sigma Aldrich, München
Hoechst 33342	Molecular Probes, Leiden, Netherlands
Isoflurane	DeltaSelect, Pfullingen
LB-medium (Luria/Miller)	Roth, Karlsruhe
Luciferin	Caliper Lifesciences, Rüsselsheim

MacConkey Agar	Roth, Karlsruhe
Natural silk 5-0	Catgut, Markneukirchen
Natural silk 4-0	Ethicon, Norderstedt
Ovalbumin (OVA), grade V	Sigma Aldrich, München
Paraformaldehyde (PFA)	Fluca, Buchs
Phosphate buffered saline (PBS)	Biochrom, Berlin
Phosphate buffered saline (PBS) solution	Sigma Aldrich, München
RPMI 1640 medium	Sigma Aldrich, München
Sodium azide (NaN_3)	Sigma Aldrich, München
Sodium bicarbonate (Na_2HCO_3)	Sigma Aldrich, München
Sodium chloride, 0.9%	B. Braun, Melsungen
Sodium hydroxide (NaOH)	Merck, Darmstadt
Sucrose	Roth, Karlsruhe
Tachocomb®	Nycomed, Konstanz
Tissue-Tek® O.C.T™	Sakura, Netherlands
Trisodium citrate ($Na_3C_6H_5O_7$)	Sigma Aldrich, München
Trypane blue (0.4%)	Lonza, Köln
Trypsin/EDTA	Lonza, Köln
Triton X-100	Sigma Aldrich, München

3.1.5 Buffers, media and solutions

FCS (fetal calf serum)

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

PBS (phosphate buffered saline)

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

0.5 M EDTA

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

FACS buffer

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

4% (w/v) PFA solution

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

30% (w/v) sucrose solution

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

50 mM luciferin solution

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

Türk´s solution

10% (v/v) acetic acid in 0,4% trypane blue. Stored at room temperature

3.1.6 TLR ligands

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

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

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

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

Lipopolysaccharide (LPS) was purchased from Invivogen, dissolved in 0.9% NaCL at a concentration of 1µg/µl. Aliquots were stored at -20°C

Substance	Description	Reference
LPS ultrapure	*E.coli* O111:B4	(Poltorak et al., 1998)

3.1.7 Peptides

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

Peptides	MHC haplotype	Amino acid sequence
$OVA_{254-267}$ (S8L)	H-$2k^b$	SIINFEKL
Influenza peptides	H-$2k^b$	ASNENMETM and SSLENFRAYV
HSV peptide	H-$2k^b$	SSIEFARL

3.1.8 Antibodies

Immunohistology antibodies

The following antibodies were used in immunohistology of spleens. Anti-Siglec-1 antibody was kindly provided by Prof. Kraal, VU University Medical Center, Amsterdam, Department of Molecular Cell Biology and Immunology

Antigen	Species	Clone	Source	Conjugate
Biotin			Invitrogen	Streptavidin-AlexaFluor®488
Biotin			Invitrogen	Streptavidin-AlexaFluor®647
CD11c	Hamster	N418	eBioscience	Biotin
GFP	Goat	Polyclonal	Gene Tex	Biotin
Siglec-1	Rat	SER-4	Prof. Kraal, Amsterdam	AlexaFluor®488
Rat	Goat		Invitrogen	AlexaFluor®647
Hamster	Goat		Jackson ImmunoResearch	
MxA		M143	Prof. Haller, Freiburg	

Fluorochrome conjugated antibodies used in FACS

The following antibodies were purchased from eBioscience or BD Biosciences (if not otherwise stated) for flow cytometric analysis of murine molecules expressed at the cell surface or intracellularly. All antibodies were labeled with a fluorochrome (FITC, PE, PE-Cy7, PerCP-Cy5.5, APC, Al647, APC-Cy7, Pacific Blue, Al405) and employed at previously determined concentrations.

Antigen	Isotype	Clone	Annotation
CD11b	rat IgG2b, κ	M1/70	= Mac-1, integrin αM chain
CD11c	hamster IgG	N418	= integrin αX chain
CD16/32	rat IgG2b, κ	2.4G2	= anti $Fc^{\gamma}R$ III + II
CD4	rat IgG2b, κ	GK1.5	
CD8a	rat IgG2a, κ	53-6.7	= Ly-2
F4/80	rat IgG2a, κ	BM8	
Gr-1	rat IgG2a, κ	RB6-8C5	
S8L/H-$2K^{b}$ Pentamers			purchased from Proimmune

3.1.9 Viruses and bacteria

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

Pathogen	Description	Source (Reference)
AdOVA	E1 and E3 deleted recombinant adenoviral vector expressing the full length OVA protein under the CMV promoter	Dr. Andreas Untergasser; Prof. Dr. Ulrike Protzer
AdLOG	E1 and E3 deleted recombinant adenoviral vector expressing luciferase, OVA-derived peptide SIINFEKL, and GFP	Prof. Dr. Thomas Tüting (Schweichel et al., 2006)
E.coli	K12 laboratory strain	Institute stock
E.coli lux	Recombinant Escherichia coli expressing luxABCD genecluster	Dr. Ulrich Dobrinth

HSV-1	Wild type Herpes simplex virus type 1	Dr. Christoph Coch; Nico Busch
Influenza virus	Wild type Influenza A PR8/H1N1 virus	Dr. Christoph Coch; Nico Busch
LmOVA	Recombinant Listeria monocytogenes expressing the full length OVA protein	Prof. Dr. Klaus Pfeffer; Dr. Stefanie Scheu (Pope et al., 2001)
Listeria monocytogenes	EGD strain	Prof. Trinad Chackraborty
MCMV-OVA	Mouse cytomegalovirus expressing the full length OVA protein	Prof. Dr. Percy Knolle (Sacher et al., 2008)

pHrodo™ *E.coli* Bioparticles® conjugate was purchased from Molecular Probes™, Invitrogen, Karlsruhe. *E.coli* Bioparticles® get visible upon phagocytosis, as they are conjugated to pHrodo™, a dye that is sensitive to acidic pH. Preparation of *E.coli* Bioparticles® was performed according to the manufacturer's instructions. Briefly 2 ml provided "uptake buffer" were added to the lyophilized product. The solution was briefly vortexed and sonicated for 10 min. until homogenous dispension.

3.1.10 Mouse strains

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

Strain	Description	Source (Reference)
CD11c-DTX	DC-specific expression of the diphtheria toxin receptor	Steffen Jung (Jung et al., 2002)
CD14 $^{-/-}$	deficient in CD14	PD Dr. Georg Baumgarten (Haziot et al., 1996)
IFNAR $^{-/-}$	deficient in the type I IFN receptor	Prof. Dr. Rainer Zawatzky (van den Broek et al., 1995)
IL-10 GFP	Reporter mice expressing GFP under the IL-10 promoter	Prof. Dr. Hermann Wagner (Bouabe et al., 2008)
IL-10 $^{-/-}$	deficient in IL-10	Prof. Dr. Achim Hörauf (Kuhn et al., 1993)
IRF3/7 $^{-/-}$	deficient in IRF3 and IRF7	Prof. Dr. Hermann Wagner
IRF7 $^{-/-}$	deficient in IRF7	Prof. Dr. Thomas Decker; Prof. Dr. Mathias Müller (Honda et al., 2005)
MXA	regained expression of MXA protein by back-crossing	PD. Dr. Jörg Wenzel (Wenzel et al., 2005b)

MyD88 $^{-/-}$	deficient in MyD88	Prof. Dr. Hermann Wagner (Adachi et al., 1998)
TLR2 $^{-/-}$	deficient in TLR2	Prof. Dr. Hermann Wagner (Takeuchi et al., 1999)
TLR4 $^{-/-}$	deficient in TLR4	Prof. Dr. Hermann Wagner (Hoshino et al., 1999)
TLR9 $^{-/-}$	deficient in TLR9	Prof. Dr. Hermann Wagner (Hemmi et al., 2000)
TNFRI/II	deficient in TNF receptor I and II	Dr. Horst Bluethtmann (Pfeffer et al., 1993)
TRIF $^{-/-}$	deficient in TRIF	Prof. Dr. Hermann Wagner (Yamamoto et al., 2003a)

3.2 Methods

3.2.1 Experimental treatment of mice

Pathogens and other reagents were adjusted in 0.9% NaCl or PBS for experimental injection of mice. Intravenous (i.v.) and intraperitoneal (i.p.) injections were performed with a volume of 200 µl, intraportal (i.po.) injections with a volume of 50 µl.

3.2.1.1 Infection with viruses

Mice were infected i.v. with $5x10^9$ virus particles of different recombinant adenoviruses
Mice were infected i.v. with $1x10^4$ PFU Herpes simplex virus type 1 and $1x10^5$ or $1x10^6$ PFU Influenza A PR8/H1N1 virus.

3.2.1.2 Infection with recombinant Listeria monocytogenes

Recombinant Listeria monocytogenes expressing OVA (LmOVA) were cultured overnight in Brain Heart Infusion Broth shaking at 37°C. Colony forming units (CFU) of LmOVA were quantified by McFarland standard. The OD was measured at 600 nm and CFU/ml was calculated and the indicated number of bacteria was injected i.v. in 200µl PBS.

3.2.1.3 Infection with *E.coli*

Recombinant Escherichia coli expressing the lux operon (*E.coli* lux) or wild type *E.coli* were cultured for four hours in Luria-Bertani broth containing 20 mg/l Ampicillin. Colony forming units (CFU) of *E.coli* were quantified by McFarland standard. The OD was measured at 600 nm and CFU/ml was calculated and the indicated number of bacteria was injected i.v. in 200µl PBS

3.2.1.4 Immunization with OVA-coupled DEC205, Siglec-1 and anti- CD40

Mice received 3 µg OVA-coupled anti-DEC205 or anti-Siglec-1 in combination with 20 µg anti-CD40 antibody intravenously

3.2.1.5 Colon ascendens stent peritonitis (CASP)

By usage of a scalpel the cannula of a peripheral venous catheter with a diameter of 16G is engraved all around at about 2 mm above the tip of the cannula. This cannula is then used as a stent which is transplanted into the colon ascendens. After anesthesia of mice with 2% Isofluorane and disinfection of the skin with 70% ethanol the abdominal skin and subsequent the peritoneum is opened with scissors. The cecum, the colon ascendens and the terminal ileum are carefully laid open and placed on top of the abdominal wall. Now the peripheral venous catheter is introduced into the colon ascendens, the indentation of the cannula is fixed with silk at the intestinal wall and the needle is retracted. The peripheral venous catheter is cropped at 1 mm above the serosa and a small drop of gut content is released through the stent by gently pressing the cecum. Next the gut is placed back into its anatomical correct position and peritoneum, and skin is sutured.

3.2.1.6 Splenectomy

Mice were anesthetized with Isofluorane and underwent laparotomy. The spleen was exposed, the ateria splenica was ligated with silk and divided between the spleen and the ligation. After careful removal of the pancreatic tissue a loop of silk is drawn over the spleen and posterior the vena splenica is ligated by tighten the loop. The spleen is removed, peritoneum, and skin is sutured.

3.2.1.7 Injection of *E.coli* into the portal vein

Herewith mice underwent laparotomy and the portal vein was exposed. *E.coli* was injected in a volume of 50µl with a 33 gauge needle into the portal vein and homeostasis was achieved pressing a small piece of TachoComb® on the site of injection.

3.2.2 Microbiological methods

3.2.2.1 Determination of bacterial load

A single cell suspension was prepared from organs by pressing the organs through a sterile sieve (mesh size 250 µm). Cell suspension of liver was resuspended in 40 ml PBS, spleen and lung were resuspended in 10 ml PBS and mesenteric lymph nodes in 5 ml PBS. 100 µl of the cell suspension are plated on selective MacConkey Agar and after 24 h incubation at 37°C CFU are calculated per organ or per g tissue respectively.

3.2.2.2 Listeria in vitro killing assay by macrophages

Analysis was performed with the help of Dr. Zeinab Abdullah. Single cell suspension was prepared from the spleen of mice injected with UV killed E. coli or PBS by passing through cell strainer. Cell suspension was plated in 12 ml of antibiotic free medium (RPMI + 10% FCS) in 10 cm plates for 2 hrs at 37 °C followed by removal of non adherent cells by using warm PBS. Adherent cells (macrophages and DCs) were harvested by trypsinization. Cells concentration was adjusted to $2x10^6$ cells/ml in RPMI medium and 250 µl of cell suspension was plated in 48 well plates. Listeria monocytogenes was harvested at the log phase by centrifugation at 4000 rpm for 10 min at RT. The pellet washed twice with PBS and resuspended in PBS

OD600 measured and CFU was adjusted to $2x10^7$ CFU/ ml in antibiotic free medium. Medium was aspirated from the cells and 250 µl of the Listeria was added per well and plates incubated at 37°C with rotation for 30 min.. A sample of the injected Listeria was cultured to control the exact infection dose. After 30 min of incubation cells were washed twice with PBS containing 100µg/ml gentamycin to eliminate extracellular bacteria and cells were then incubated in 250µl medium (RPMI + 10% FCS+ 50µg/ml gentamycin). Following 30, 60, 120 and 240 min after infection, wells were washed twice with PBS (no antibiotic) and 150µl of 0.1% triton X (in H_2O) were added per well in order to lyse cells. After 5 min incubation on ice cells lysates were scraped and resuspended thoroughly and five serial dilutions of 1: 10 in PBS were prepared. 100µl of the last three dilutions were plated on BHI agar plate and incubated 24 h at 37°C followed by determination of the CFU.

3.2.3 Flow cytometric analysis (FACS)

Flow cytometry (fluorescence activated cell sorting, FACS) was used to examine the expression of different surface molecules and to determine the generation of endogenous OVA-specific CD8 T cells by S8L/H-2K^b pentamer staining. Data were acquired using a FACS Canto II and subsequently analyzed using the FlowJo software.

3.2.3.1 Surface staining of molecules

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

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

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

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

Cytotoxic activity of endogenous antigen-specific CD8 T cells was measured in spleens five days post pathogen infection by performing an *in vivo* cytotoxicity assay (Feuerer et al., 2003). Briefly, splenocytes from syngeneic donor mice were divided into two fractions. One fraction was pulsed with 1 µM of the specific H-2K^b peptides SIINFEKL (OVA), ASNENMETM/SSLENFRAYV (Influenza virus), or SSIEFARL (HSV-1 virus) for 20 minutes at 37°C. Subsequently, peptide-loaded cells were labeled with 1 µM of CFSE by incubation for 15 minutes at 37°C (CFSE^{high}, target cells). The second fraction was not pulsed with peptide and labeled with 0.1 µM CFSE (CFSE^{low}, reference cells). Upon labeling, cells were washed extensively and counted. Both populations were mixed in a 1:1 ratio (CFSE^{high}/ CFSE^{low}) and 1x10^7 cells were injected i.v. into recipient mice. Spleens were removed four hours later and homogenized by passing through a metal sieve. After centrifugation for 10 minutes at 486 x g, splenocytes were resuspended in 5 ml MACS buffer. Lysis of peptide-loaded cells was measured by flow cytometric analysis. Unimmunized naive mice served as a control. The percentage of specific lysis was calculated using the following formula:

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

3.2.5 Histology

3.2.5.1 Histological analysis of MxA expression

Sections and staining was kindly performed by Jörg Wenzel, Department *of Dermatology university of Bonn*. In short, MxA labeling was performed on paraffin-embedded tissue sections (4 mm). Sections were incubated with anti-MxA-antibody (dilution 1 : 100) overnight at room temperature after heat antigen retrieval. Secondary labeling was performed using the LSAB2 kit (DAKO) (Wenzel et al., 2005a).

3.2.5.2 Immunohistology of the spleen

After excision spleens were fixated for 30 min. in 1% PFA and transferred into PBS containing 30% (w/v) sucrose. After spleens had sunken to the bottom of the tubes they were

shortly washed with PBS to remove excess sucrose. After careful removal of moisture spleen were embedded in fresh O.C.T.™ medium and frozen at -80°C.

Sectioning and staining were performed on the same day in order to keep background fluorescence low. Spleens were cut into sections of 7 µm and were blocked in 5% (v/v) mouse serum in PBS for 5 min. at room temperature. For staining, all antibodies were diluted in 2% (v/v) mouse serum in PBS. All antibodies were used in previously determined concentrations. Between different staining steps, sections were thoroughly washed with PBS. Following incubation for 45 min. with anti-SER-4 and anti-CD11c, sections were stained with anti-rat antibody and anti-hamster antibody for 30 min.. After blocking for 15 min. in 20% (v/v) rat serum in PBS, sections were incubated for 45 min. with biotinylated anti-GFP and for 30 min. with Streptavidin-AlexaFluor®488 conjugate. Nuclei were stained by incubation of sections with 1 µg/ml DAPI for 10 min..

3.2.6 *In vivo* imaging of bioluminescence

Mice were analyzed at indicated time points after injection of recombinant AdLOG immediately before measurement of bioluminescence using the real-time IVIS Imaging System, mice were injected i.p. with 200 µl luciferin solution. Mice infected with *E.coli* lux were analyzed without injection of luciferin. Analysis was performed under inhalational anesthesia with isoflurane. Data were acquired using the Living Image 2.50 software.

3.2.7 Determination of cell number

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

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

3.2.8 Statistics

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

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

4 Results

4.1 Bacterial translocation and its impact on adaptive immune responses

Toll-like receptors (TLR) enable the vertebrate´s immune system to recognize structurally conserved molecules derived from pathogens. TLR stimulation by their respective ligands leads to activation of the immune system and generally results in immunity (Janeway and Medzhitov, 2002). Therefore, locally administered TLR ligands, such as complete Freund´s adjuvant, are commonly used as immunopotentiator. However, we and other groups have shown that TLR ligands administered systemically induce immunoregulatory processes, which result in suppression of antigen-specific immune responses (Mellor et al., 2005; Wingender et al., 2006).

The human gut harbors approximately $1x10^{14}$ bacteria; tenfold more than the number of eukaryotic cells in an adult's body. Therefore, the gut represents a critical barrier between this huge reservoir of endotoxins and the entire body. The gut barrier becomes leaky under certain conditions, for example in inflammatory bowl diseases (IBD) or as a complication occurring after abdominal surgery. Subsequent inflammation leads to the breakdown of the gut barrier. As a consequence endotoxins as well as bacteria are released into the periphery resulting in sepsis.

Since it is known that sepsis is a common complication of abdominal surgery we investigated in the present study if circulating bacteria might have analogous effects on the generation of murine adaptive immune responses.

4.1.1 Dissemination of *E.coli* lux after CASP

As a common animal sepsis model we used the colon ascendens stent peritonitis (CASP) technique. In this model a stent is placed colon ascendens in direction to colon transversum, which leads to a subsequent release of the gut content into the peritoneum. Sham treated mice underwent laparotomy and the gut was touched with cotton swabs.
At first we determined the distribution of bacteria in C57BL/6 wild type mice after CASP. Besides the classic microbiological techniques, such as determination of colony forming

units per organ, we assessed the distribution of bacteria by *in vivo* imaging of bioluminescent luciferase expression at different time points after CASP using the *in vivo* imaging system (IVIS). Mice were fed with luciferase expressing E. coli (*E.coli* lux) one hour prior to gut manipulation. The distribution of bacteria was assessed by *in vivo* imaging of bioluminescent luciferase expression at different time points after CASP using the *in vivo* imaging system (IVIS).

