The Long-time Consequences of Systemic Inflammation and Sepsis on T-cell Immunity

Dissertation

To Fulfil the
Requirements for the Degree of
„doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the Council of the Faculty
of Biology and Pharmacy
of the Friedrich Schiller University Jena

by Diplom-Biochemiker

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Datum der öffentlichen Disputation: 29.09.2015

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II Summary

Systemic Inflammatory Response Syndromes (SIRS), including *sepsis*, describe a broad spectrum of immunological disorders with a very heterogeneous clinical manifestation. A SIRS caused by infectious triggers is defined as sepsis and with mortality rates exceeding 30% and totally 56000 deaths *per annum*, septic syndromes are the third most common causes of death after cardiovascular diseases and cancer in Germany. Acute episodes of SIRS and sepsis are characterised by an excessive and de-regulated inflammatory host response leading to organ and tissue damage and most fatally to death. In addition, the uncontrolled release of pro- and anti-inflammatory immune mediators is the root of immune suppressive states in patients at acute and post-acute stages of the disease. Malfunction of T-cells, a major component of adaptive immunity, has been shown to contribute to acute disease-induced immunosuppression but very little is known about the functional state of T-cells at post-acute and late stages of SIRS and sepsis and its potential implication with late morbidity and mortality.

The present thesis provides an in-depth analysis of T-cell immunity at post-acute of SIRS and sepsis. Four different murine disease models were employed to account for the large clinical heterogeneity of the syndromes allowing drawing eligible conclusions for the situation in human patients. The data presented here show that SIRS and sepsis lead to protracted systemic loss of T-cells but do not induce persistent cellular defects in adaptive T-cell function. T-cell activation and responses were intensively characterised by the examination of activation marker up-regulation, T-cell proliferation capacity and detailed T-cell receptor signalling studies. T-cell analyses were extended by the employment of secondary infection models allowing to investigate antigen-specific effector T-cell responses on multiple levels, including cytokine production and activation marker up-regulation. *Ex vivo* and *in vivo* effector T-cell studies in background of secondary infections confirm that SIRS and sepsis do not induce protracted inherent alterations in T-cell function.

In sum, systemic inflammation and sepsis induce a profound persistent loss of naïve T-cells thereby affecting T-cell immunity, but do not compromise T-cell function on a cellular level at post-acute stages of the disease. These findings shift the focus from T-cell immune-stimulatory therapies in sepsis to other aspects of adaptive T-cell immunity, e.g. antigen presentation or prevention of T-cell apoptosis.

III Zusammenfassung

Systemische Inflammatorische Response Syndrome (SIRS), einschließlich *Sepsis*, beschreiben ein breites Spektrum an immunologischen Erkrankungen mit sehr heterogenem klinischen Erscheinungsbild. Ein SIRS mit nachgewiesenem infektiösen Ursprung wird als Sepsis definiert und stellt mit einer Sterblichkeit von über 30% und jährlich insgesamt 56000 Todesfällen die dritthäufigste Todesursache nach Herz-Kreislauf- und Krebserkrankungen in Deutschland dar. Die Pathophysiologie von SIRS und Sepsis ist gekennzeichnet durch unkontrollierte systemische Wirtsimmunreaktionen, die zu Gewebs- und Organschädigungen führen und die Ursache sowohl akuter als auch dauerhafter Störungen des Immunsystems darstellen. Insbesondere T-Zellen, als zentrale Komponente des adaptiven Immunsystems, weisen in der Akutphase der Krankheit eine Vielzahl von funktionellen Störungen auf, die zum immunsupprimierten Zustand der Patienten und damit zur sepsis-induzierten Mortalität beitragen. Sehr wenig ist aber darüber bekannt, ob T-Zellen zelluläre Defekte in post-akuten Krankheitsphasen aufweisen und ob diese potentiellen Störungen zur anhaltenden Immunsuppression beitragen, die mit erhöhter post-akuter Sterblichkeit assoziiert ist.