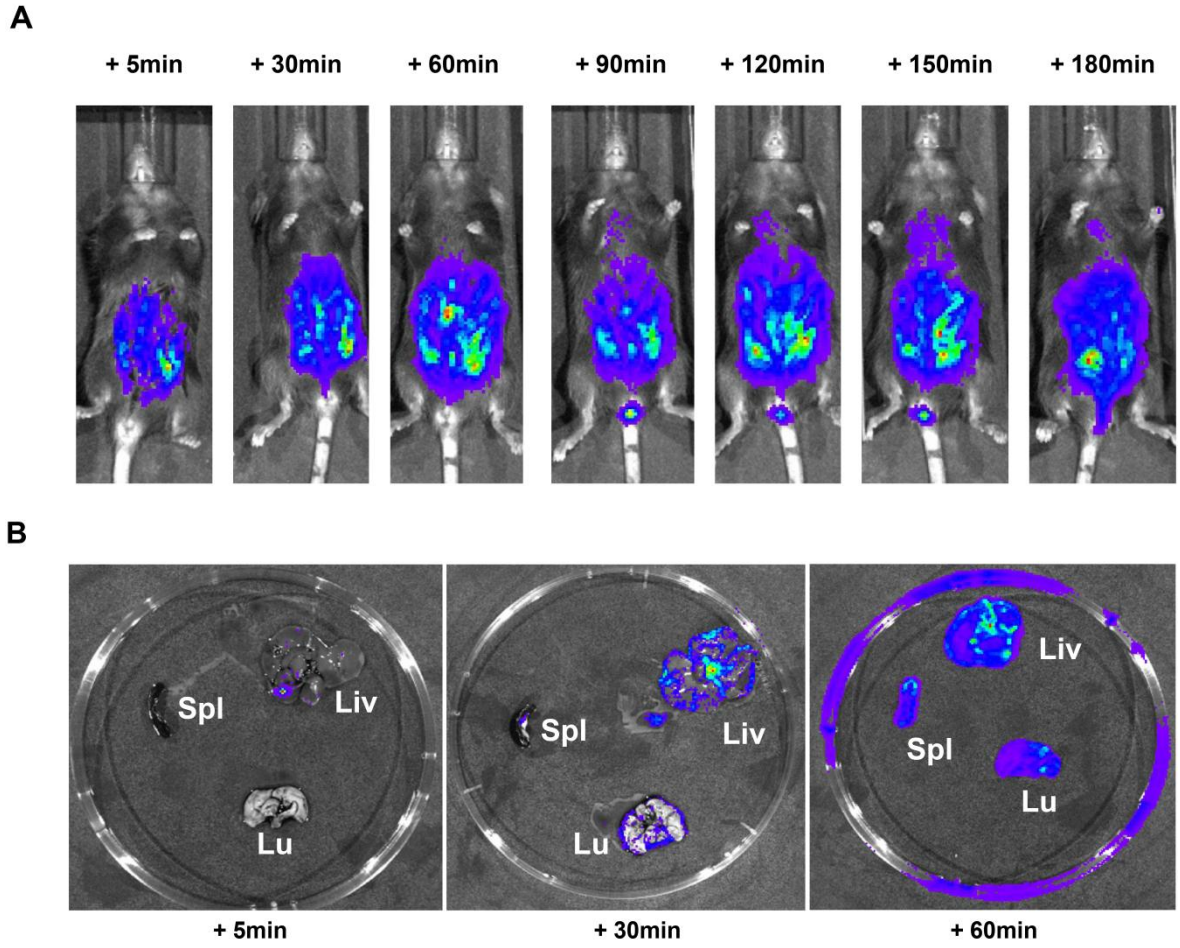

Figure 4.1 Distribution of *E.coli* lux after CASP
1x1011 *E.coli* lux were given orally per gavage to wild type C57BL/6 mice one hour before CASP procedure. (A) Bacterial distribution in mice was analyzed at indicated time points by *in vivo* imaging of bioluminescent luciferase expression using IVIS. (B) Organs were isolated and bacterial expression of luciferase was determined in spleen (Spl), liver (Liv) and lung (Lu) 5min., 30min., and 60min. after CASP procedure.

As expected bacteria disseminated in the organism very fast. Five minutes after the CASP procedure bacteria were detected in the peritoneum, 30 minutes after surgical treatment bacteria were found in the liver. 60 minutes after CASP, *E.coli* lux reached lung and spleen. Mice were septic after 150 minutes as bacteria could be found in all organs (Figure 4.1 **A** and **B**).

Next we investigated the immune status of mice surviving CASP sepsis. Therefore, mice that had survived CASP were immunized intravenously (i.v.) 24 hours after surgery with 5x109 virus particles recombinant adenovirus expressing ovalbumin (AdOVA). Following infection the OVA-derived MHC class I peptide SIINFEKL (S8L) is presented on H-2k^{b} and peptide-specific CD8^{+} T cells are activated and subsequently undergo expansion and differentiate into cytotoxic T lymphocytes (CTL). Five days post infection OVA-specific cytotoxicity was analyzed in spleens of mice using an *in vivo* cytotoxicity assay. Untreated mice generated a strong CTL response upon AdOVA infection whereas mice that underwent CASP almost completely lack an effective CTL response (Figure 4.2 A). To investigate whether CASP procedure itself or the translocating bacteria contribute to the suppression of CTL responses we injected either fecal suspension or *E.coli* intraperitoneally (i.p.). Mice treated with feces or *E.coli* i.p. showed a strong reduction of cytotoxic T cell activity after adenoviral challenge (Figure 4.2). Furthermore i.v. application of *E.coli* revealed that systemic distribution of bacteria lead to suppression of antigen-specific CTL responses (Figure 4.2).

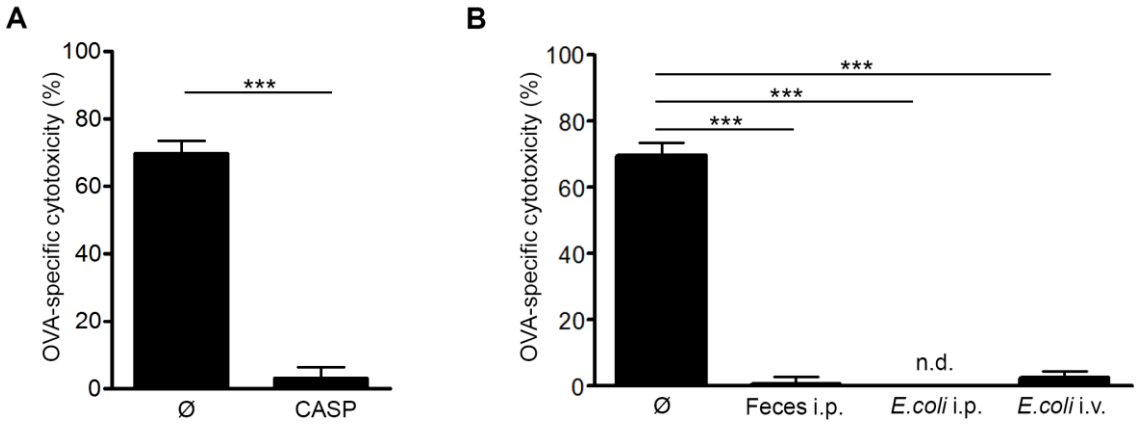

Figure 4.2 Mice surviving CASP lacked antigen-specific CTL responses against a subsequent AdOVA infection
C57BL/6 wild type mice were infected 24 hours after **(A)** CASP procedure or **(B)** after having received fecal suspension intraperitoneally (i.p.), 1x10^{7} *E.coli* i.p. or 1x10^{6} *E.coli* intravenously (i.v.) with 5x10^{9} virus particles AdOVA. OVA-specific CTL responses were determined in spleens five days post AdOVA infection. Representative data of two independent experiments are shown with **(A)** n=5 mice per group and **(B)** n=3 mice per group.

4.1.2 Bacterial translocation after sham treatment

To exclude that the surgical procedure itself affects the induction of CTL responses we performed sham operations. Mice having undergone non-invasive sham operation (OP) were examined for bacterial translocation using a classical microbiological technique.

24 hours after mock treatment organs were isolated and homogenates of the organs were prepared. Cell suspensions were plated on McConkey agar and after overnight incubation colony forming units (CFU) were determined. Surprisingly even sham operation led to translocation from the gut to distant organs. We detected vast amounts of gram negative bacteria in liver, lung and, minor amounts in mesenteric lymph nodes. The spleen was affected in only few animals and the bacterial burden of those was low. Living bacteria were absent in blood (Figure 4.3). However bacterial load was very different between mice.

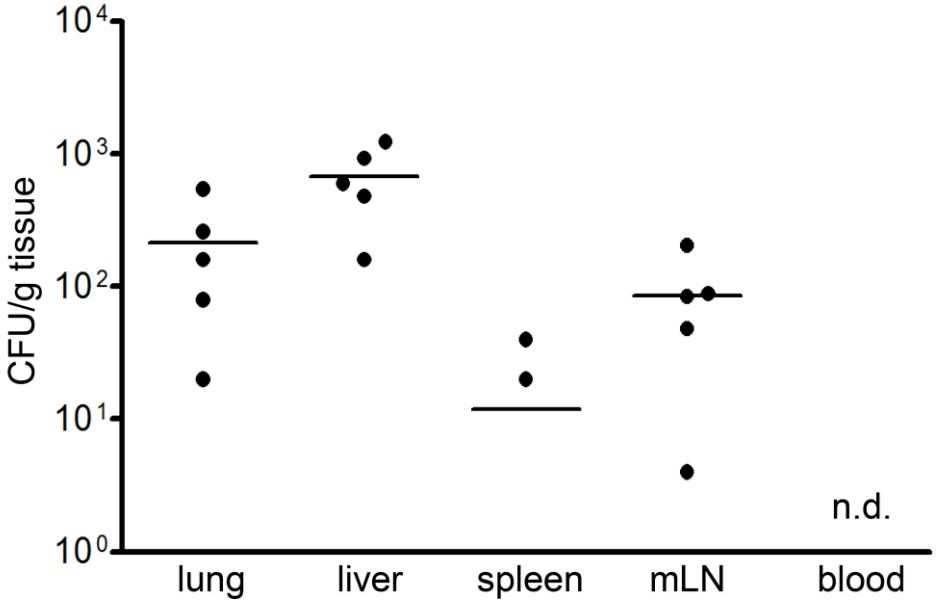

Figure 4.3 Bacterial loads of organs 3h after sham treatment
Blood and cell suspensions of lung, liver, spleen, and mesenteric lymph nodes (mLN) of sham treated C57BL/6 wild type mice were plated on MacConkey Agar. After 24 hours incubation at 37°C colony forming units (CFU) of gram negative bacteria were determined and calculated per gram tissue. Data presented as mean +/- SEM with n=5 mice per group. Representative data from one out of three independent experiments are depicted. (n.d.=not detectable).

To determine if mice, which had undergone sham treatment, were able to mount antigen-specific CTL responses, we intravenously infected mice with $5x10^9$ virus particles AdOVA. Five days after immunization OVA-specific CTL responses were quantified in spleens by using an *in vivo* cytotoxicity assay.

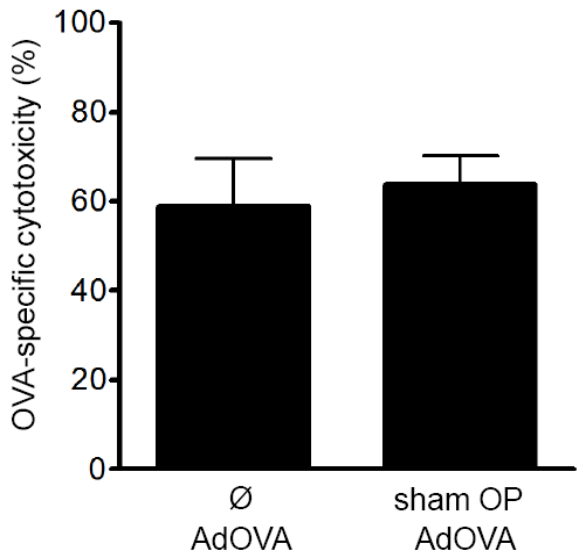

Figure 4.4 Sham treated mice induced strong CTL responses upon infection with AdOVA
C57BL/6 wild type mice were infected 24 hours after sham treatment with AdOVA and OVA-specific CTL responses were determined in spleens five days post AdOVA infection. Data are presented as mean +/- SEM with n=3 mice per group. Representative data out of two independent experiments are depicted.

Interestingly, we observed a different distribution of bacteria in animals having undergone CASP compared to sham treated mice. In the CASP model bacteria spread very fast resulting in a systemic distribution, while in sham treated mice bacteria where mainly found in the liver. Interestingly, in contrast to the CASP model, in which CTL responses were impaired, sham treatment and bacterial translocation did not impair the induction of cytotoxic T cell responses. (Figure 4.4)

4.2 *E.coli*-mediated immune suppression is time and dose dependent

The observed suppression of adaptive immunity by bacteria was surprising as TLR ligands are known to be immunostimulatory and generally result in the activation of immunity (Akira et al., 2006). Therefore, we asked whether adaptive immune suppression was a general effect mediated by circulating bacteria and whether it was time and dose dependent. To this end mice were injected i.v. with $1x10^7$ *E.coli* seven days (d-7), five days (d-5) or one day (d-1) prior to infection with adenovirus expressing luciferase, ovalbumin, and GFP (AdLOG). Five days after immunization with adenovirus antigen-specific cytotoxic T cell responses were assayed in spleens. OVA-specific CTL responses were significantly impaired if *E.coli* was administered five days prior to AdLOG infection, whereas CTL responses were completely abolished if *E.coli* was given one day before adenoviral challenge.

Administration seven days prior to adenovirus infection had only a minor influence on the generation of CTL responses (Figure 4.5 **A**). Thus, suppression of CTL responses by systemic *E.coli* depended on the time point of *E.coli* injection.

To monitor a possible impact of different bacterial doses on the induction of CTL responses we injected three different doses of *E.coli* ($1x10^5$, $5x10^4$ and $1x10^4$) intravenously into mice one day prior to AdLOG infection. A clear dose dependency of *E.coli*-mediated CTL suppression was recognized. $1x10^5$ bacteria almost completely inhibited the generation of CTL responses and CTL activities were still remarkably reduced if $5x10^4$ *E.coli* were injected. Even $1x10^4$ bacteria significantly impaired the induction of OVA-specific cytotoxicity (Figure 4.5 **B**).

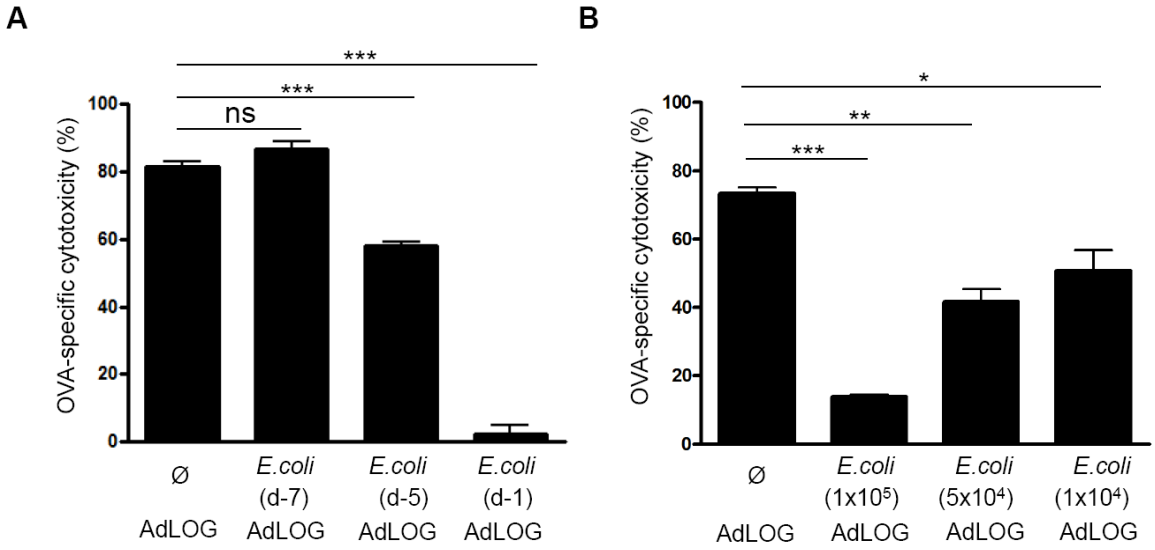

Figure 4.5 *E.coli* mediated suppression was time and dose dependent
(A) Wild type mice were left untreated or infected intravenously with $1x10^7$ *E.coli* on day 7, 5 or 1 before infection with AdLOG. **(B)** Wild type mice were infected with $1x10^5$, $5x10^4$ or $1x10^4$ *E.coli* one day prior infection with AdLOG. Five days after adenoviral infection OVA-specific CTL responses were determined. Representative data are shown as mean +/- SEM out of three individual experiments with n=3 mice per group. (ns=not significant)

So far, we demonstrated that systemic antigen-specific CTL responses against adenovirus were inhibited if mice had previously been infected with *E.coli*. Based on these findings that systemic *E.coli* led to the suppression of subsequent CTL responses, we wondered whether the lack of cytotoxic activity was due to tolerization (Steinman et al., 2003) or rather due to an absent generation of antigen-specific effector CD8 T cells. Moreover, we investigated how a subsequent injection of *E.coli* influenced the generation of CTL responses against an already established adenovirus infection. To address these questions mice received an intravenous injection of *E.coli* either one day before or one day after ade-

novirus infection. CTL responses were analyzed in spleens five days post adenoviral challenge. The generation of endogenous OVA-specific CD8 T cells was assessed by flow cytometric analysis of S8L/H-2Kb specific pentamers.

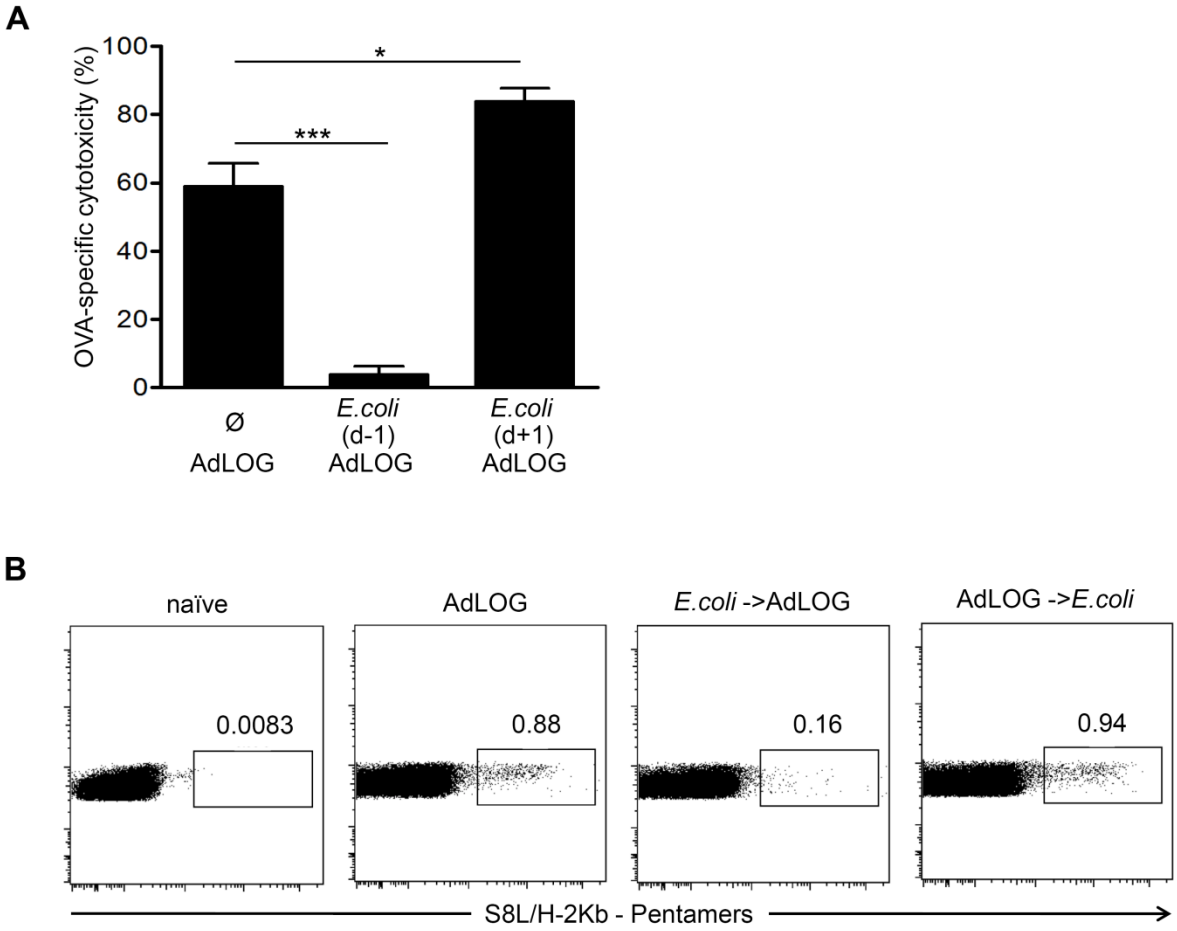

Figure 4.6 *E.coli* suppressed or stimulated CTL responses depending on the time point of adenoviral infection
C57BL/6 wild type mice were infected with $5x10^5$ *E.coli* either one day prior or one day after adenoviral infection. **(A)** CTL responses were determined in spleens five days after viral infection and are depicted as mean +/- SEM with n=3 mice per group. **(B)** The percentage of endogenous OVA-specific CD8 T cells was assayed by S8L/H-2Kb pentamer staining of splenocytes and subsequent flow cytometric analysis. Dot plots are shown for one representative mouse out of three mice. Representative data of at least two independent experiments are shown.