Die vorliegende Arbeit stellt eine umfassende Tiermodell-Studie zur T-Zell-Immunität in der post-akut Phase von SIRS und Sepsis dar. Um der Heterogenität der Krankheitsbilder gerecht zu werden und die klinische Relevanz der Studie zu erhöhen, wurden vier unterschiedliche experimentelle Mausmodelle verwendet. Die Ergebnisse dieser Studie zeigen, dass SIRS und Sepsis zu anhaltend verringerten T-Zellzahlen führen, aber keine zellulären Defekte in der adaptiven T-Zell-Antwort induzieren. Die Funktion der T-Zellen wurde eingehend anhand der Expression von Aktivierungsmarkern, der T-Zell-Proliferation und auf Ebene der T-Zell-Rezeptor-Signaltransduktion charakterisiert. Für weiterführende Analysen wurden sekundäre Infektionsmodelle verwendet, um antigen-spezifischen Immunantworten auf der Ebene von Effektor T-Zellen zu untersuchen. Funktionelle *ex vivo* und *in vivo* Studien im Hintergrund von sekundären Infektionen bestätigen, dass SIRS und Sepsis keine anhaltenden zellulären Defekte in der T-Zell-Funktion induzieren.

Durch die Befunde der vorliegenden Arbeit verschiebt sich der Fokus klinischer Forschung von T-Zell-stimulatorischen Therapien auf andere Aspekte der adaptiven Immunantwort zur Behandlung von immunsupprimierten Sepsispatienten.

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VI List of Abbreviations

AICD	Activation-induced cell death
AKT	Protein kinase B
AP-1	Activator protein 1
APC	Antigen-presenting cell
BID-1	BH3 interacting-domain death agonist
BTLA	B- and T-lymphocyte attenuator
CARS	Compensatory anti-inflammatory response syndrome
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming units
CLP	Cecal ligation and puncture
CON	Control
CRAC	calcium release-activated calcium channels
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAG	Diacylglycerol
DAMP	Danger-associated molecular pattern
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-related kinase
FACS	Fluorescence-activated cell sorting
FADD	Fas-Associated protein with Death Domain
FCS	Fetal calf serum
FoxP3	Forkhead box P3
Fura-2 AM	Fura-2-acetoxymethyl ester
GM-CSF	Granulocyte macrophage colony-stimulating factor
GOT	Aspartate aminotransferase
GPT	alanine transaminase
GTP / GDP	Guanosine triphosphate / Guanosine diphosphate
HMGB1	High-mobility group protein B1
ICOS	Inducible T-cell co-stimulator

ICU	Intensive care unit
IκB	Inhibitor of NfκB
IL	Interleukine
IP_3	Inositol 1,4,5-trisphosphate
iNOS	Inducible nitric oxide synthase
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
JNK	c-Jun N-terminal kinase
LAT	Linker of activated T-cells
LCMV-Arm	Armstrong strain of *Lymphocytic choriomeningitis virus*
LCP-2	Lymphocytic cytosolic protein 2
LCK	Lymphocyte-specific protein tyrosine kinase
LDH	Lactate dehydrogenase
LM	Listeria monocytogenes
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibilty complex
MIF	Macrophage migration-inhibitory factor
NFAT	Nuclear factor of activated T-cells
NfκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NLS	Nuclear localisation sequence
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCI	Peritoneal contamination and infection
PD-1	Programmed cell-death 1
PFU	Plaque forming units
PI_3-kinase	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKCθ	*Protein kinase Cθ*
PLCγ1	Phospholipase C γ1
PRR	Pattern recognition receptors
PUMA	p53 up-regulated modulator of apoptosis

PVDF	Polyvinylidene fluoride
RasGRP1	Ras guanyl-releasing protein 1
ROS	Reactive oxygen species
RT	room temperature
SDS	Sodium dodecyl sulfate
SEM	Standard error of mean
SH2	Src homology 2
SHIP	SH2-containing inositol phosphatase
SHP	SH2-containing phosphatase
SIRS	Systemic inflammatory response syndrome
SLP76	SH2 domain containing leukocyte protein of 76kDa
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-β
Th	T-helper
TLR	Toll-like receptor
TNFα	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Tris	tris(hydroxymethyl)aminomethane
ZAP-70	ζ-chain associated protein 70