Compared to mice infected with AdLOG only, administration of *E.coli* one day before adenoviral infection resulted in suppression of OVA-specific cytotoxic activities, whereas CTL responses were significantly enhanced in mice that had been infected one day after adenovirus injection (Figure 4.6 **A**). In line with these results, the generation of endogenous OVA-specific CD8 T cells was remarkably impaired as the percentage of OVA-

specific CD8 positive T cells was clearly reduced if *E.coli* was given prior to adenovirus infection. The percentage of OVA-specific CD8 T cells was increased if *E.coli* was given one day after adenoviral infection (Figure 4.6 **B**). Taken together, we conclude that a systemic administration of *E.coli* prior to adenoviral infection causes suppression of adaptive immunity, whereas the generation of antigen-specific CD8 T cells responses is augmented if *E.coli* was given after an adenovirus infection.

4.3 *E.coli*-mediated suppression of CTL responses is not restricted to adenovirus infection

Based on our findings that *E.coli* suppressed the CTL response against a subsequent adenoviral infection, we wondered if this phenomenon was restricted to adenoviral vectors. We examined if CTL responses were impaired against distinct pathogens as well by a previous injection of *E.coli*. Wild type mice were infected i.v. either with $1x10^3$ CFU OVA-expressing *Listeria monocytogenes* (LmOVA), $1x10^5$ PFU Herpes simplex virus type I (HSV-1) or $1x10^5$ PFU Influenza A PR8/H1N1 virus.

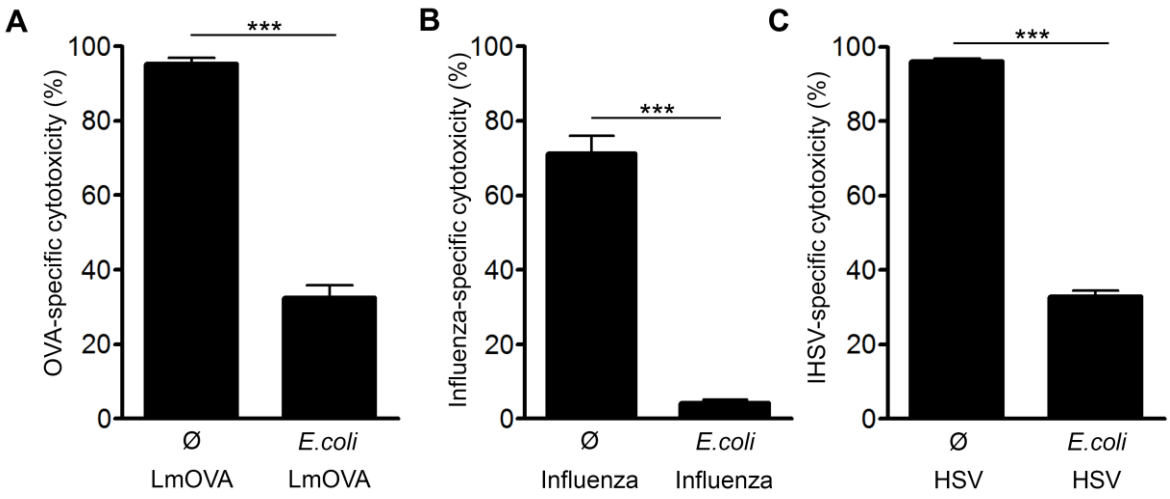

Figure 4.7 *E.coli* mediated suppression of CTL responses against various pathogens
C57BL/6 wild type mice were infected with $5x10^3$ CFU LmOVA **(A)**, $1x10^5$ PFU Influenza APR/8/H1N1 **(B)** or $1x10^4$ PFU HSV-1 one day after intravenous injection of $5x10^5$ *E.coli*. Antigen-specific CTL responses were determined in spleens five days after infection. Results are presented as mean +/- SEM and representative data of two independent experiments are shown.

Indicated groups received an i.v. injection of $5x10^5$ *E.coli* one day prior to infection. We detected strong antigen-specific CTL responses against LmOVA, HSV-1 or Influenza vi-

rus, which were significantly suppressed in mice previously infected with *E.coli* (Figure 4.7). Thus suppression of antigen-specific CTL responses by bacteremia is not restricted to adenoviral infection.

4.4 Different distribution of *E.coli* after CASP and sham treatment is due to the retention of gut-derived bacteria in the liver

Although bacterial translocation from the gastrointestinal tract is a well described phenomenon, the route of translocating bacteria is still unknown (Berg, 1985, 1990, 1992, 1995; Deitch and Berg, 1987a; Deitch et al., 1990a). With respect to the anatomy of the gut two distinct routes appear possible: first, translocation via the mesenteric lymph nodes or second, dissemination via the gut draining blood vessels. Although the bacterial load of the mesenteric lymph nodes was high we could detect tenfold more bacteria in the liver (Figure 4.3). Therefore we hypothesized that translocation mainly occurred via the blood route. Based on these results, we asked if the liver was able to retain bacteria, which had translocated from the intestinal tract and thus prevented systemic distribution of gut-derived bacteria.

4.4.1 The liver retains bacteria after intraportal injection

As mentioned before, sham treatment led to very variable extents of bacterial translocation in mice. To ensure standardized experimental conditions, which allowed studying the effect of bacterial translocation into the liver we directly injected a defined number of *E.coli* into the portal vein. We then compared bacterial load after i.v. injection with intraportal (i.po.) administration.

While systemic application of *E.coli* via the tail vein led to comparable amounts of bacteria in liver, spleen and blood, injection of *E.coli* into the portal vein resulted in a higher bacterial load in the liver and reduced bacterial loads in spleen, lung and blood (Figure 4.8 **A**). Thus, it could be speculated that the liver has the capability to retain bacteria translocated from the gut, thereby preventing a systemic and thus splenic distribution of bacteria.

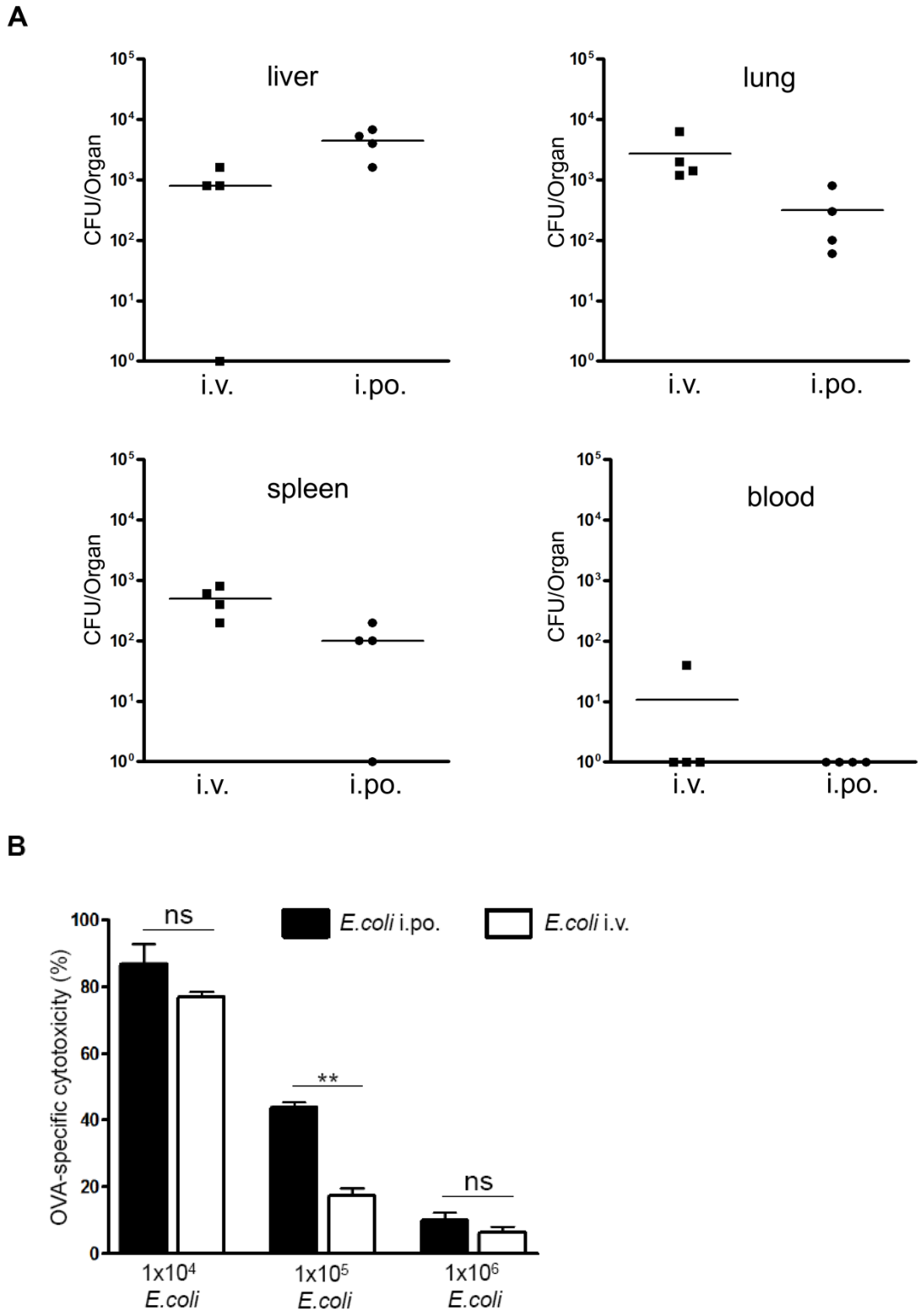

Figure 4.8 Different distribution of *E.coli* after intravenous or intraportal injection.
(**A**) C57BL/6 wild type mice were injected with $5x10^6$ *E.coli* either i.v. or i.po.. 18h after infection, CFU were assayed in liver, lung, spleen and blood. Representative data are shown from two independent experiments with n=4 mice per group. (**B**) $1x10^4$, $1x10^5$ or $1x10^6$ CFU of *E.coli* were injected either i.v. or into the portal vein (i.po.) of C57BL/6 wild type mice one day prior to infection with AdOVA. OVA-specific cytotoxicity was determined on day five after adenoviral challenge in spleens. Data presented as +/- SEM of two independent experiments with n=3 mice. (ns=not significant)

We have recently shown that the spleen is essential for the induction of adenovirus-specific CTL responses (Backer et al., 2010). Furthermore suppression of CTL responses correlates with the amount of bacteria reaching the spleen (Figure 4.5 **B**). Thus, in order to analyze a dose dependency of the CTL suppression, we injected *E.coli* in different concentrations either i.v. or i.po.. Indeed, intravenous injection caused suppression in a dose dependent manner, whereas mice that had received an i.po. injection of *E.coli* generated significantly higher CTL responses than intravenously treated mice (Figure 4.8 **B**). We therefore conclude that the liver could retain bacteria entering via the portal vein, thus preventing a systemic distribution and subsequent immunosuppression.

4.4.2 *E.coli* is efficiently taken up by liver resident macrophages and bacterial clearance is associated with TNF secretion and signaling.

To investigate if the accumulation of *E.coli* in the liver was either a passive effect or an active retention including the uptake of bacteria we made use of a specific fluorescent dye. The pHrodo™ dye is a specific sensor for endocytosis, which is non fluorescent at neutral pH and fluoresces in acidic environments, for example in endophagosomes. Here we injected pHrodo™ *E.coli* Bioparticles into the portal vein of mice and the liver was removed one hour after injection. A flowcytometric analysis of liver cells was performed. As shown in Figure 4.9, pHrodo™ *E.coli* Bioparticles were taken up within the liver. To discriminate which cell type takes up the pHrodo™ *E.coli* Bioparticles we performed flowcytometric analysis of liver cells and splenocytes. As we were interested in resident cells and their ability to phagocytose bacteria, we eliminated blood born phagocytic cells, such as neutrophile granulocytes, by perfusion of the liver and spleen with PBS. We observed that predominantly hepatic CD11b positive cells had phagocytosed the particles whereas CD11c positive cells had taken up the particles to a lesser extent (Figure 4.9).

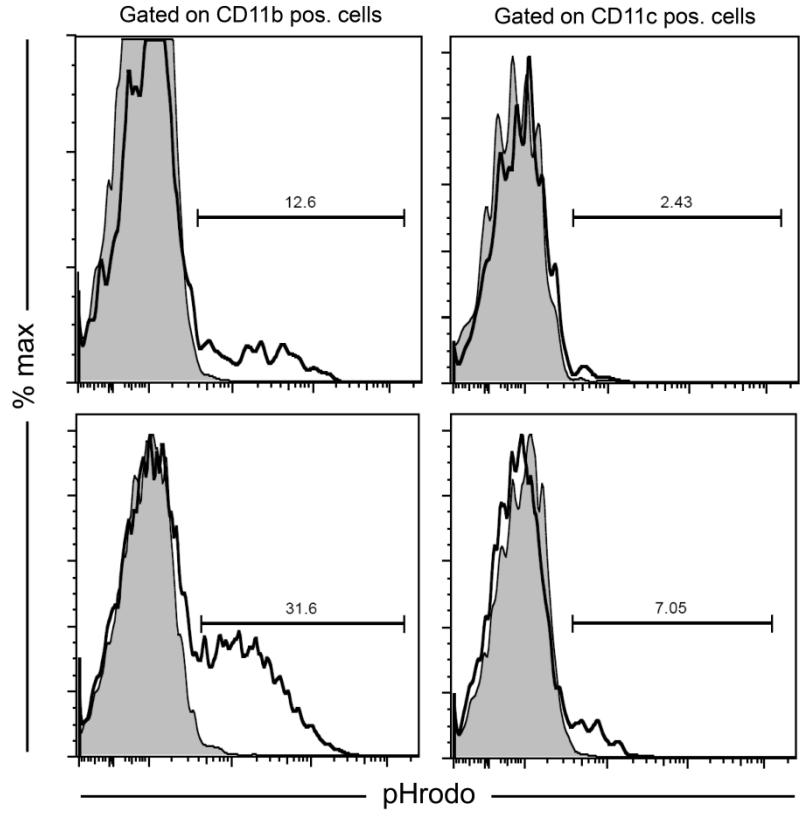

Figure 4.9 CD11b positive cells phagocytosed *E.coli* particles predominantly in liver and spleen.
C57BL/6 wild type mice were injected i.po. with 200µl of pHrodo™ *E.coli* Bioparticles. One hour after injection mice were sacrificed and perfused with collagenase solution. Liver and spleen cells were purified, stained for CD11b or CD11c and analyzed for particle uptake by flow cytometric analysis. Representative histograms of one mouse out of four are shown.

As Kupffer cells constitute 80-90% of tissue macrophages present in the body, they display the most prominent resident macrophage population, which is well known to potently clear opsonized pathogens from the circulation (Bilzer et al., 2006; Fox et al., 1987). Therefore, we investigated the role of Kupffer cells in bacterial clearance. To analyze whether these liver resident macrophages contributed to bacterial clearance we injected *E.coli* i.po. into wild type mice or into mice that had been depleted from macrophages with clodronate liposomes. Bacterial clearance was determined 3 or 18 hours after injection of bacteria. In wild type mice bacteria were cleared to 90% in all organs after 18 hours upon injection (Figure 4.10). However, depletion of macrophages resulted in an increase in bacterial load in all organs (liver, spleen, and lung). Particularly bacterial load in the liver of clodronate liposomes treated mice was 1000 fold higher after 18h than in untreated mice (Figure 4.10)

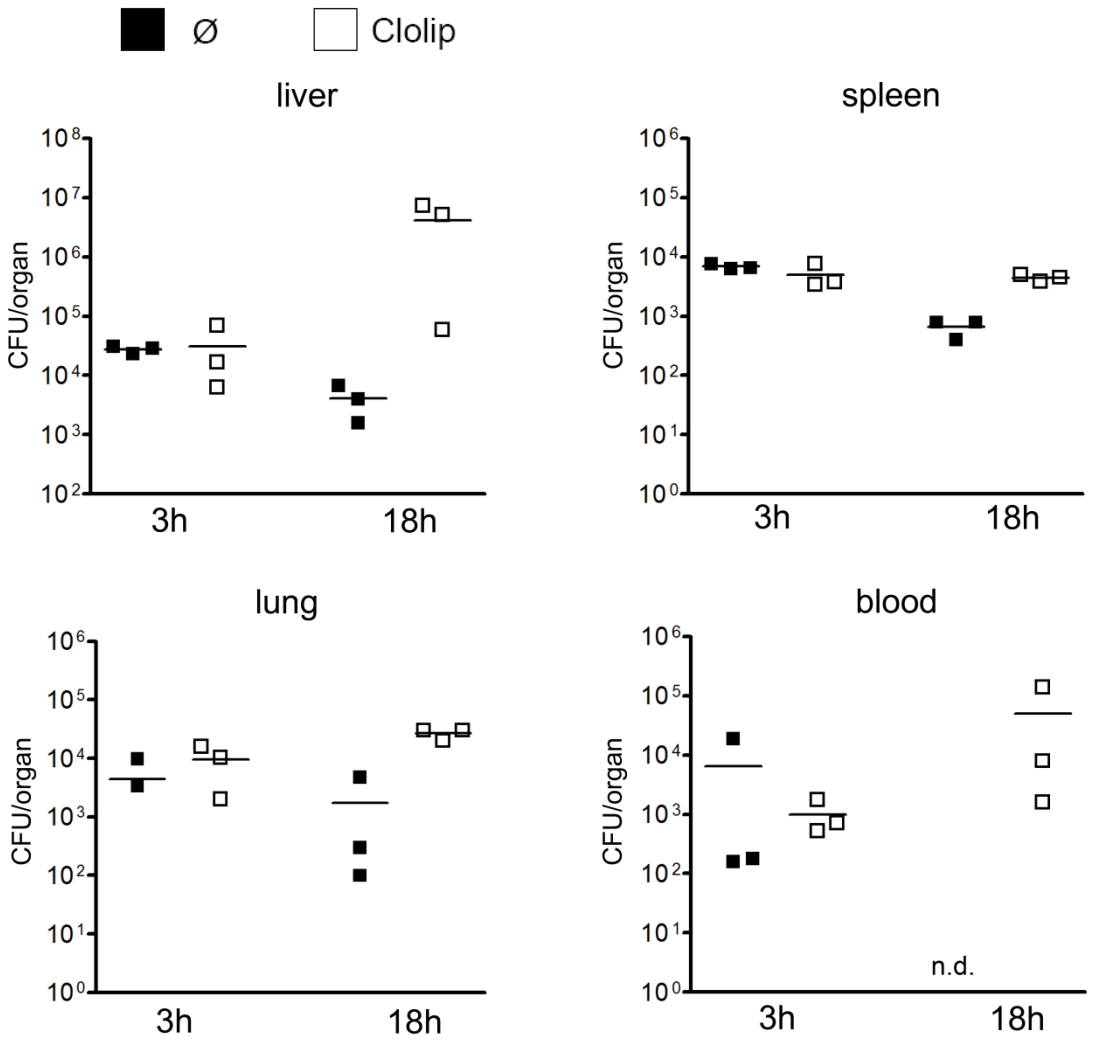

Figure 4.10 Bacterial clearance after i.po. injection of *E.coli* depended on macrophages
Mice were infected with 1x10^6 *E.coli* intraportally alone (filled squares) or received 200µl clodronate liposomes (Clolip) i.v. 24h prior to *E.coli* infection (open squares). Bacterial load was determined after 3 or 18 hours. After blood samples were taken, mice were perfused with PBS and lung, spleen, and liver were removed. Organ suspensions were prepared and plated on McConkey agar. 24 hours later colony forming units were counted on plates. Representative data of three independent experiments are shown with n=3 mice per group. (n.d.=not detectable)

The most potent inflammatory mediator secreted by Kupffer cells upon stimulation with LPS and other bacterial structures is the tumor necrosis factor (TNF) (Hoedemakers et al., 1995). Therefore we investigated the role of TNF in bacterial clearance. Wild type and TNF receptor I and II deficient mice (TNFRI/II) or mice treated with Infliximab, an anti-TNF antibody, were infected i.p. with *E.coli* and bacterial burden was calculated as CFU in different organs 18h post infection. Blockade of secreted TNF or inhibition of TNF signaling via TNF receptors resulted in an increase of bacterial burden mainly in lung and liver compared to wild type mice (Figure 4.11).