1 Introduction

1.1 Systemic Inflammatory Response Syndrome and Sepsis

1.1.1 Systemic Inflammatory Response Syndromes and Sepsis: definitions

The *Systemic Inflammatory Response Syndrome* (SIRS) describes a multi-system inflammatory state characterised by the vigorous activation of the immune system and the profound release of cytokines and other immune modulators. The syndrome is associated with tissue damage and/or organ damage and can lead to death. It can be caused by non-infectious triggers, such as trauma, burns, surgical complications or pancreatitis. By definition, SIRS induced by infections with bacteria, viruses, fungi and other micro organisms is termed *sepsis*. In order to standardise the terminology of these highly diverse syndromes, precise definitions were proposed by a consensus conference in 1992 and are now widely accepted in the community (Fig. 1) [1]. A systemic inflammatory response syndrome is present by manifestation of two or more of the following conditions: (i) body temperature greater than 38°C or less than 36°C; (ii) heart rate greater than 90 beats per minute; (iii) tachypnea (> 20 breaths per minute) or hyperventilation ($PaCO_2$ of less than 32 mm Hg); (iv) altered leukocyte counts in blood (< 4,000 or > 12,000 cells / mm^3 blood) and (v) presence of more than 10% immature neutrophils in blood. A SIRS caused by the presence of a confirmed infection is defined as sepsis. Sepsis associated with organ/tissue failure and hypoperfusion abnormalities is defined as *severe sepsis*. *Septic shock* represents the most life-treating septic condition when a sepsis-induced hypotension occurs along with severe sepsis. However, these rather unspecific definitions do not entirely meet the complex clinical manifestation of the syndromes. Currently, physicians and clinical scientist discuss to extend the definition guidelines by including the occurrence of immunological and organ dysfunction parameters (e.g. C-reactive protein or plasma creatinine) that better indicate the presence of a systemic inflammation and/or sepsis associated with organ damage.

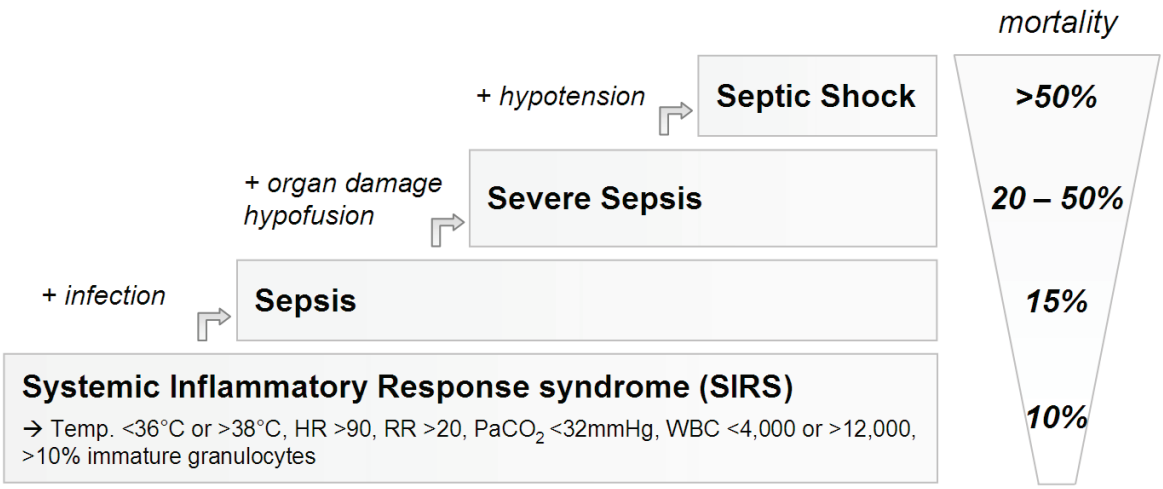


Figure 1. Systemic Inflammatory Response Syndromes and Sepsis - definitions
Systemic Inflammatory Syndromes (SIRS), including septic syndromes describe a broad spectrum of immunological disorders with a very heterogeneous nature and clinical manifestation. To standardise the terminology of the syndromes precise definitions were proposed by a consensus conference in 1992 that are widely expected now [1]. The mortality rates correlate with disease severity, ranging from 10% in sterile SIRS and 15% in non-severe sepsis up to 80% in septic shock. Abbreviations: Temp.: body temperature; HR: heart rate; RR: breaths per second; $PaCO_2$: CO_2 partial pressure in blood; WBC: white blood cell count.

1.1.2 Epidemiology of systemic inflammation and sepsis

Systemic Inflammatory Response Syndromes are the most common complications on intensive care units (ICUs) worldwide. According to an epidemiological cohort study, SIRS affects more than 50% of all ICU patients. More dramatically, more than 80% of surgical ICU patients develop a SIRS without a documented infection. The 28-day mortality rate of patients suffering from SIRS (excluding septic cases) is about 10% [2]. The same study shows that approximately 35% of all SIRS patients in ICUs develop sepsis and its subsets.

For sepsis, a large cohort study from 2001 shows that septic syndromes are common and fatal conditions accounting for more than 200,000 deaths in the United States of America [3]. In total, approximately 750,000 cases of sepsis and 198,000 cases of severe sepsis *per annum* were reported. The incidence of sepsis is steadily escalating due to a rise of major surgery, ageing population and the wide-spread use of antibiotics and immune modulators [2]. Although there is a great variability in mortality, the overall sepsis-related mortality is estimated to be 30% [3]. It has been shown that mortality rates correlate with disease severity, ranging from 15% in non-severe sepsis up to 80% in septic shock (Fig.1) [2].

Similar morbidity and mortality rates are observed in Germany [4]. Importantly, SIRS and sepsis do not affect human populations indiscriminately. Infants (aged < 1 year) and elderly people show the highest incidences for sepsis, with over 50% of all septic patients being older than 65 years. Although the overall incidence does not differ in female and male populations, it has been shown that from 30 years onwards, woman exhibits rates of that observed in men 5 years younger. The same gender/age correlation is reported for mortality rates [3].

1.1.3 Pathophysiology of sepsis

Sepsis-induced pathophysiology is a combination of a complex network of processes induced by both the excessive systemic host immune response as well as the invading infectious trigger. The acute inflammatory response (see 1.2) in systemic inflammation and sepsis is the trigger of a cascade of disease processes ultimately leading to tissue / organ damage and most fatally to death.

Early disease-induced alterations affect the coagulation and complement machinery, the endothelial-vascular system, metabolic regulation as well as the autonomic nervous system [5, 6]. Subsequently, these malevolent alterations induce damages in virtually all tissues and organs of the organism. In cases of severe sepsis organ failure occurs most frequently in respiratory (45.8%), cardiovascular (24.4%), renal (22%), haematological (20.6%) and central nervous (9.3%) systems [3].

Furthermore, recovery and quality of life of septic patients is negatively influenced by persistent disease-induced myopathies [7], chronic pain [8], encephalopathy [9], posttraumatic stress disorder [10] and immunological disorders [11]. As a result, patients who survived an acute episode of sepsis have a significantly lowered health-related quality of life [12] associated with increased rates of death years after the initial insult [13].