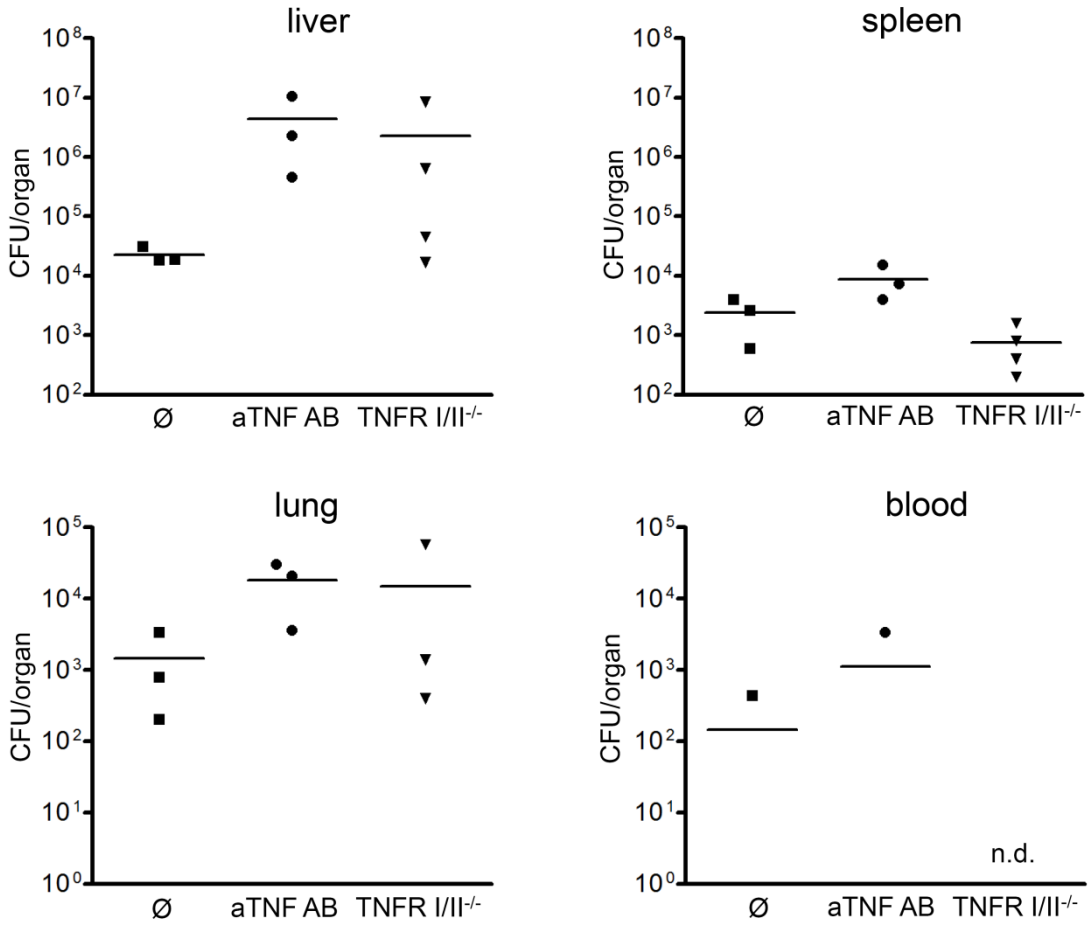

Figure 4.11 Blocking of secreted TNF or TNF signaling resulted in an increase in bacterial burden especially in liver and lung
Wild type mice, mice deficient in TNFR I and II (TNFR $^{-/-}$) or mice treated with 100µg anti-TNF antibody (aTNF AB) (Infliximab) were infected with 1x10^6 *E.coli* i.po. Bacterial load was determined 18h after infection. After blood samples had been taken mice were perfused with PBS and lung, spleen, and liver were homogenized. Suspended organs were plated on McConkey Agar. Colony forming units were counted on plates after 24h of incubation. Representative data of two independent experiments are shown with n=3 mice per group. (n.d.=not detectable)

In summary, we conclude from our data that macrophages and most likely Kupffer cells play a crucial role in the retention and clearance of gut-derived bacteria. Furthermore, secretion of TNF and signaling via the TNF receptor are critically involved in this process.

4.5 The spleen is essential for induction of effective CTL response to adenovirus

4.5.1 Suppression of CTL responses against a systemic adenovirus infection is mediated by systemically circulating *E.coli*

Based on our findings that bacteremia had caused suppression of the antigen-specific CTL responses we wondered whether suppression was restricted to systemic adenoviral infections or whether also local adenoviral infections were suppressed by *E.coli*. Therefore, we infected mice i.v. with $5x10^5$ *E.coli*. Then, one group of mice received a systemic injection of adenovirus (i.v.), while the second group was infected intranasally. To ensure that intranasal administration of AdLOG exclusively resulted in a local infection of the lung those animals were analyzed for expression of luciferase in the IVIS system. Intranasal application of AdLOG exclusively resulted in a tracheobronchial infection, whereas AdLOG given i.v. infected the liver and spleen (Figure 4.12 **C**). Therefore, a clear distinction between the locus of CTL induction could be made. Five days after immunization, CTL responses of i.v. and of intranasally infected mice were determined in the spleen and lung draining lymph nodes, respectively. Cytotoxic activities were significantly reduced in mice that had been infected systemically with AdLOG but not in mice infected intranasally (4.12 **A**, **B**). Thus we concluded that suppression of CTL responses exclusively occurs if both *E.coli* and adenovirus are systemically circulating and induction of local immunity is not impaired by bacteremia.

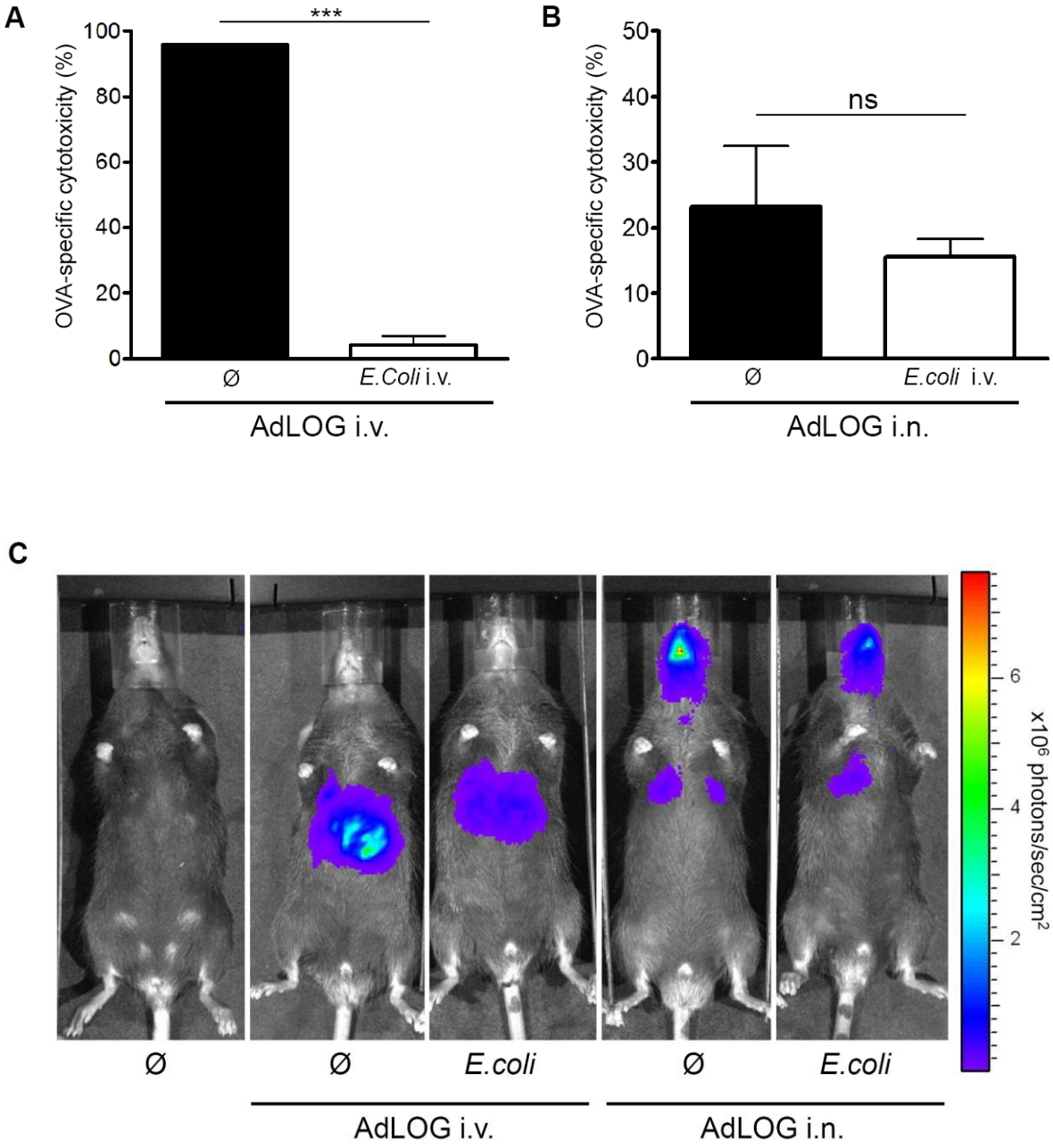

Figure 4.12 Induction of local immunity was not affected by bacteremia
C57BL/6 wild type mice were infected with $1x10^6$ *E.coli* i.v. or left untreated. 24 hours later mice were infected either **(A)** intravenously or **(B)** intranasally with AdLOG. OVA-specific CTL responses were assessed five days after adenoviral infection in **(A)** spleens or **(B)** in the tracheobronchial lymph node. **(C)** Adenoviral burden was analyzed two days after infection with AdLOG by *in vivo* imaging. Representative data are shown as mean +/- SEM (n=4 mice per group) of two independent experiments. (ns=not significant)

4.5.2 Splenic DCs and macrophages contribute to the generation of antigen-specific cytotoxic T cells upon adenoviral infection.

Since the spleen is a secondary lymphoid organ, which is known to be involved in the induction of CTL responses we wanted to elucidate the importance of the spleen in our experimental setup. We analyzed splenectomized mice for their ability to induce CTL responses upon systemic adenovirus infection. Splenectomized and sham treated mice were infected three days after surgery with AdOVA. Five days after infection antigen-specific CTL responses were quantified in blood. As shown in Figure 4.13, splenectomized mice were not capable to induce a CTL response upon systemic infection with adenovirus. Although adenovirus mainly infected the liver upon a systemic injection (Figure 4.12), importantly the generation of CTL responses strictly relied on the spleen.

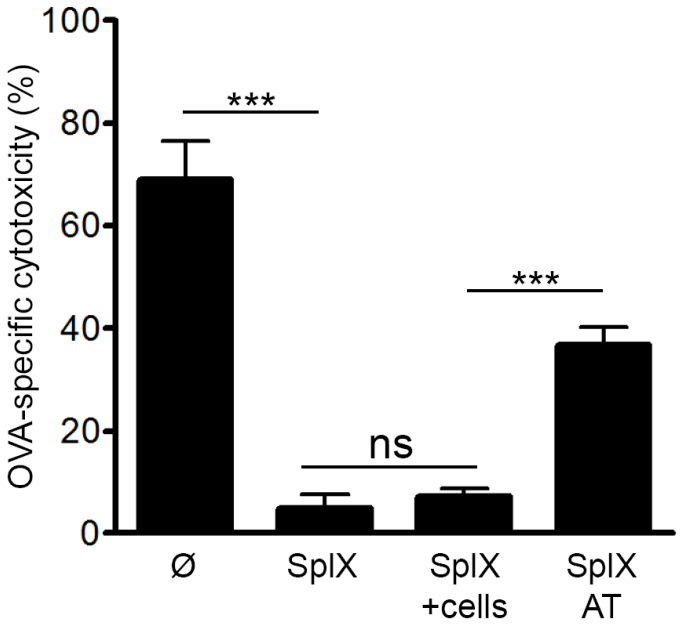

Figure 4.13 The spleen and an intact splenic structure were essential for the generation of efficient CTL responses after systemic adenoviral infection
C57/BL6 wild type or splenectomized (SplX) mice were infected three days after surgery by an intravenous injection of AdOVA. One group of splenectomized mice was reconstituted with single-cell suspensions of splenocytes (SplX+cells) one day prior to adenoviral infection. Another group received splenic autotransplants, which were placed into the renal capsule seven days before adenoviral challenge (SplX AT). OVA-specific cytotoxicity was determined five days after infection in blood. Data are presented as mean +\- SEM. Data from two independent experiments are shown.

In the following experiments we asked the question whether it was possible to rescue the CTL response by reconstituting splenectomized mice with single-cell splenocyte suspensions, given i.v. one day prior to infection with AdOVA. However, regardless of whether splenectomized mice were reconstituted with splenocytes or left untreated these animals

failed to induce adenovirus-specific CTL responses (Figure 4.13). These results indicate that an intact splenic architecture is essential for the induction of adenoviral CTL responses. To confirm this hypothesis splenectomized mice received autologous splenic autotransplants, which were placed into the renal capsule seven days prior to AdOVA infection. The induction of OVA-specific CTL responses was measured in these mice by performing an *in vivo* cytotoxicity assay five days post AdOVA infection. CTL responses were inhibited in splenectomized mice, whereas the re-transplantation of small pieces of the spleen partially restored the capability to generate a CTL response (Figure 4.13). Taken together, these results imply that efficient antigen-specific CTL responses upon systemic adenoviral infections were exclusively generated in the spleen and that an intact splenic structure is a prerequisite for efficient CTL induction.

Based on our data that the spleen and its unique architecture were essential for the initiation of a strong CTL response, we wondered which splenic cell types were mainly involved in the induction of these CTL responses. It is known that macrophages in the marginal zone efficiently take up blood born antigens and they are considered to be involved in the induction of CTL responses (Backer et al., 2010). In addition, immature, CD8+DEC205+ dendritic cells (DCs) present in the marginal zone are professional antigen-presenting cells, which exhibit a crucial role in initiating primary T cell responses (Belz et al., 2005; de Haan et al., 2000). To study a possible role of these two cell subsets for the induction of CTL responses in response to adenoviral infection we depleted either macrophages or DCs. Macrophages were depleted by an i.v. injection of clodronate liposomes one week before AdLOG infection. To deplete splenic DCs we made use of a transgenic mouse expressing the diphtheria toxin receptor under the CD11c promoter (CD11cDTR). CD11c positive cells were depleted by injection of diphtheria toxin (DTX) one day prior to adenoviral infection. Mice depleted of macrophages (Figure 4.14 **A**) or dendritic cells (Figure 4.14 **B**) were not capable to induce CTL responses upon adenoviral infection, whereas control mice treated with PBS-formulated clodronate liposomes or wild type mice injected with DTX generated a strong OVA-specific CTL response. From these results we concluded that both macrophages and dendritic cells are essential for the stimulation of a potent antigen-specific CTL response.

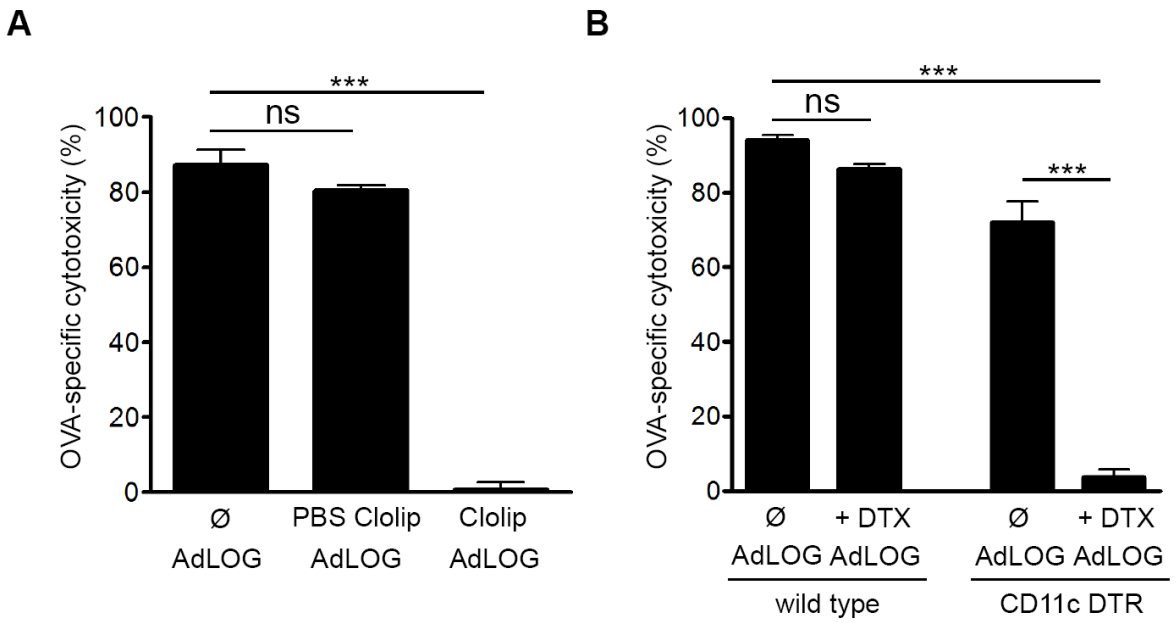

Figure 4.14 Mice depleted of either macrophages or dendritic cells failed to induce adenovirus-specific CTL responses
(A) C57BL/6 wild type mice were depleted from macrophages with clodronate liposomes (Clolip) or left untreated. One day after depletion mice were infected with AdLOG and CTL activity was determined five days after adenoviral infection. **(B)** C57BL/6 wild type mice and CD11cDTR mice, were infected with AdLOG alone or received an intraperitoneal injection of 800ng diphtheria toxin (DTX) one day prior to challenge with AdLOG. OVA-specific cytotoxicity was determined in spleens five days post adenovirus challenge. Data are presented as mean +/- SEM with n=3 mice per group from two independent experiments.(ns=not significant)

To further investigate which cell type might be influenced in its capability to respond adequately to adenoviral infection in the presence of *E.coli*, we analyzed the distribution of *E.coli* as well as the cellular infection pattern in spleens of adenovirus with and without *E.coli* pretreatment. To determine the influence of *E.coli* on adenovirus infection, mice were infected with GFP-expressing adenovirus alone or were pretreated with *E.coli* one day prior to adenoviral infection (Figure 4.15 **A**). Moreover, in order to evaluate the localization of *E.coli* in the spleen by histology, an additional group of mice received an i.v. injection of pHrodo™ *E.coli* Bioparticles. From all groups of mice, spleens were isolated for histological analysis one hour post injection. PHrodo™ *E.coli* Bioparticles were predominantly found in the marginal zone of the spleen, where they co-localized with metallophilic marginal zone macrophages (MMMs) (Figure 4.15 **B**)

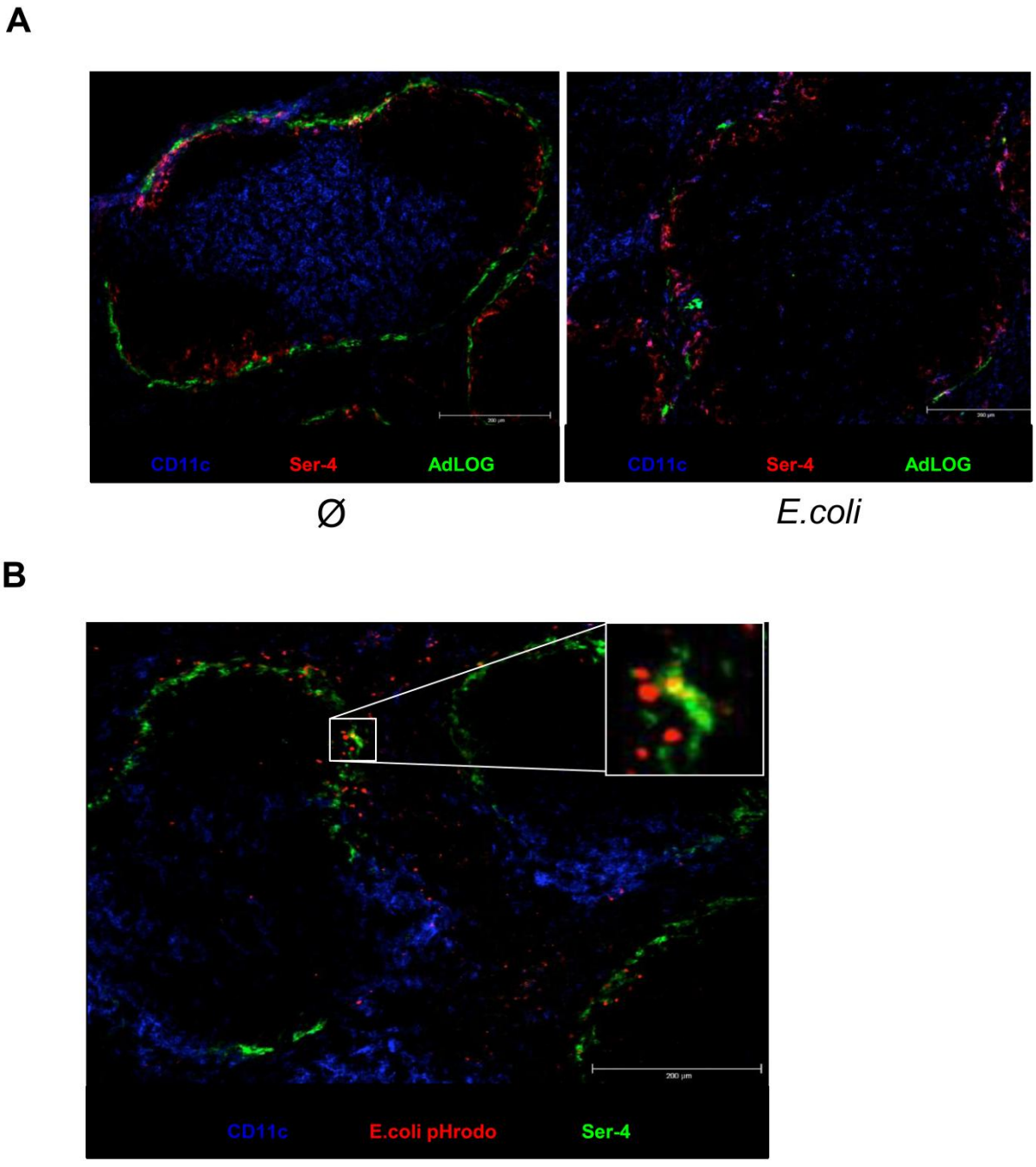

Figure 4.15 Systemic *E.coli* were taken up in the marginal zone of the spleen and diminished adenoviral burden.
(A) C57BL/6 wild type mice were infected with $5x10^7$ *E.coli* or left untreated. On the following day mice received $1x10^{11}$ VP AdLOG intravenously and spleens were removed for histological analysis. Cryo sections were prepared and stainings for CD11c to identify DCs (blue) and for Ser-4 to identify metallophilic marginal zone macrophages (red) were performed. Expression of GFP by AdLOG infected cells is displayed in green. **(B)** 200µl of pHrodo™ *E.coli* Bioparticles were administered intravenously and cryo sections were prepared one hour after infection. DCs were stained with CD11c (blue) and metallophilic marginal zone macrophages with Ser-4 (green). PHrodo™ *E.coli* Bioparticles showed red fluorescence upon uptake.