1.2 The inflammatory response in systemic inflammation and sepsis

1.2.1 The acute pro-inflammatory response

An acute infection with bacteria, viruses and other microorganisms induces a rapid innate immune host response to fight and eliminate the invading pathogens. The inflammatory innate response involves cells, mediators and processes that are tightly regulated to prevent

host damage. With the help of *pattern recognition receptors* (PRRs), innate immune cells, such as monocytes / macrophages, dendritic cells and granulocytes recognise pathogens by the presence of pathogen signature molecules in fluids or on the invader's surface. *Toll-like receptors* (TLRs) are the most prominent pattern recognition receptors for sensing these *pathogen-associated molecular patterns* (PAMPs) including nucleic acids, lipids, microbial proteins as well as cell wall components such as lipopolysaccharide (LPS) [14]. Additionally, necrotic death of cells induced by infection or the host response leads to the release of cellular components such as ATP, nucleic acids, heat shock proteins and others. These compounds are referred to as *alarmines* and further enhance the deregulated innate immune response in sepsis by additional triggering of PPRs. Endogenous alarmines and exogenous PAMPs are collectively defined as *damage-associated molecular patterns* (DAMPs) and are crucial triggers of the malevolent septic host response [15].

In acute episodes of systemic inflammation and sepsis very high levels of DAMPs, either from invading microorganisms or damaged host tissues promote an excessive inflammatory response characterised by the activation of the complement system and hyper-activation of cellular innate responses [6]. Moreover, vigorous activation of pattern recognition receptors in early sepsis leads to the up-regulation of an array of proteins that mediate the host response to the septic trigger. During the acute phase response high levels of pro-inflammatory *interleukin*-(IL)-*1β*, *tumor necrosis factor* α (TNFα) and IL-6 are released by innate immune cells inducing a damaging pro-inflammatory response [16]. These cytokines are crucial activators of the coagulation cascade, *inducible nitric oxide synthase* (iNOS), vascular endothelium, *C-reactive protein* and the complement system. The initial pro-inflammatory cytokines induce the production and secretion of additional inflammatory mediators, such as the pro-inflammatory cytokines *macrophage migration-inhibitory factor* (MIF), *high-mobility group protein B1* (HMGB1) and IL-17A as well as the highly inflammatory anaphylatoxins C5a [6]. Consequently, the imbalanced cytokine release, often referred to as cytokine storm, converts the otherwise beneficial local inflammation to a damaging systemic inflammation.

1.2.2 Immunosuppression in systemic inflammation and sepsis – the *Compensatory Anti-inflammatory Response Syndrome* (CARS)

In addition to the strong pro-inflammatory response in SIRS and sepsis, clinicians on ICUs had noted immunosuppressive states in patients suffering from systemic inflammation

caused by trauma or burns since the 1970s [17, 18]. The impaired immune response in this patient cohort has been early linked with hyporesponsive states of immune cells but detailed mechanisms and processes were unknown at that time. Moreover, many clinical trials of therapies designed to inhibit the pro-inflammatory cascade in SIRS and sepsis had no beneficial effects in human patients [19]. The tested anti-inflammatory approaches included glucocorticoids, agents active against endotoxins, anti-TNFα antibodies as well as inhibitors of nitric oxide synthase and prostaglandin synthesis. Taken together, these findings clearly indicated that hyper-inflammation is not the sole pathogenic mechanism in acute systemic inflammation and sepsis.

Research over the past decades revealed that many septic patients exhibit two, oftentimes concomitant, inflammatory states in acute stages of the disease: SIRS and a *Compensatory Anti-inflammatory Response Syndrome* (CARS) [20]. Importantly, CARS and SIRS represent to separate states involving distinct mediators, mechanisms and cellular components. It is important to note that CARS does not necessarily represent a regulated mechanism to compensate the excessive pro-inflammatory host response, although the term CARS suggests this interpretation. In fact, CARS, introduced in 1996 by R. Bone, includes all mechanisms and processes that result in impaired host immune responses, such as leukocyte apoptosis, release of anti-inflammatory cytokines, decreased monocytes / macrophage responses or impaired antigen presentation [21]. Along with hyper-inflammation, anti-inflammatory processes in sepsis contribute to sepsis-induced morbidity and mortality in acute stages of the disease. Furthermore, many patients who survived an acute episode of systemic inflammation or sepsis enter a protracted state of immune hyporesponsiveness characterised by impaired pathogen clearance, increased susceptibility to secondary (often noscomial) infections and viral reactivation [22-24]. The markedly increased long-term mortality after sepsis is noticeably associated with infection-caused deaths even years after the initial septic insult [13].

1.2.3 Mechanisms of SIRS- and sepsis-acquired immune deficiency

In recent years, cellular and molecular mechanisms of the anti-inflammatory response syndrome have been elucidated and it became clear that CARS involves an equally complex network of cells and processes as the systemic (pro-) inflammatory response. The early loss of immune cells via apoptosis is one of the key mechanisms of CARS and affects immunity in acute and post-acute stages of sepsis. In particular, the loss of lymphocytes

including T-cells, B-cells and natural killer cells (NK cells) in various organs contributes to immunosuppression in sepsis [25]. Altered apoptotic cell death was also reported in cells of the innate immune system such as neutrophils and monocytes / macrophages, although with contradictory data. Depending on patient cohorts or animal models employed, decreased or increased susceptibility to sepsis-induced apoptosis was described for phagocyte cell populations [26]. Importantly, dendritic cells, as professional antigen-presenting cells and linkers of innate with adaptive immune responses, are highly susceptible to apoptosis in sepsis [27].

The profound early apoptotic death of cells induces phagocytosis of the apoptotic particles by macrophages to prevent the uncontrolled release of alarmines. It has been shown that the ingestion of apoptotic particles leads to altered cytokine expression patterns in macrophages resulting in anti-inflammatory / immunosuppressive responses. Phagocytosis of a large number of apoptotic bodies in SIRS / sepsis leads to diminished production of *granulocyte macrophage colony-stimulating factor* (GM-CSF), IL-1, IL-8, IL-10 and TNFα, while enhancing the production of TGF-β (*transforming growth factor-β*), prostaglandin E2 and platelet activating factor [28].

The abundant systemic or focal release of cytokines associated with anti-inflammatory functions has been described in human patients as well as rodent animal models. Elevated serum levels of IL-10, IL-1 receptor antagonists and soluble TNFα receptor were detected in patients with severe sepsis [29]. Interestingly, the same patients also showed increased levels of TNFα, IL-1β and IL-6 which are linked to the pro-inflammatory state in systemic inflammation and sepsis. Importantly, elevated serum levels of TGF-β were also detected in human patients with sepsis contributing to CARS [30]. TGF-β is known to suppress antigen-dependent activation and effector function of lymphocytes and to drive generation of suppressive regulatory T-cells from activated T-cells.

Furthermore, impaired cellular functions of neutrophils and monocytes / macrophages have been described for SIRS / sepsis patients as well as in animal studies. Neutrophils, as major cellular components of the innate immune response, exhibit defects in bacteria clearance, *reactive-oxygen-species* (ROS) production and recruitment to inflammation sites associated with the profound loss of chemotactic activity. In line with these observations, macrophage responses to bacterial compounds and secretion of pro-inflammatory cytokines are compromised in sepsis [31]. Taken together, episodes of systemic inflammation and sepsis are characterised by the disturbance of immune homeostasis

triggering both pro- and anti-inflammatory processes early after onset of the diseases. The excessive and de-regulated inflammatory cascades are the root of disease-related acute and protracted immunosuppression (Fig. 2).