As seen in figure 4.15 **A** adenovirus mainly infected cells in the marginal zone. A strong reduction in adenoviral burden was noticed in mice pretreated with *E.coli* one day before AdLOG infection (Figure 4.15 **A**).

To exclude that *E.coli* had simply impaired the ability of MMM to be infected with adenovirus and to ensure that cells of the marginal zone gained equal amounts of antigen, wild type and septic mice were injected i.v. with soluble OVA together with a stimulatory anti-CD40 antibody to ensure activation of DCs. As mice showed impeded CTL responses upon immunization after *E.coli* treatment we concluded that bacteria-mediated suppression was not only to the result of a reduced viral antigen load but rather depended on additional mechanisms (Figure 4.16 **A**).

MMMs are essential for cross-presentation of blood-borne antigens by splenic dendritic cells (Backer et al., 2010). To investigate whether macrophages or dendritic cells were compromised in their function in response to a previous injection of *E.coli* we made use of OVA-coupled antibodies which directly target the antigen to these specific cell types (Backer et al., 2010). Using this approach OVA is targeted either to MMMs by coupling to a Siglec-1 antibody (SIGLEC-1-OVA) or to CD8+DEC205+ DCs by coupling to an antibody directed against DEC205 (DEC205-OVA).

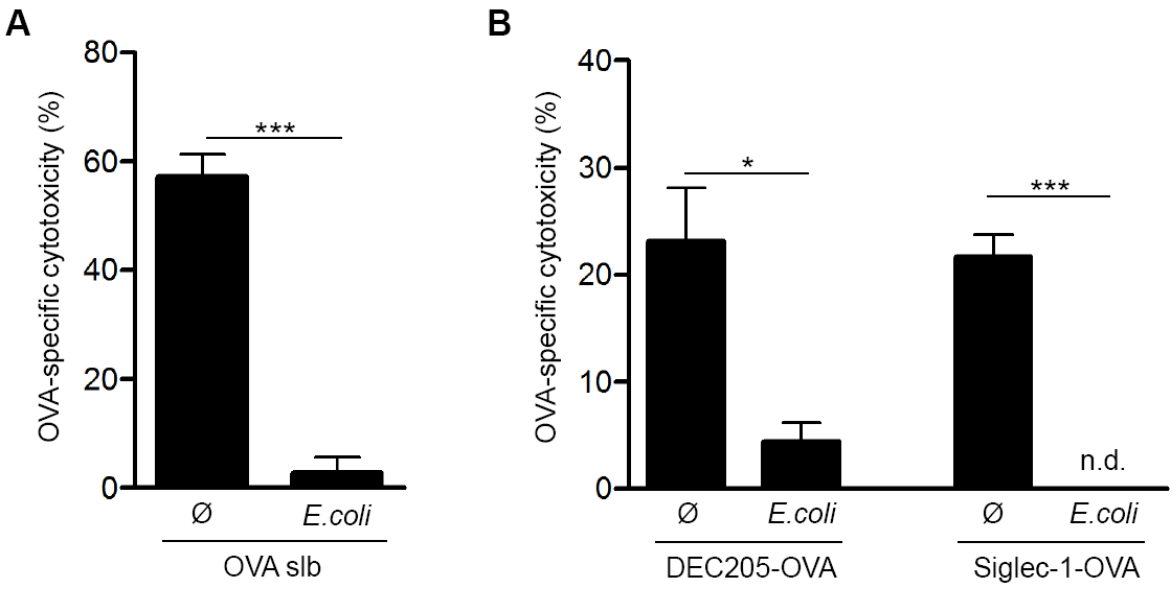

Figure 4.16 Suppression was not due to reduced adenoviral burden and affects both macrophages and dendritic cells
C57BL/6 mice were injected intravenously with 25µg activating aCD40 mAb (1C10) and **(A)** 100µg Ovalbumin (OVA slb), **(B)** 5µg DEC205-OVA or Siglec-1-OVA alone or received $1x10^5$ *E.coli* one day prior to immunization. CTL responses were determined in spleens on day five after immunization. Each bar presents the mean +/- SEM with n=3 mice per group. Representative data of two independent experiments are shown. (n.d.=not detectable)

If administered to wild type mice OVA coupled to DEC205 and Siglec-1 induced a CTL response. In contrast, CTL responses were significantly reduced or even absent in mice treated with *E.coli* one day prior to immunization with OVA-coupled DEC205 or Siglec-1 (Figure 4.16 **B**). From these results we concluded that DCs as well as MMM were affected by bacteremia as they were not able to induce CTL responses after exposure to *E.coli*.

4.6 IL-10 is not associated with *E.coli*-mediated suppression of CTL responses

IL-10 is known to be a very potent anti-inflammatory cytokine. IL-10 is expressed by many cells of the adaptive immune system such as a broad variety of T cells and B cells as well by cells of the innate immune system, for instance DCs, macrophages, mast cells, NK cells, eosinophiles and neutrophils upon stimulation with PAMPs (Siewe et al., 2006; Zhang et al., 2009). As we observed suppression of CTL responses by *E.coli*, we speculated whether IL-10 production might be stimulated upon *E.coli* administration and whether it might play a role in immune suppression. Moreover, we intended to identify which cell types produced IL-10 in the spleen upon systemic exposure to *E.coli*.

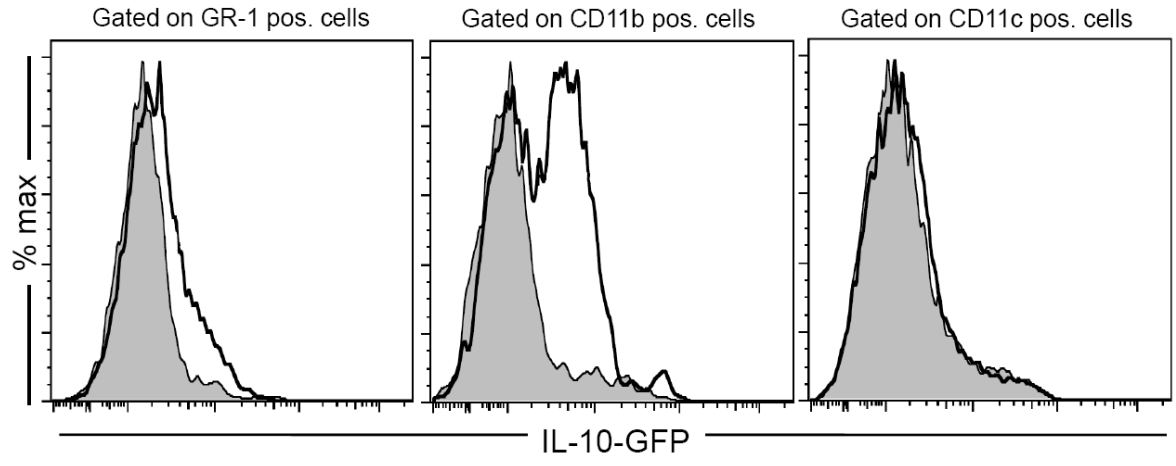

Figure 4.17 Splenic Macrophages produced IL-10 upon *E.coli* stimulus
IL-10 GFP-reporter mice left untreated (tinted histograms) or received $5x10^5$ *E.coli* intravenously (open histograms). One day post infection IL-10-GFP expression was examined by flowcytometric analysis of splenocytes. Granulocytes were identified by GR-1^{pos}, Macrophages by CD11b^{pos} and F4/80^{pos}, and dendritic cells by CD11c^{pos}. Representative histograms of one mouse out of three mice are shown.

To this end, we made use of IL-10 knockin reporter mice, which express GFP under the IL-10 promoter (Bouabe et al., 2008).

We assayed GFP expression in splenoctyes by flowcytometric analysis 24h after systemic exposure to *E.coli*. Macrophages ($CD11b^{pos}$) were identified as the main producers of IL-10 after exposure to *E.coli* while DCs ($CD11c^{pos}$) and Granulocytes ($GR\text{-}1^{pos}$) did not produce IL-10 (Figure 4.17). As we know that macrophages were essential in induction of potent CTL responses (Figure 4.14 **B**) and IL-10 was secreted by macrophages we speculated whether IL-10 was involved in the suppression of CTL responses.

To this end, we infected C57BL/6 wild type mice or IL-10 ko mice either with AdLOG alone or injected them with $5x10^5$ *E.coli* i.v. one day before adenoviral infection. OVA-specific cytotoxicity was measured in spleens five days after infection. *E.coli*-mediated suppression of OVA-specific CTL response was still observed in IL-10 deficient mice indicating that IL-10 was not involved in the suppression of CTL responses by *E.coli* (Figure 4.18).

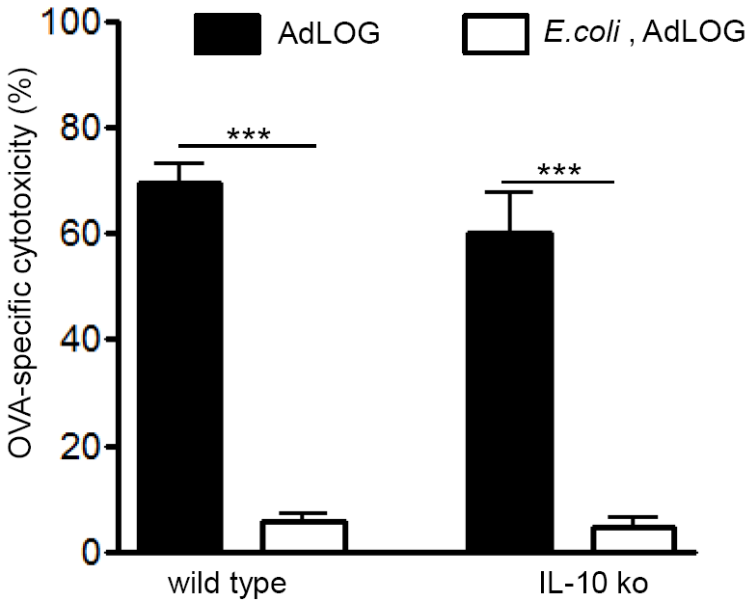

Figure 4.18 IL-10 was not involved in *E.coli*-mediated CTL suppression
C57BL/6 wild type mice or mice deficient in IL-10 were infected with AdLOG alone or received $5x10^5$ *E.coli* one day prior to adenoviral infection. OVA-specific CTL responses were determined five days post AdLOG infection in the spleen. Data are presented as mean +/- SEM (n=3)

4.7 *E.coli* suppresses antigen-specific CTL responses in a TLR 4 and TRIF dependent manner

4.7.1 TLR4 signaling promotes suppression of CTL responses upon bacteremia

E.coli contains a broad variety of TLR ligands, such as CpG recognized by TLR9, peptidoglycan sensed by TLR2 and LPS, which is recognized by TLR4 (Medzhitov et al., 1997). It has been recently shown that TLR4 and its downstream signaling play a crucial role in the immunophathology of sepsis (Beutler and Rietschel, 2003). Blockade of TLR4 by antibodies renders mice resistant towards septic shock (Roger et al., 2009). Therefore, we asked if TLR signaling was involved in the suppression of CTL responses as well. To study the contribution of TLR ligands to sepsis-mediated immunosuppression we used mice deficient in TLR2, TLR9 or TLR4 and analyzed their capability to generate effective CTL responses upon adenoviral infection and a previous systemic exposure to *E.coli*. Wild type and TLR-deficient mice were infected intravenously with $5x10^5$ *E.coli* one day prior to adenoviral infection. Antigen-specific T cell responses were determined in spleens five days after AdLOG challenge. CTL suppression was still obtained in TLR2- and TLR9-deficient mice, indicating that *E.coli*-mediated CTL suppression was independent of TLR2 and TLR9 signaling (Figure 4.19 **A** and **B**). However, no inhibition of antigen-specific CTL responses by *E.coli* was observed in TLR4-deficient mice. These results clearly demonstrated that *E.coli*-mediated CTL suppression was exclusively dependent on TLR4 (Figure 4.19 **C**).

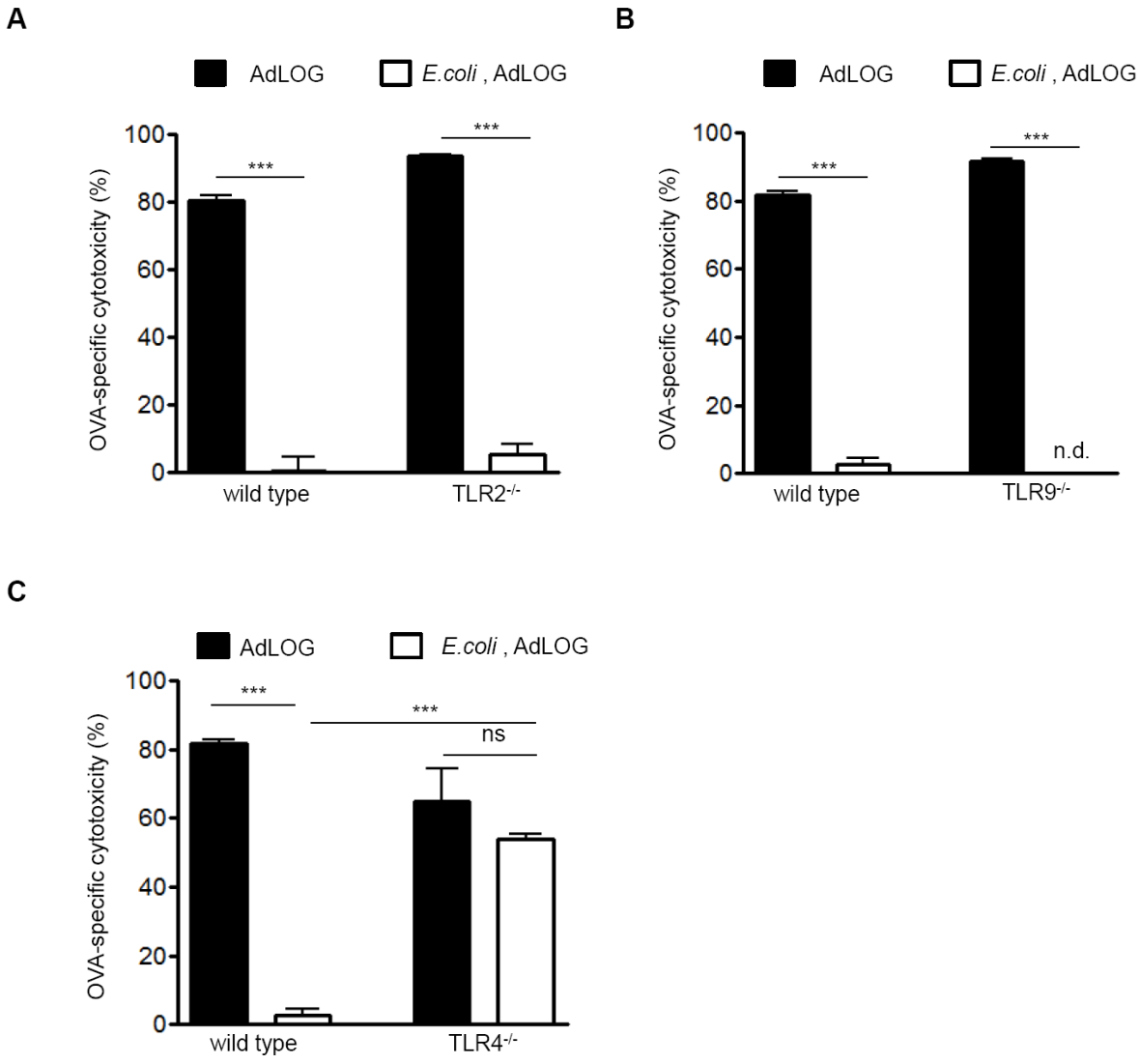

Figure 4.19 *E.coli*-mediated suppression exclusively depended on TLR4 signaling
C57BL/6 wild type mice or mice deficient in TLR2 **(A)**, TLR9 **(B)** or TLR4 **(C)** were injected with $5x10^5$ *E.coli* intravenously one day before infection with AdLOG. OVA-specific cytotoxic T cell activities were quantified in spleens on day 5 after adenoviral infection. Data are shown as mean +/- SEM with n=3 mice per group. Representative data of at least two independent experiments are presented.

4.7.2 CD14 is not involved in *E.coli* mediated CTL suppression

Since TLR4 exclusively mediated the suppression of antigen-specific CTL responses after infection with *E.coli*, we speculated that LPS was the main suppressive constituent of *E.coli*. LPS can be transferred to TLR4 by the LPS binding protein (LBP) and CD14 which therefore play important roles in promoting innate immunity to gram negative bacteria (Kitchens and Thompson, 2005). Moreover, it is known that CD14 can promote or attenuate cell responses towards LPS (Kitchens et al., 2001) and is consequently an attractive

clinical target to prevent septic shock caused by endotoxin. As monoclonal antibodies directed at CD14 have been tested in phase I clinical trials (Reinhart et al., 2004) we asked for the relevance in our model of sepsis-induced immunosuppression. Thus, we infected mice deficient in CD14 with $5x10^5$ *E.coli* i.v. one day prior to adenoviral infection. On day five after AdLOG infection antigen-specific CTL responses were measured in spleens. Astonishingly, although CD14 seems to be a potent regulator of endotoxin shock it was clearly not involved in *E.coli*-mediated CTL (Figure 4.20).

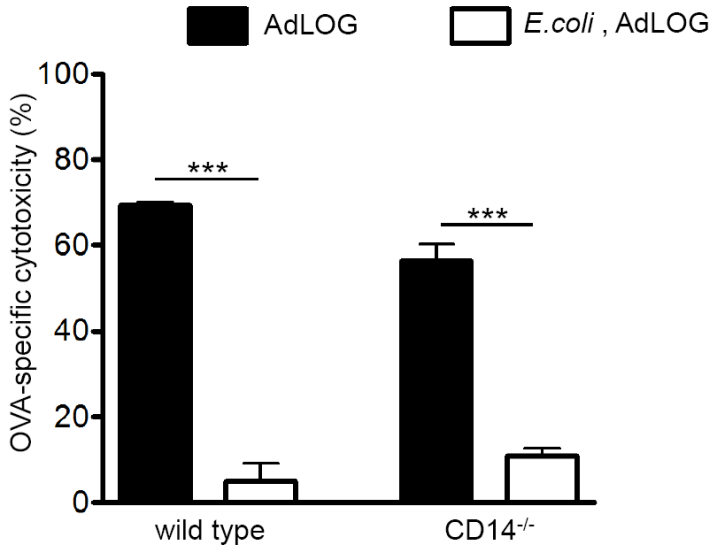

Figure 4.20 CD14 was not involved in *E.coli*-mediated CTL suppression
C57BL/6 wild type mice and mice deficient in CD14 were left untreated or given $5x10^5$ *E.coli* intravenously one day before infection with AdLOG. Five days after adenoviral infection OVA-specific cytotoxic CTL responses were assessed in spleens. Data are presented as mean +/- SEM with n=3 wild type mice per group and n=5 CD14 deficient mice per group.