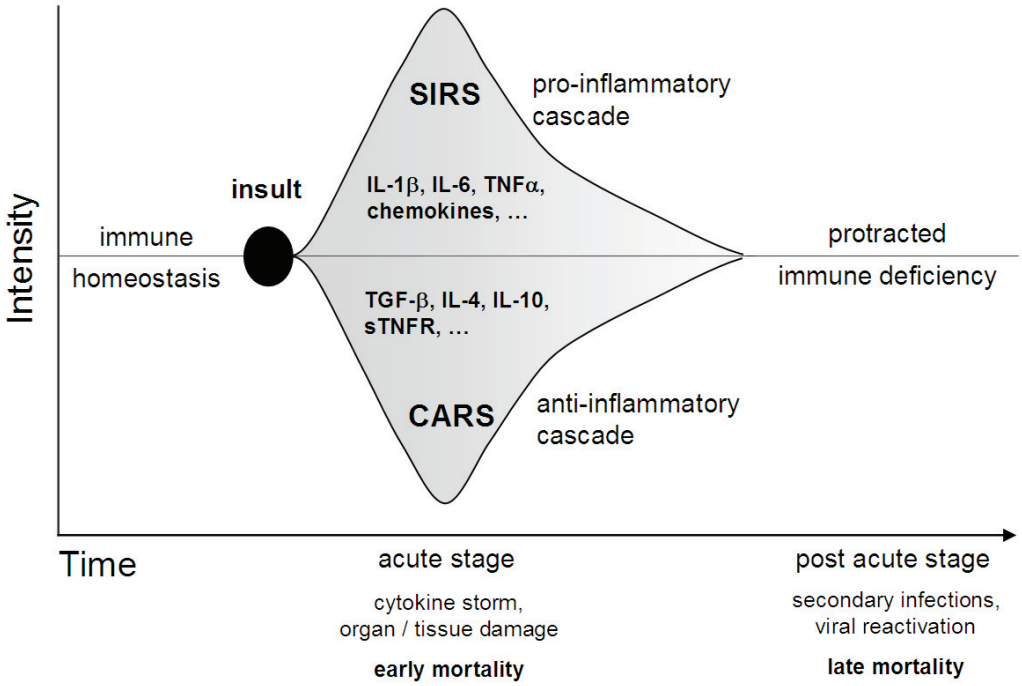


Figure 2. The cytokine storm in systemic inflammation and sepsis
Schematic overview of the inflammatory response in systemic inflammation and sepsis. Acute episodes of systemic inflammation and sepsis lead to the disturbance of immune homeostasis through the de-regulated excessive release of cytokines inducing pro- and anti-inflammatory cascades associated with early mortality. Systemic pro-inflammatory responses (SIRS) lead to the activation of immune cells enhancing the initial inflammatory response and ultimately resulting in organ / tissue damage. Concomitant anti-inflammatory immune responses (CARS) lead to acute immunosuppression affecting acute immune responses and thus clearance of the infectious sepsis trigger. Post-acute stages of systemic inflammation and sepsis are characterised by protracted immunosuppression leading to viral reactivation and increasing the risk for secondary (often noscomial) infections associated with post-acute late mortality and reduced health-related quality of life.

1.3 T-cells

1.3.1 T-cells - cellular components of the adaptive immune system

The present thesis focuses on the role and function of T-cells at post-acute stages of systemic inflammation and sepsis. T-cells (or T-lymphocytes) are crucial cellular components of the immune system involved in numerous immune responses against

virtually all pathogens, including viruses, bacteria, fungi and multi-cellular parasites. Together with B-cells, T-cells are the main cellular components of the lymphocyte compartment promoting antigen-dependent (adaptive) immune responses, though T-cells are additionally involved in early innate responses during infections. T-cells feature unique antigen-specific surface receptors that recognise foreign antigens presented in complexes with MHC molecules (*major histocompatibility complex*) on the surface of antigen-presenting cells (APCs), such as macrophages, granulocytes and dendritic cells.

Antigen-stimulated T-cells mediate both cellular and humoral effector responses. Cell-mediated immunity includes direct killing of pathogens or infected cells by cytotoxic T-cells and the activation of phagocytes by T-helper cells. As part of the humoral T-cell response, activated T-helper cells stimulate B-cells to produce antibodies against the invading pathogen. Malfunction of T-cells is associated with many disease like immune-deficiency, auto-immune diseases and cancer.