4.7.3 TRIF exclusively contributes to suppression of CTL responses

Based on our finding that TRL4 is the main receptor involved in CTL suppression by *E.coli* we wondered which adaptor molecule downstream of TLR4 was responsible for the suppression. In most TLRs except TLR3 downstream signaling is mediated by the TIR domain containing adaptor molecule MyD88 (Akira et al., 2006). The second TLR adaptor molecule is the TIR-domain-containing adapter-inducing interferon-β (TRIF) which initiates downstream signaling of TLR3 and TLR4. In contrast to MyD88, TRIF can activate NF-κB and additionally interferon regulatory factor 3 (IRF3) which activates the interfe-

ron-β transcription. TLR4 is kind of special among the TLRs because it is the only TLR that is able to recruit both adaptor molecules, MyD88 and TRIF. Therefore we investigated if MyD88 and TRIF promoted the suppression of CTL responses upon TLR4 stimulation.

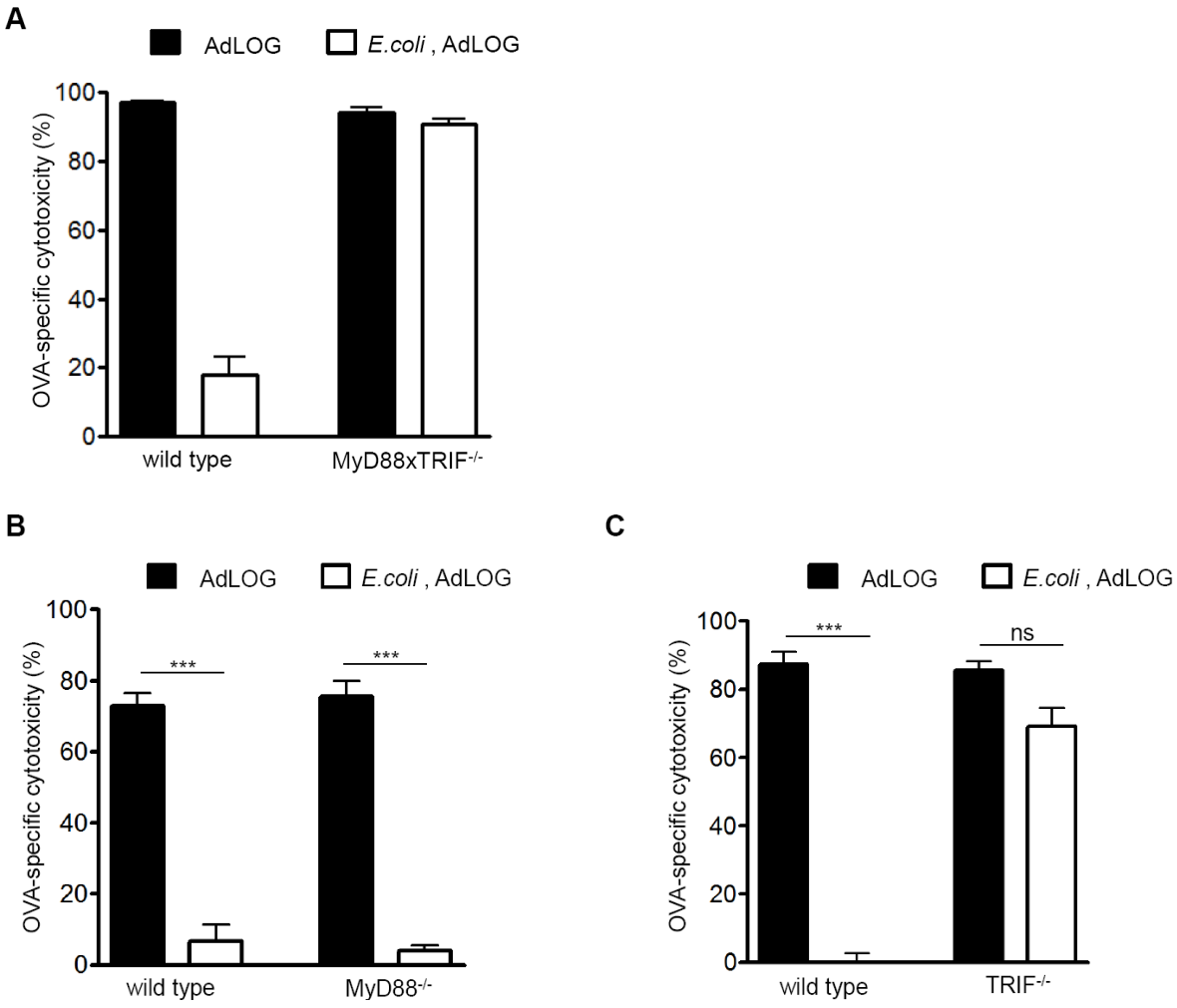

Figure 4.21 *E.coli* suppressed adeno-specific CTL responses in a TRIF dependent manner
C57BL/6 wild type mice or mice deficient either in (**A**) MyD88xTRIF (**B**) MyD88 or (**C**) TRIF were infected intravenously with $5x10^5$ *E.coli* or left untreated. One day after induction of bacteremia mice were infected with $5x10^9$ virus particles AdLOG. OVA-specific cytotoxicities were quantified in spleens five days post infection. Data are displayed as mean +/- SEM with n=3 mice per group. Representative data of at least two independent experiments are depicted.

Inhibition of TLR4 downstream signaling clearly contributed to *E.coli* mediated suppression of adenovirus-associated CTL responses as mice deficient in both MyD88 and TRIF showed no suppression (Figure 4.21 **A**). Surprisingly, MyD88 did not contribute to *E.coli*-mediated suppression of adenovirus-specific CTL responses. Instead CTL suppression was

exclusively induced by TRIF signaling, as only mice lacking TRIF were able to induce strong CTL responses after systemic pretreatment with *E.coli* (Figure 4.21 **B** and **C**).

4.7.4 Type I interferons are induced after bacteremia and contribute to suppression of CTL responses

Since the observed inhibition of CTL responses by *E.coli* was completely dependent on TRIF signaling, we further studied the role of TRIF regulated genes and its downstream products. It is well known that LPS-stimulated interferon-β (IFN-β) induction via TLR4 is entirely TRIF dependent (Yamamoto et al., 2003b) and TRIF is identified as a regulator of interferon (IFN) transcription via IRF3 and IRF7 (Fitzgerald et al., 2003b). For that reason we examined if TRIF signaling via IRF3 and IRF7 and consequently type I IFNs were involved in the observed absence of CTL responses upon systemic *E.coli* exposure. To first address the question whether type I IFNs were induced upon infection with *E.coli*, we made use of mice expressing a functional human myxovirus resistance protein (MxA), a protein, which is normally lacking in most inbred mouse strains (Shuai, 1994). MxA is a GTPase that accumulates to high levels in the cytoplasm of interferon-treated cells and can be stained in histology by a specific antibody (Pavlovic et al., 1995; Wenzel et al., 2005b). Thus, detection of MxA correlates with type I IFN production.

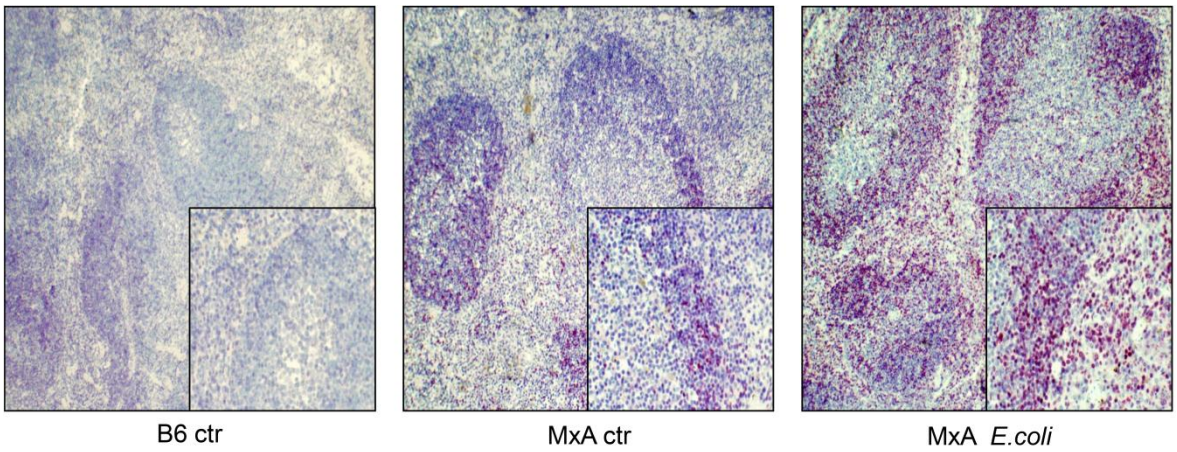

Figure 4.22 MxA protein was expressed in the spleen after infection with *E.coli*
C57BL/6 wild type mice or mice expressing the MxA protein were infected with $5x10^5$ *E.coli* i.v.. One day after bacterial infection histological sections were performed from formalin-fixed, paraffin-embedded spleens. Standard haematoxylin and eosin staining followed by immunohistochemistry for MxA protein was done. Shown is one mouse out of three for each group respectively. Original magnification x200 and x400 small panel.

MxA expressing mice were infected with $5x10^5$ *E.coli* and histology of the spleens was performed one day after infection. Systemic administration of *E.coli* led to robust expression of MxA protein in the marginal zone as well as in the white pulp of the spleen (Figure 4.22) suggesting that type I IFNs were indeed induced upon systemic *E.coli* in the spleen.

As we could show that type I IFNs were induced after challenge with *E.coli* we wondered whether mice that lack the IFN-α receptor (IFNAR) and therefore cannot respond to type I IFNs in general, show immunosuppression after bacterial infection and subsequent adenoviral challenge. We infected IFNAR1/2 deficient mice with $5x10^5$ *E.coli* one day prior to AdLOG infection. Cytotoxic activity of CD 8 T cells was measured on day five after adenoviral challenge in spleens.

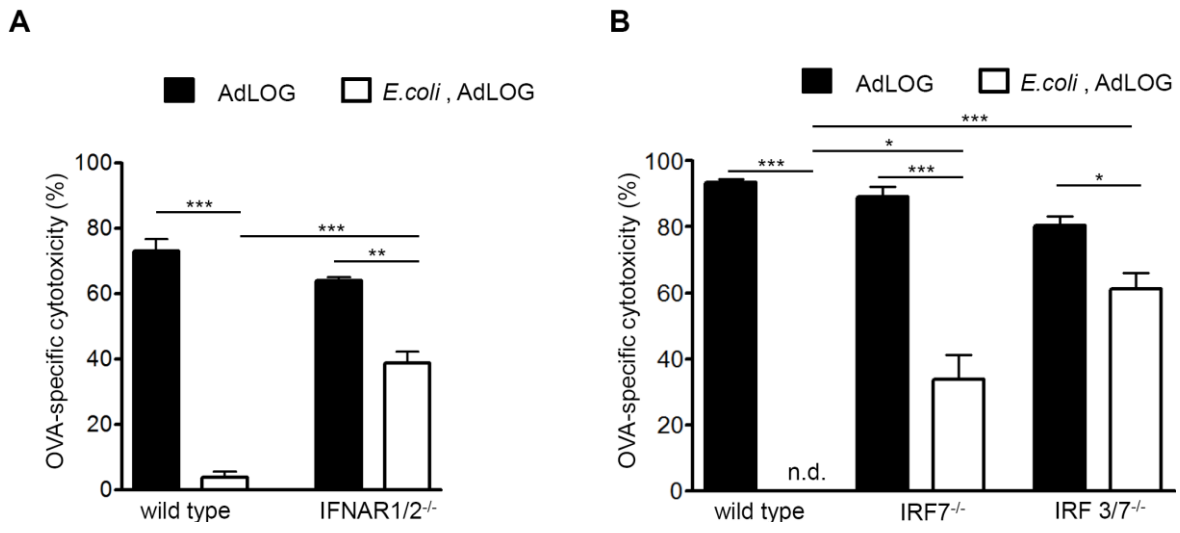

Figure 4.23 IFNs were essentially involved in the suppression of antigen-specific CTL response by *E.coli*
C57BL/6 wild type mice and mice deficient in the IFNAR1 and IFNAR2 **(A)**, IRF7 or IRF3/7 **(B)** were infected with $5x10^5$ *E.coli* one day before immunization with AdLOG. The activity of OVA-specific cytotoxic CD 8 T cells was determined five days after adenoviral infection in spleens. Data presented as +/- SEM with at least n=3 mice. Representative data are shown of two independent experiments. (n.d.=not detectable)

Mice lacking both chains (1 and 2) of the IFNAR showed reduced antigen-specific cytotoxicity after pretreatment with *E.coli*. However compared to wild type mice the CTL response was significantly enhanced which proposed involvement of the type I interferon signaling pathway in mediating dampening of CTL responses after *E.coli* stimulus (Figure

4.23 **A**). Furthermore upon binding of IFN to its receptor, the transcription factors IRF3 and IRF7 are activated and translocate into the nucleus where they exert their function. Compared to wild type mice, mice lacking IRF7 as well as mice lacking both IRF3 and IRF7 showed an enhanced capability to induce a CTL response (Figure 4.23 **B**). Thus IRF3 and IRF7 signaling were both involved in *E.coli* mediated suppression of antigen-specific CTL responses. Additionally these data suggest that the amplification of the type I interferon signal via the IFNAR 1 and 2 and IFR 7 is involved in the suppression of CTL responses in the same manner as the initial induction of type I IFNs.

4.8 Regulation of adaptive and innate immune responses by TLR ligands

It is known that stimulation of pattern recognition receptors (PRR) on professional antigen presenting cells (APC), such as DCs is a prerequisite for the induction of functional adaptive immune responses (Blander and Medzhitov, 2006). Upon TLR stimulation DCs alter their phenotype from an immature to a mature status, which is characterized by secretion of cytokines, up-regulation of MHC molecules and their ability to efficiently prime T cell responses (Inaba et al., 1993; Ludewig et al., 1998; Reis e Sousa et al., 1997). Thus it was striking that mice deficient in both MyD88 and TRIF signaling and hence lacking complete TLR signaling were still able to induce a strong antigen-specific CTL response upon infection with adenovirus (Figure 4.21 **A**). First, to determine whether only CTL responses against adenovirus were independent of TLR signaling or whether it could be extended to other viral infections, we infected MyD88xTRIF deficient mice with other viruses such as Influenza virus, mouse cytomegalovirus (MCMV-OVA), or herpes simplex virus type 1(HSV). Antigen-specific cytotoxic responses were determined in spleens on day five after viral infection. Interestingly, also for these viruses TLR signaling was not mandatory for the induction of effective antigen-specific CTL responses, as MyD88xTRIF deficient mice were able to generate CTL responses as efficient as wild type mice against Influenza virus, MCMV and HSV-1 (Figure 4.24)

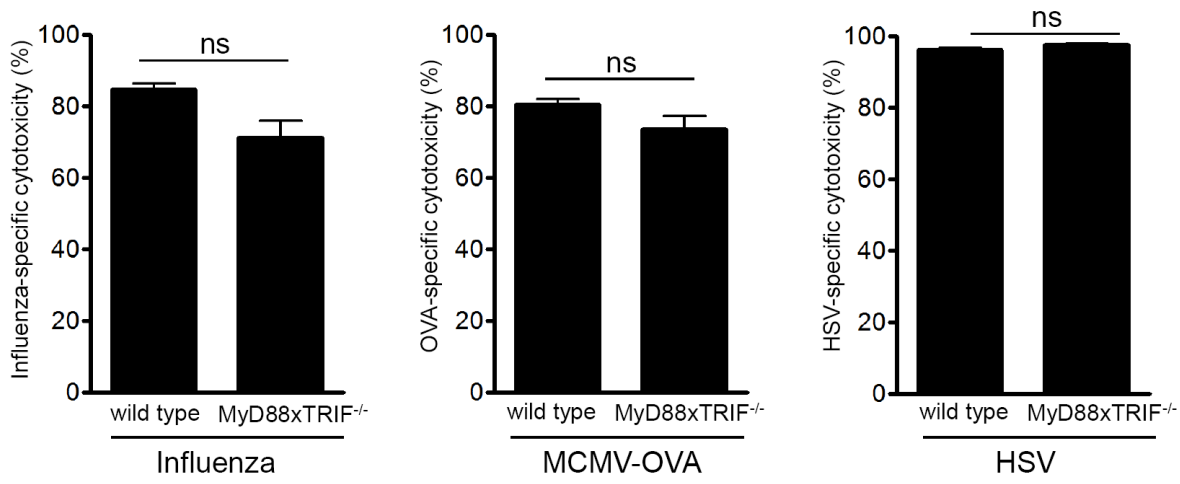

Figure 4.24 MyD88xTRIF deficient mice were able to induce strong CTL responses against different viral infections as efficient as wild type mice.
C57BL/6 wild type mice and mice deficient in both MyD88 and TRIF were infected either with $1x10^5$ PFU Influenza virus, $2x10^5$ PFU MCMV-OVA or $1x10^4$ PFU HSV i.v.. Five days after immunization antigen-specific CTL responses were determined in spleens. Data are presented as mean +/- SEM with n=3 mice per group.

Based on these findings we further hypothesized that TLR signaling was not required for *generation* of effective CTL responses in general but instead played a crucial role in *modulating* CTL responses. This hypothesis was further supported by the fact that *E.coli* treatment and TLR4 signaling could dampen or enhance CTL responses whether TLR signaling occurred before or after viral infection (Figure 4.6). Despite their ability to induce maturation of DCs and subsequent induction of adaptive immunity, TLR ligands also trigger the innate immune system by promoting the release of pro-inflammatory cytokines as well as by enhancing phagocytosis of pathogens (Akira, 2006; Akira et al., 2006; Blander and Medzhitov, 2004). For example, TLR2 signaling is strongly associated with the release of nitric oxide (NO) and stimulation of TLR4 is reported to initiate the release of defensines (Lehrer and Ganz, 2002; Thoma-Uszynski et al., 2001). Additionally macrophages are efficiently activated by TLR ligands. TLR stimulation leads to secretion of pro-inflammatory cytokines by macrophages themselves as well as DCs (Akira et al., 2006).

Therefore, we analyzed the importance of TLR signaling on innate immunity. Wild type and MyD88xTRIF deficient mice were infected with a lethal dose of *Listeria monocytogens* alone or were injected i.v. one day prior to listeriosis with *E.coli*. Wild type mice that had not been infected with *E.coli*, rapidly died from day four on due to listeriosis (Figure 4.25 **A**). In contrast, a previous injection of *E.coli* rendered mice resistant to a detrimental infection with *Listeria monocytogenes*. This protection required signaling via TLRs, as

MyD88xTRIF deficient mice were susceptible to listeriosis irrespective of whether they received a previous injection of *E.coli* or not.

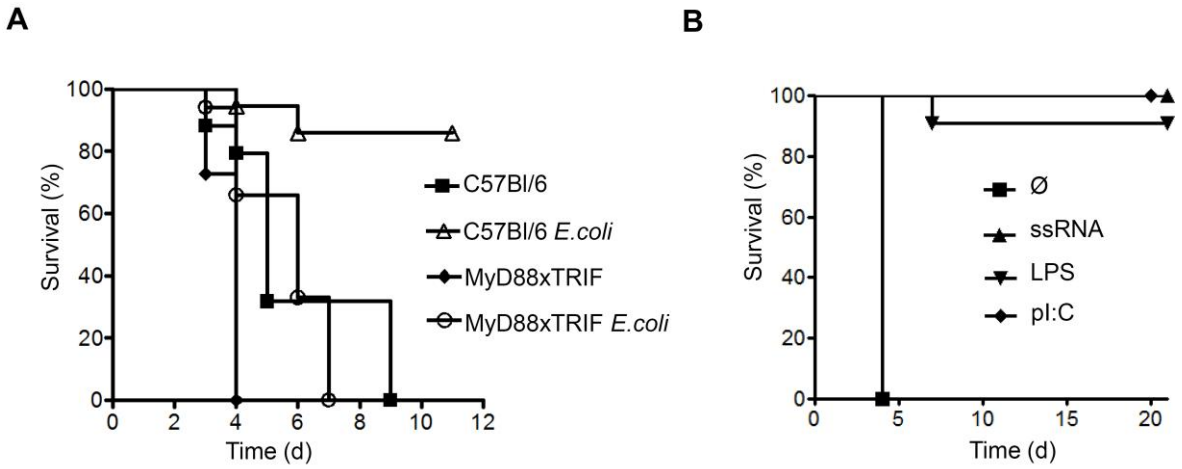

Figure 4.25 Mice previously infected with *E.coli* showed less susceptibility to listeriosis, enhanced survival and bacterial clearance in a TLR signaling dependent manner
Analysis of survival of mice after infection with $5x10^4$ CFU *Listeria monocytogenes*
(A) C57BL/6 wild type mice or mice deficient in MyD88 and TRIF were infected with $5x10^5$ *E.coli* prior to administration of Listeria or left untreated. **(B)** C57BL/6 mice were injected with 20µg ssRNA complexed in DOTAP, 1µ LPS or 50µg pI:C respectively one day prior to infection with Listeria. Survival of mice was assayed once a day. Experiment was performed with **(A)** n=9 mice per group and **(B)** n=6 mice per group.

To investigate if the protective effect of *E.coli* during Listeria infection was restricted to bacterial TLR ligands, we treated C57BL/6 wild type mice with different bacterial or viral TLR ligands prior to Listeria infection. To this end ssRNA recognized by TLR7 and TLR8, LPS which binds to TLR4, and the ligand for TLR3 pI:C were injected i.v. one day before infection with a lethal dose of *Listeria monocytogenes*. All mice that had received TLR ligands prior to infection with Listeria were significantly protected and survived the infection, whereas all untreated wild type mice died on day four (Figure 4.25 **B**).

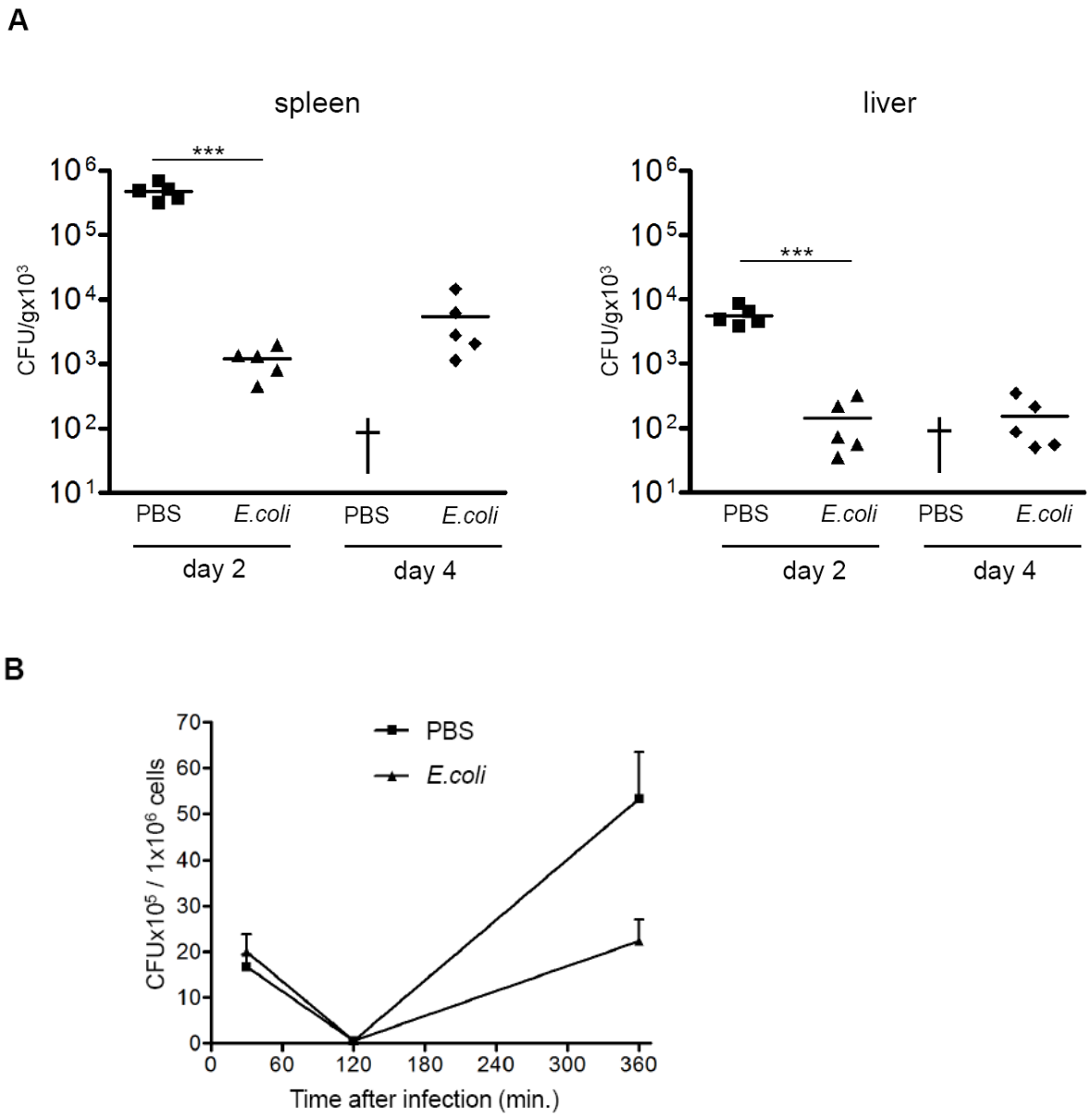

Figure 4.26 *E.coli* pretreatment enhanced capability of macrophages to kill Listeria *in vivo* and *ex vivo*

(**A**) C57BL/6 mice were treated with PBS or $5x10^5$ *E.coli* one day prior to infection with $1x10^5$ Listeria. Bacterial load was determined in spleens and livers of mice two and four days after infection with Listeria and is displayed as CFU per gram tissue. (**B**) Macrophages were isolated from spleen of mice infected with $5x10^5$ *E.coli* or left untreated. Isolated macrophages were infected *in vitro* with $1x10^5$ Listeria and bacterial burden was determined at indicated time points.

These results clearly showed that application of TLR ligands in general was protective in an infection model of *Listeria monocytogens*. Next we analyzed if the enhanced survival of mice pretreated with TLR ligands was due to an increased clearance of bacteria. Accordingly, we assessed the bacterial load in spleens and livers after two and four days post infection in mice, that had been left untreated or were injected i.v. with UV-killed *E.coli*. Mice that were infected with UV-killed *E.coli* one day prior to Listeria infection

showed improved clearance of Listeria in spleens and livers after two hours (Figure 4.26 **A**). In mice treated with PBS no bacterial load could be determined since they died from listeriosis on day three and four. To further investigate if the enhanced clearance of Listeria was due to an improved activity of macrophages, we isolated splenic macrophages from mice treated with UV-killed *E.coli* or PBS on the day before isolation. Macrophages were infected *ex vivo* with Listeria monocytogenes and CFU per $1x10^6$ cells was determined after two and six hours. Macrophages isolated from mice preatreted with *E.coli* killed Listeria much better than macrophages from control mice (Figure 4.26 **B**). From this observation we concluded that triggering of TLR signaling on the one hand could modulate CTL responses but at the same time could enhance innate immunity, such as activation of macrophages.

4.9 Summary of chapter 4

Our data presented here showed that high numbers of bacteria rapidly disseminated to distant organs in a mouse model for sepsis (CASP), whereas sham treatment led to bacterial translocation only to a minor extent. Most importantly, mice that underwent CASP showed a significant impairment of CTL responses against subsequent infections with viral and bacterial pathogens in contrast to sham treated mice which efficiently generated antiviral cytotoxic T cell responses. This suppression was characterized by reduced numbers of antigen-specific CD8 T cells which did lacked antigen-specific cytotoxicity and correlated with the amount of bacteria that had reached the spleen. Moreover, only CTL responses towards subsequent systemic infections were inhibited, whereas the generation of already established CTL responses was stimulated and induction of local immunity was unaffected. We revealed that Kupffer cells and TNF within the liver contributed to the retention and clearance of gut-derived bacteria, thereby preventing systemic distribution of bacteria and in consequence immune suppression.

Furthermore, our data indicated that antigen-specific CTL responses against systemic infections were mainly induced in the spleen and required an intact splenic architecture. Efficient induction of adaptive CD8 T cell responses in the spleen depended on CD8+DEC205+ DCs and metallophilic marginal zone macrophages (MMMs). Both DCs and MMMs were affected by bacteremia as they were not able to induce CTL responses

after exposure to *E.coli*. We identified TLR4 downstream signaling via TRIF and a subsequent expression of type I IFNs to be involved in mediating the suppression of antigen-specific CTL responses. Furthermore, we revealed that TLR signaling is not necessarily required for the initiation of CTL responses, but rather is indispensable for induction of innate immune responses. Thus, our results imply a dual role of TLRs, as the anatomical site, dose and time point of TLR ligand application determined if a systemic adaptive immune response was stimulated or suppressed.

5 Discussion

Sepsis, systemic inflammatory response syndrome (SIRS), and multi-organ failure are serious clinical problems because of high patient morbidity and mortality. One factor thought to be important in the etiology of SIRS is a failure of the intestinal barrier resulting in bacterial translocation and subsequent bacteremia causing sepsis (Nieuwenhuijzen et al., 1996a; Pastores et al., 1996). Whereas the influence of sepsis on the innate immune system is well described, the counter regulatory mechanisms and the impact on the adaptive immune system are still largely unknown.

The aim of this thesis was to analyze the influence of gut-derived bacteria on the induction of systemic immune responses. Disintegration of the gut barrier led to profound release of intestinal bacteria resulting in a rapid systemic dissemination. Here we showed that bacteremia caused suppression of adaptive immune responses against subsequent viral and bacterial pathogens, which was characterized by reduced numbers of antigen-specific cytotoxic T cells and a lack of antigen-specific cytotoxicity. Interestingly, a systemic distribution was not always observed upon bacterial translocation from the gut as the liver exhibited a filter function thereby preventing the dissemination of gut-derived bacteria, development of bacteremia and subsequent immunosuppression. Importantly, the induction of local immunity at peripheral sites was not affected by bacteremia, contradicting the current opinion of a general, systemic immunosuppression following sepsis. Moreover, bacteremia exclusively suppressed the generation of subsequent adaptive immune responses, whereas already established CTL responses were further stimulated by systemic bacteria. Although *E.coli* stimulated a broad variety of TLRs, suppression was solely dependent on TLR4 activation. Neither TLR4 downstream signaling via MyD88 nor expression of the potent suppressive cytokine IL-10 contributed to the observed immunosuppression after bacteremia. Instead signaling via TRIF and subsequent expression of type I interferons (IFNs) were critically involved in *E.coli*-mediated CTL suppression.

Suppression of systemic CTL responses strictly relied on bacteria being present in the spleen. We demonstrate here that the induction of adaptive immune responses against systemic infections, such as infection resulting from blood-born adenovirus, was initiated in the spleen. Furthermore, macrophages and DCs in the marginal zone were critically involved in the generation of adaptive immunity. It is known that metallophilic marginal

zone macrophages (MMMs) play an important role in the induction of CTL responses as they efficiently take up blood borne antigens and transfer them to cross-presenting DCs (Backer et al., 2010). Exposure of these cell types to *E.coli* impaired their ability to induce adaptive immunity to subsequent adenoviral infection thus pointing out the central role of splenic macrophages and DCs in the induction of immunosuppression after sepsis.

The observed suppression of adaptive immunity was striking as pathogens are known to be potent activators of the innate immune system by stimulation of pattern-recognition receptors (PPRs) such as Toll-like receptors (TLRs). In addition, activation of the innate immune system is known to be essential for the initiation of an effective adaptive immune response (Akira et al., 2006; Iwasaki and Medzhitov, 2004). Here we showed that activation of innate immunity indeed strictly depended on TLR signaling, whereas such signaling was not required for triggering of effective adaptive immune responses. Activation of TLRs on the one hand led to effective induction of innate immunity, but on the other hand exerted mechanisms which could rather regulate adaptive immune responses in a paracrine manner. The results presented in this thesis clearly demonstrate a dual role of TLRs: the anatomical site, dose and time point of TLR ligand application determined whether an adaptive immune response was stimulated or suppressed.

5.1 The liver retains translocated bacteria thereby inhibiting spleen-dependent suppression of adaptive immune responses during sepsis

The gut is the largest border surface of the body and plays an important role in digestion and absorption of nutrients. Overall the intestine harbors > 10^{14} bacteria with at least 500−1,000 species (Gill et al., 2006; Sonnenburg et al., 2004). Therefore the intestinal epithelium represents a critical barrier between the huge reservoir of endotoxins and the cells of the body (Magnotti and Deitch, 2005). Breakdown of this gut barrier leads to translocation of bacteria and subsequent dissemination to distant organs, such as mesenteric lymph nodes, liver, lung and spleen (Caso et al., 2009; Deitch and Berg, 1987b; Deitch et al., 1986; Schulte-Herbruggen et al., 2009).

In this study we reported that the integrity of the gut barrier could be disrupted by mechanical stress during abdominal surgery. Abdominal surgery, mimicked by a sham operation, resulted in bacterial translocation and bacteria were found predominantly in liver, lung, and mesenteric lymph nodes (Figure 4.3). Bacterial translocation was even worse in a common

model of poly-microbial sepsis, namely the ascendens stent peritonitis (CASP), in which gut content is released through a stent into the peritoneal cavity. *In vivo* imaging experiments revealed that bacteria disseminated very fast and were detected in comparable numbers in all organs after 150 min. (4.1 **A** and **B**). This is in line with clinical observations, where perforation of the gut, as observed for example in leaky anastomosis or ruptured appendix, subsequently leads to sepsis (Komen et al., 2008).

We and others have revealed that systemic exposure to TLR ligands suppresses adaptive T cell immunity (Mellor et al., 2005; Wingender et al., 2006). Likewise, we investigated if bacteria, which have translocated from the gut were also able to inhibit the induction of CTL responses. Mice that underwent CASP lacked effective CTL responses (Figure 4.2). In contrast, mice that underwent abdominal surgery were not impaired in their ability to induce CTL responses upon adenoviral infection, despite of bacteria being present in several organs (Figure 4.3 and Figure 4.4). We assume that this difference is due to varying amounts of bacteria being present in the spleen. This hypothesis was further substantiated by the fact that the degree of suppression strictly correlated with the number of injected bacteria (Figure 4.5 **B**). Mice injected with *E.coli* i.po., where only few bacteria reached the spleen, generated significantly stronger CTL responses compared to mice infected with the same amount of bacteria i.v., where high amounts of bacteria were detected in the spleen (Figure 4.8 **B**). These data provide strong evidence that suppression of CTL responses depends on high numbers of bacteria reaching the spleen while low amounts of bacteria do not negatively influence CTL response since they do not reach the spleen. Considering the differences in bacterial distribution after i.v. and i.po. application of *E.coli* (Figure 4.8 **A**), these data clearly imply that the liver might play an important role in the retention of bacteria. Indeed, it has been described that the liver represents an important barrier which trap and clear bacteria as well as endotoxins that translocate from the gut via the vascular route and enter the liver through the portal vein (Gregory et al., 2002; Gregory et al., 1996; Gregory and Wing, 2002; Jacob et al., 1977). This retention and clearance is attributed to resident liver macrophages, namely Kupffer cells, which line the hepatic sinusoids and are found in greatest numbers in the periportal area (Fox et al., 1987). In line with that we observed that *E.coli* bioparticles, injected into the portal vein, were efficiently taken up by Kupffer cells (Figure 4.9) and that mice were incapable to retain and clear i.po. injected bacteria after depletion of Kupffer cells by clodronate liposomes (Figure 4.10). In addition, we demonstrated that TNF was critically involved in the clearance and retention

of gut-derived bacteria as neutralization of secreted TNF or inhibition of TNF signaling via the TNF receptor resulted in an increase of bacterial burden mainly in the liver (Figure 4.11). As the biological effects of TNF vary from inducing cell death to promoting cell regeneration it will be difficult to identify a single effect of TNF on the bacterial clearance in the liver (Baker and Reddy, 1998; Leist et al., 1995a; Leist et al., 1995b). One possibility how TNF mediates its antibacterial effect might be by enhancing the adhesion of neutrophils within the liver. TNF has been shown to directly exert the expression of epithelial cell-derived neutrophil-activating protein-78 (ENA-78) as well as the integrin α5β1, which contribute to adhesion of neutrophils (Keates et al., 1997; Sun et al., 2010; Yoshimura et al., 1987). Along this line, neutrophil-Kupffer cell interaction has been described to be critically involved in host defense against systemic bacterial infection (Gregory et al., 1996; Gregory and Wing, 2002). Neutrophils accumulate within the liver sinusoids and kill bacteria onsite by the formation of neutrophil extracellular traps (NETs) which are build up by rapid release of DNA (Clark et al., 2007; Yousefi et al., 2008). Moreover the interaction between Kupffer cells and neutrophils is beneficial as neutrophils synthesize and secrete soluble factors that decrease the release of pro-inflammatory cytokines like IL-6 or TNF by Kupffer cells (Daley et al., 2005; Holub et al., 2009). Modulating the release of pro-inflammatory cytokines allows the inflammation to stay local thereby avoiding life-threatening systemic inflammatory response syndromes with concurrent clearance of bacteria within the liver. Future experiments need to be designed in order to analyze the exact role of TNF in the clearance of bacteria in the liver. Overall our findings underline the importance of TNF in the clearance of bacteria in the liver and thereby contributes to the maintenance of homeostasis.

It is discussed that bacterial translocation from the gut is the origin of sepsis and subsequent multi organ dysfunction syndrome (MODS) (Nieuwenhuijzen et al., 1996a, b; Pastores et al., 1996). In contrast our data suggest that bacterial translocation does not always lead to sepsis and subsequent MODS. The induction of CTL responses is not inhibited if low amounts of translocating bacteria are trapped and cleared within the liver. However, breakdown of this filter function by vast amounts of translocated bacteria or in the absence of macrophages or TNF, respectively, results in a systemic dissemination of bacteria and subsequent suppression of spleen-dependent CTL responses.

5.2 Mechanisms of *E.coli*-mediated suppression of adaptive immune responses

In general, stimulation of innate immune system by TLR ligands initiates the generation of subsequent adaptive immunity by causing functional maturation of antigen-presenting cells. However, we could show that a systemic exposure to *E.coli* resulted in the suppression of subsequent adaptive immunity against various viral and bacterial pathogens (Figure 4.7). This *E.coli*-mediated suppression was characterized by a reduction in the number of antigen-specific T cells that led to lack of specific cytotoxicity (Figure 4.6 **B**). These findings are in line with several reports showing that systemic exposure to TLR ligands suppresses subsequent adaptive immune responses (Mellor et al., 2005; Wingender et al., 2006). Interestingly, we observed that the dosage and time point of *E.coli* infection played a crucial role in the determination whether CTL responses against other pathogens were stimulated or suppressed. Low doses of *E.coli* did not influence the induction of CTL responses against a subsequent adenovirus infection, whereas doses greater than $1x10^4$ *E.coli* significantly reduced CTL responses (Figure 4.5 **B**). Moreover, the suppressive effect of *E.coli* lasted for about five days, as injection of *E.coli* seven days before infection with adenovirus did not inhibit antigen-specific cytotoxicities (Figure 4.5 **A**). In contrast, administration of *E.coli* after infection with adenovirus boosted the generation of antigen-specific CTL responses. Both cytotoxic activity and the numbers of antigen-specific CD8 T cells were enhanced in mice treated with *E.coli* after adenoviral infection (Figure 4.6 **A** and **B**) indicating that systemic bacteria did not negatively influence already induced antigen-specific CTL responses. This finding supports the current opinion postulating immunostimulatory properties of TLR ligands (Akira et al., 2006; Iwasaki and Medzhitov, 2004). Taken together, we demonstrate that the time point of bacteria being present in the spleen determines whether antigen-specific CTL responses are suppressed or even enhanced.

5.2.1 Suppression of adaptive immunity is associated with the spleen and relies on TLR4-dependent TRIF signaling

Next we were interested in analyzing the impact of bacteremia on the induction of local immunity. Interestingly, systemic injection of *E.coli* did not impair the induction of local immunity in the lung against adenovirus, whereas CTL responses were absent in the spleen after a systemic adenovirus infection. Thus, suppression of CTL responses seems to be a

phenomenon affecting systemic immunity, whereas local immunity at peripheral sites is not altered. Furthermore, it rules out the importance of the spleen in induction and regulation of adaptive immunity towards systemic infections.

Based on these findings, the molecular mechanisms underlying the suppression of CTL responses by *E.coli* were unveiled. Cells mainly sense bacteria via TLRs and components of *E.coli* stimulate a broad variety of TLR ligands, such as CpG which is sensed by TLR9, peptidoglycan which binds to TLR2 and LPS that stimulates TLR4. TLR4 and its downstream signaling pathways play a dominant role in promoting sepsis and solely the blockade of TLR4 was shown to protect mice from septic shock (Beutler and Rietschel, 2003; Hoshino et al., 1999; Roger et al., 2009). We analyzed the role of TLRs in our model of immunosuppression. However, neither TLR9 nor TLR2 contributed to the suppression as mice deficient in these TLRs still showed diminished CTL responses upon bacteremia and subsequent adenoviral infection. This was unexpected as *E.coli* provides TLR2 and TLR9 ligands and systemic application of TLR2 or TLR9 ligands before adenoviral infection was shown to potently suppress antigen-specific CTL responses (unpublished findings by Beatrix Schumak) (Mellor et al., 2005; Wingender et al., 2006) (Figure 4.19 **A** and **B**). Interestingly, suppression by *E.coli* was solely mediated by TLR4, as mice deficient in TLR4 were capable to mount strong CTL responses upon infection with *E.coli* and subsequent immunization with adenovirus (Figure 4.19 **C**). Although CD14 is known to be critically involved in recognition of LPS (Kitchens and Thompson, 2005) it did not significantly contribute to suppression by *E.coli* (Figure 4.20). The role of CD14 and its interaction with TLR4 and the myeloid differentiation factor 2 (MD-2) is controversially discussed in literature. While observations by Jiang et al. showed that CD14-independent binding of LPS is restricted to so called "rough" LPS, recent publications have shown evidence of direct interaction of LPS with MD-2 (Jiang et al., 2005; Park et al., 2009). Furthermore, the publication of Jiang et al. claimed that CD14 is a prerequisite for TRIF signaling after stimulation of TLR4 by LPS. These findings contradict our observation that immunosuppression after bacteremia was CD14 independent, but strictly depended on TLR4 and TRIF signaling. To further elucidate the role of CD14 or MD-2 in the observed immunosuppression, CD14 deficient mice will be treated with various types of LPS in future experiments.

Based on these key findings we investigated the influence of TLR4 induced expression of IL-10 in suppression of adaptive immune responses. Stimulation of TLR4 and subsequent activation of NFκB leads to the expression of pro- as well as anti-inflammatory cytokines

(Boonstra et al., 2006; Hacker et al., 2006). IL-10 is one of the most potent anti-inflammatory cytokines released by broad variety of immune cells upon stimulation with pathogen-associated molecular pattern (PAMP) and primarily targets antigen-presenting cells (Pestka et al., 2004a; Siewe et al., 2006; Zhang et al., 2009). Using IL-10 transgenic reporter mice, we revealed that IL-10 was produced mainly by splenic CD11b positive cells upon *E.coli* injection (Figure 4.17). This is in line with findings demonstrating that optimal LPS-induced IL-10 production by macrophages require the induction of TLR dependent signaling pathways as well as the production of type I IFNs (Boonstra et al., 2006; Chang et al., 2007). Although IL-10 has a strong capacity to inhibit adaptive T cell responses (Moore et al., 2001), it was not involved in the bacteremia-mediated suppression of adaptive immune responses in our model, as mice deficient in IL-10 were still impaired in their ability to generate strong CTL responses after *E.coli* treatment (Figure 4.18). IL-10 is produced as part of the homeostatic response to infection and inflammation thus playing a critical role in limiting the duration and intensity of immune reactions. As IL-10 did not contribute to *E.coli*-mediated immunosuppression this indicates that the relevant mechanisms allowing the development of T cell immunity in the spleen are not influenced by IL-10 in general.

5.2.2 *E.coli*-mediated suppression of CTL responses requires TRIF and type I interferon signaling

Based on these key findings we investigated the relevance of TLR4 downstream signaling in suppression of adaptive immune responses. TLR4 signals via two adaptor molecules MyD88 and TRIF. While signaling via MyD88 initiates the expression of pro- as well as anti-inflammatory cytokines, activation of TRIF predominantly leads to recruitment of transcriptions factors, namely IRFs, which induce the expression of type I IFNs (Boonstra et al., 2006; Hacker et al., 2006). In accordance with our findings that the absence of TLR4 prevented immunosuppression, mice deficient in both adaptor molecules MyD88 and TRIF showed no *E.coli*-mediated suppression of adenovirus-specific CTL responses (Figure 4.21). This result further confirmed the general role of TLR4 in the suppression of CTL responses after systemic application of *E.coli* (Figure 4.21 **A**). Furthermore, MyD88 did not contribute to the suppressive effect of *E.coli* whereas inhibition of TRIF signaling prevented the induction of mechanisms leading to suppression of CTL responses toward adenovirus after systemic exposure to *E.coli* (Figure 4.21 **B** and **C**). TRIF is identified as a

regulator of IFN transcription by recruiting IRF3 and IRF7 (Fitzgerald et al., 2003b). In line with the literature we could show that type I IFNs were expressed after i.v. injection of *E.coli* and cells in the spleen responded to these cytokines (Figure 4.22) (Decker et al., 2005). Along this line, we demonstrated that type I IFNs were critically involved in *E.coli*-mediated immunosuppression, as mice lacking the IFN-α receptor (IFNAR) as well as mice deficient in IRF7 or IRF7 and IRF3 generated significantly enhanced CTL responses after bacteremia and subsequent adenoviral infection compared to wild type mice (Figure 4.23). These data imply an equivalent contribution on immunosuppression of early expressed type I interferons initiated by IRF3 on the one side and the production of IFNs induced by the amplification loop which is triggered by IRF7 on the other side. This is astonishing because type I IFNs are generally associated with immune stimulation, as type I IFNs are known to enhance DC maturation and activation as well as differentiation of CD8 T cells by providing signal 3 (Curtsinger et al., 2005; Le Bon et al., 2003; Luft et al., 1998; Montoya et al., 2002). Thus, type I IFNs display an essential link between early innate and subsequent adaptive immune responses (Biron, 2001; Bogdan et al., 2004). In contrast to the stimulatory capacity, also anti-inflammatory functions of type I IFNs have been reported in several models of autoimmunity such as colitis or systemic lupus erythematosus (Hron and Peng, 2004; Katakura et al., 2005). Furthermore, effector T cells, macrophages, as well as DCs are sensitized to apoptosis upon exposure to type I FNs (Lehner et al., 2001; Merrick et al., 1997; Stockinger et al., 2002). In addition, it has been reported that these double sided effects of type I IFNs apply to alterations in antigen cross-presentation of DCs depending on their maturation. Exposure of mature DCs to type I IFNs results in upregulation of co-stimulatory molecules such as CD40L or IL-12 (Gautier et al., 2005; Luft et al., 1998). In contrast, immature conventional DCs activate STAT1 in response to type IFNs which results in significant inhibition of these co-stimulatory molecules, thus accounting for the inhibition of CD8 T cell activation (Longman et al., 2007).

Thus based on our findings that blockade of type I IFNs expression and signaling prevented immunosuppression, type I IFNs are a potential candidate to affect splenic macrophages or DCs in our model. To further elucidate the role of type I IFNs in suppression of CTL responses after bacteremia, experiments in mice with Cre-mediated cell type-specific IFNAR deficiency need to be performed.

5.3 The crosstalk of splenic macrophages and DCs is crucial for induction and suppression of CTL responses

Since the spleen as a secondary lymphoid organ is known to be critically involved in the induction of CTL responses we investigated the role of the spleen in our model of adenoviral infection (Backer et al., 2010; Balazs et al., 2002; Mebius and Kraal, 2005; Oehen et al., 2002). After splenectomy mice were not able to induce adenovirus-specific CTL responses any more. The ability to mount a CTL response upon adenoviral infection could partially be restored by re-transplantation of the spleen into splenectomized mice (Figure 4.13) This clearly reveals that the spleen is required for effective induction of systemic immunity against adenoviral infections. Importantly, we demonstrated that reconstitution of splenectomized mice with splenic single cell suspensions failed to rescue the ability to induce CTL responses. Thus, the lack of CTL responses is not due to the absence of splenic cells but rather the consequence of a lack of particular compartments within the spleen that promote development of T cell immunity.

In collaboration with the group of Joke de Haan we have recently shown that macrophages in the marginal zone efficiently take up blood borne antigens and transfer them to CD8+ splenic DCs, leading to an efficient cross-presentation and priming of CTL responses (Backer et al., 2010). Moreover, DCs present in the marginal zone play an essential role in priming CTL responses (Belz et al., 2005; de Haan et al., 2000). Based on these findings we analyzed the role of marginal metallophilic macrophages (MMMs) and CD8+ splenic DCs in the induction of adenovirus-specific immune responses. Depletion experiments revealed that both MMMs and DCs are essential for an effective CTL response upon adenoviral infection (Figure 4.14). Interestingly, DCs alone were not efficient to induce CTL responses. As there is a close collaboration between macrophages and DCs in the marginal zone of the spleen we further analyzed the impact of *E.coli* on these two cell populations. Histological analysis revealed that adenovirus predominantly infected MMMs (Backer et al., 2010; Qiu et al., 2009)and macrophages in the marginal zone efficiently phagocytosed *E.coli* (Figure 4.15). To circumvent the need of macrophages in the induction of CTL responses we immunized mice with an OVA-coupled antibody (DEC205-OVA), which directly targeted the antigen to CD8+ DCs. Depletion of macrophages with clodronate liposomes and subsequent immunization of mice with DEC205-OVA led to a strong CTL response (Backer et al., 2010). Nevertheless, CTL responses were significantly reduced in mice pretreated with *E.coli* and subsequent DEC205-OVA immunization. The same out-

come was observed if the antigen was directed to MMM by Siglec-1-OVA antibody (Figure 4.16). As in both cases CTL responses were suppressed after systemic *E.coli* we conclude that both macrophages as well as DCs are affected by exposure to *E.coli*. So far, we were not able to determine whether macrophages release mediators upon *E.coli* stimulation which subsequently suppresses the DCs or if macrophages themselves lack their ability to hand over antigen to the DCs. For this reason it would be interesting to analyze the ability of mice depleted of macrophages to induce CTL responses after bacteremia and immunization with DEC205-OVA. This experiment could be performed in transgenic mice expressing the diphtheria toxin receptor under the CD11b promoter or by depletion of macrophages by clodronate liposome application. Alternatively cell-type specific TLR4 deficient mice could be used to reveal the cell type which is initially responsible for the induction of suppression.

Furthermore we observed that reduced numbers of DCs were present in the marginal zone after exposure to *E.coli* compared to untreated mice (Figure 4.15). On one hand this could be due to depletion of DCs by apoptosis or, on the other hand due to DC-migration from the marginal zone into the T cell zone of the spleen after exposure to TLR ligands (De Smedt et al., 1996; Hotchkiss et al., 2002; Wen et al., 2008). In the latter case DCs would not be able to gain the antigen from macrophages located in the marginal zone due to spatial separation. Although we cannot exclude depletion of DCs so far we have preliminary data underlining the theory that DCs migrate and could not reach the antigen.

The late phase of sepsis is characterized by immune suppression (Docke et al., 1997; Heidecke et al., 1999) caused by profound depletion of immune effector cells (Hotchkiss and Karl, 2003; Hotchkiss et al., 2003; Hotchkiss et al., 2001). Here we report that immunosuppression in sepsis is not exclusively due to a profound depletion of lymphocytes and secretion of anti-inflammatory cytokines but rather due to a failure of spleen-resident macrophages and DC to induce CTL responses.

5.4 TLR-signaling determines the outcome of CTL responses

DCs constitutively sample their microenvironment and phagocytose pathogens as well as host apoptotic cells (Greenberg and Grinstein, 2002; Ravichandran, 2003; Savill et al., 2002).The induction of functional adaptive immune responses requires the activation of professional antigen presenting cells, such as DCs (Blander and Medzhitov, 2006). Upon

TLR stimulation DCs alter their phenotype from an immature to a mature phenotype, which is characterized by secretion of cytokines, up-regulation of MHC molecules and their ability to efficiently prime T cell responses (Inaba et al., 1993; Ludewig et al., 1998; Reis e Sousa et al., 1997). *In vivo*, two major receptor families play prominent roles: TLRs and tumor necrosis factor (TNF) receptors, mainly CD40 (De Smedt et al., 1996; Hawiger et al., 2001; Sparwasser et al., 2000). Without this stimulation, DCs differentiate into a tolerogenic phenotype and induce tolerant or regulatory T cells (Steinman et al., 2003). Therefore efficient induction of CTL responses strictly correlates with close association of antigen and TLR ligands in one cellular compartment as CTL activity was increased, if antigens were coupled to TLR-L directly (Blander and Medzhitov, 2006; Heit et al., 2007). In contrast to these studies, our findings revealed that TLR signaling through MyD88 and TRIF is not required for efficient induction of CTL responses. Mice lacking entire MyD88 and TRIF still induced profound antigen-specific CTL responses upon infection with a broad variety of viral infections such as adenovirus, influenza virus, MCMV, and HSV (Figure 4.21 **A** and 4.24). This finding was not completely unexpected, as virus can be recognized by the inflammasome or intracellular PRRs such as RIG-I or MDA5 which subsequently leads to activation and maturation of DCs (Franchi et al., 2010; Kato et al., 2006; Yoneyama et al., 2004). Based on these findings, we hypothesize that TLR signaling is not required for generation of effective CTL responses in general but instead plays a crucial role in modulating CTL immunity. This hypothesis was further supported by the fact that *E.coli* treatment and TLR4 signaling could dampen or enhance CTL responses, depending on whether TLR signaling was initiated before or after viral infection (Figure 4.6).

TLR ligands do not solely induce maturation of DCs and thus promote adaptive immunity, but they are also able to prime the innate immune system as well. For example, TLR2 signaling is strongly associated with the release of nitric oxide (NO) and stimulation of TLR4 is reported to initiate the release of defensines (Lehrer and Ganz, 2002; Thoma-Uszynski et al., 2001). Additionally, macrophages are efficiently activated by TLR ligands and TLR stimulation leads to secretion of pro-inflammatory cytokines by macrophages themselves as well as DCs (Akira et al., 2006). Accordingly, we demonstrated that mice treated with *E.coli* i.v. and subsequently infected with Listeria monocytogenes survive listeriosis, whereas mice that had not been infected with *E.coli* rapidly died due to listeriosis (Figure 4.25 **A**). Importantly, this protective effect was dependent on TLR signaling as mice deficient in

MyD88 and TRIF were not protected from listeriosis irrespective of whether they had been previously infected with *E.coli* or not. Moreover, the protective effect was not restricted to *E.coli* as previous application of TLR ligands to infection with Listeria was shown to protect mice from listeriosis (Figure 4.25 **B**). The improved survival was due to significantly enhanced bacterial clearance in mice pretreated with *E.coli* as these mice showed reduced bacterial burden in spleen and liver compared to untreated mice. In addition, we could ascribe the improved bacterial clearance to macrophages as they showed an enhanced ability to kill Listeria ex vivo (Figure 4.26).

These data clearly reveal a new function of TLRs in the induction and regulation of adaptive immunity. Although systemic distribution of TLR ligands has no effect on the induction of local immunity, they exert regulating effects on the induction of CTL systemic immune responses. As expected, triggering of TLRs stimulates the innate immune system, but in contrast, systemic TLR ligands inhibit the induction of subsequent CTL responses while they boost already established immunity. The group of Reis e Sousa showed that APCs cannot be activated in a paracrine manner by cytokines and only direct activation by TLRs enable APCs to potently induce immunity (Sporri and Reis e Sousa, 2005). Here we showed that the induction of immunity did not depend on TLR signaling but could also be triggered by a so far unknown mechanism. Furthermore we could demonstrate that inhibition of T cell immunity is mediated in a paracrine fashion by type I IFNs. Moreover, these data revealed that the molecular mechanisms determining suppression are dominant over the induction of immunity.

Regarding the benefits for the organism we speculate that the observed immunosuppression represents somehow an “emergency shutdown” of the adaptive immune system to prevent autoimmunity. As DCs are known to present self-antigens, this mechanism might ensure that the induction of CTL responses only takes place if antigen and stimulation by TLRs are present simultaneously, namely if antigen and TLR ligands originate from one and the same pathogen. Separation of antigen and TLR stimuli in time would imply that antigen and TLR ligands are derived from different sources, therefore increasing the risk of stimulating autoimmune responses. Increased cell death occurs in many situations such as during sepsis (Hotchkiss et al., 2003; Hotchkiss et al., 2001; Tinsley et al., 2003) and it promotes allocation of self antigens such as DNA, heat shock proteins, or high mobility group box 1 protein (HMGB1) a nuclear protein that is released passively after lose of cell membrane integrity in necrotic cells (Bianchi and Manfredi, 2007; Hof et al., 2005).

HMGB1 is taken up and cross-presented on DCs thus giving rise to the production of auto-antibodies which can be found in a high proportion of patients with various autoimmune disorders (Bondanza et al., 2004; Gauley and Pisetsky, 2009; Sobajima et al., 1998; Uesugi et al., 1998). Thus, we suggest that TLR-dependent induction of immunosuppression represents one of the physiological mechanisms to prevent potentially undesired or even harmful immune responses.

5.5 Conclusion and outlook

The findings presented in this thesis demonstrate several aspects of immunosuppression due to bacteremia. We could show that bacterial translocation per se is not harmful as the liver prevents systemic dissemination of gut-derived bacteria. Nevertheless, if bacteria reach the spleen, splenic macrophages and DCs are affected in their ability to induce CTL responses. Although we revealed that suppression in gram-negative sepsis depends on TLR4 signaling via TRIF and subsequent expression of type I interferon, further experiments have to be performed addressing the question which cell type is affected. Furthermore, it remains to be determined whether phenotypical changes of DCs or macrophages lead to immunosuppression.

Nevertheless, profound immunosuppression is a critical factor in intensive care medicine as it gives rise to opportunistic infections (Munoz et al., 1991; Mustard et al., 1991; Richardson et al., 1982). Therefore, it would be reasonable to prevent the suppression of CTL responses due to bacteremia. With respect to our data this would only make sense if given in advance to surgery of the gastrointestinal tract. Thus prophylactic pharmacological inhibition of TLR4, for example by the drug Eritoran, would prevent sepsis (Wittebole et al., 2010) and subsequent immunosuppression.

Overall these results revealed a dual role of TLRs in the induction of immunity as that the anatomical site, dose and time point of TLR ligand application determined if an adaptive immune response was stimulated or suppressed. Moreover, these results may provide insights for the development of new therapeutic approaches that might circumvent suppression of systemic adaptive immune response in septic patients.

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7 Table of Figures

8 Abbreviations

AdLOG	adenovirus expressing luciferase, ovalbumin, green fluorescent protein
AdOVA	adenovirus expressing ovalbumin
Al647	Alexa647
APC	antigen presenting cell
BCR	B cell receptor
BSA	bovine serum albumin
C	Celsius
CCL	CC-chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
cDC	conventional DC
CFU	colony forming unit
cm	centimeter
CpG	cytosine-phosphate-guanosine
CTL	cytotoxic T lymphocyte
Cre	Cre (*causes recombination*) recombinase
DC	dendritic cell
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
DTR	diptheria toxin receptor

EDTA	ethylenediaminetetraacetic acid
ENA-78	epithelial cell-derived neutrophil-activating protein-78
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
g	gram
g	gravity
GFP	green fluorescent protein
h	hours
HMGB1	high mobility group box 1 protein
HSP	heat shock protein
HSV	Herpes Simplex Virus
i.p.	intraperitoneally
i.po.	intraportally
i.v.	intravenously
IFN	interferon
IFNAR	type I interferon receptor
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
IRFBS	IRF-binding site
ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulates response element

JAK	Janus kinase
ko	knock out
l	liter
Lm	Listeria monocytogenes
LPS	lipopolysaccharide
Lux	LuxABCDE genecluster
mM	millimolar
MAPK	mitogen-activated protein kinase
MCMV	murine cytomegalovirus
MDA-5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
min	minutes
ml	milliliter
mm	millimeter
MODS	multi organ dysfunction syndrom
MxA	myxovirus resistance protein
NET	neutrophils extracellular traps
ng	nanogram
NK cells	natural killer cells
NOD	nucleotide-binding oligomerization domain
nt	nucleotides

OD	optical density
ODN	oligonucleotide
ORN	oligoribonucleotide
OVA	ovalbumin
PALS	periarteriolar lymphoid sheaths
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PFA	paraformaldehyde
PFU	plaque forming unit
POI	postoperative ileus
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene I
RNA	ribonucleic acid
rpm	rounds per minute
s.c.	subcutaneously
S8L	SIINFEKL
SEM	standard error of the mean
Siglec-1	sialic acid-binding immunoglobulin-like lectin-1
siRNA	small interfering RNA
ssRNA	single-stranded RNA
SIRS	systemic inflammatory response sysndrom
STAT	signal transducers and activators of transcription
TCR	T cell receptor

TGFβ	transforming growth factor beta
Th1	T helper 1 cells
Th2	T helper 2 cells
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Tyk	tyrosine kinase
UV	ultra violet
v	Volume
VP	virus particles
w	weight
YFP	yellow fluorescence protein
µg	microgram
µl	microliter
µm	Micrometer

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