1.3.2 T-cell subsets

The T-cell compartment consists of many subtypes with distinct phenotypes and effector functions. According to the surface expression of the co-receptors CD4 and CD8 T-cells are divided in two major subtypes: $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells. Cytotoxic $CD8^+$ T-cells mediate cellular defence against intracellular pathogens, in particular viruses [32]. Infected cells present pathogen peptides on their surface in the context of MHC-I, thereby activating antigen-specific $CD8^+$ T-cells. Activated cytotoxic $CD8^+$ T-cells kill target cells by the release of cytotoxic agents such as perforin, granzymes, granulysin and others. Moreover, effector $CD8^+$ T-cells secrete cytokines including TNFα and IFNγ to activate phagocytes that further enhance the immune response against infected cells and to remove apoptotic particles.

$CD4^+$ T-helper cells control cellular and humoral immune responses against almost all pathogens. Based on their cytokine profile activated effector $CD4^+$ T-cells are divided into different subtypes [33]. Th1 (T-helper 1) cells enhance cell-mediated immune responses through activation of macrophages, neutrophils and dendritic cells by producing pro-inflammatory cytokines, such as TNFα or IFNγ. Conversely, Th2 (T-helper 2) cells suppress the inflammatory cell-mediated Th1 response by secretion of cytokines with anti-inflammatory properties including IL-4, IL-5 and IL-10. Th2 responses mediate humoral immunity by stimulating B-cells to produce antibodies. The differentiation into either cell

type during $CD4^+$ T-cell activation is orchestrated by specific cytokines released by immune cells in response to specific pathogens. The differentiation of naïve $CD4^+$ T-cells to either Th1 or Th2 effector cells is a key mechanism to regulate distinct $CD4^+$ T-cell responses to specific types of infections. Disproportionate Th1 and Th2 responses are observed in many chronic inflammatory diseases, such as autoimmunity or allergies and are also believed to play a role in sepsis-acquired immunosuppression (see 1.4.2).

In recent years further effector $CD4^+$ T-helper cells types and their distinct roles in immunity and disease have been described. According to their cytokine profile these subtypes are referred to as Th9, Th17, Th21 or Th22. Exact mechanisms of lineage development and functional patterns *in vitro* and *in vivo* are not fully understood yet and under investigation. Since only little data are available about their potential role in sepsis-acquired immunosuppression, they are not further discussed here.

Regulatory $CD4^+$ T-cells (Tregs) represent another very heterogeneous class of $CD4^+$ T-cells that are implied to play an ambivalent role in SIRS and sepsis (see 1.4.2). Since regulatory T-cells can modulate and suppress other immune cells, they play a crucial role in tolerance to self-antigens as well as balancing and terminating adaptive and innate immune responses [34]. The most prominent type represent $CD4^+$ regulatory T-cells co-expressing the IL-2 receptor α-chain (CD25) and the transcription factor FoxP3 (*forkhead box P3*). $CD4^+/CD25^+/FoxP3^+$ Tregs constitute about 5-10% of all peripheral T-cells. By secretion of TGF-β and IL-10 they can inhibit innate and adaptive immune responses. TGF-β can reduce phagocytotic activity of macrophages and the activation / proliferation of B- and T-cells. IL-10 has been shown to suppress phagocytotic activity and IL-12 production by dendritic cells thereby blocking Th1 T-cell differentiation.

However, the above described T-cell subsets represent a very basic classification. The T-cell compartment is highly diverse and continuously expands continuously by the characterisation of new T-cell subsets.

1.3.3 The T-cell receptor and the TCR/CD3/CD247 complex

The *T-cell receptor* (TCR) is the key feature of antigen-specific T-cell immunity. Expressed on the surface of all mature peripheral T-lymphocytes the TCR recognises foreign antigen peptides bound to MHC proteins on the surface of antigen-presenting cells, such as dendritic cells, macrophages or B-cells. Structurally, the T-cell receptor consists of two membrane-anchored α- and β-protein chains, forming a disulfide bond-linked

heterodimer (Fig. 3) [35]. The great majority of T-cells express the αβ-TCR variant; though, a small fraction of T-cells (~ 5%) possess an alternate receptor that consists of γ- and δ-chains. According to their TCR variant, both populations are referred to as αβ-T-cells or γδ-T-cells.

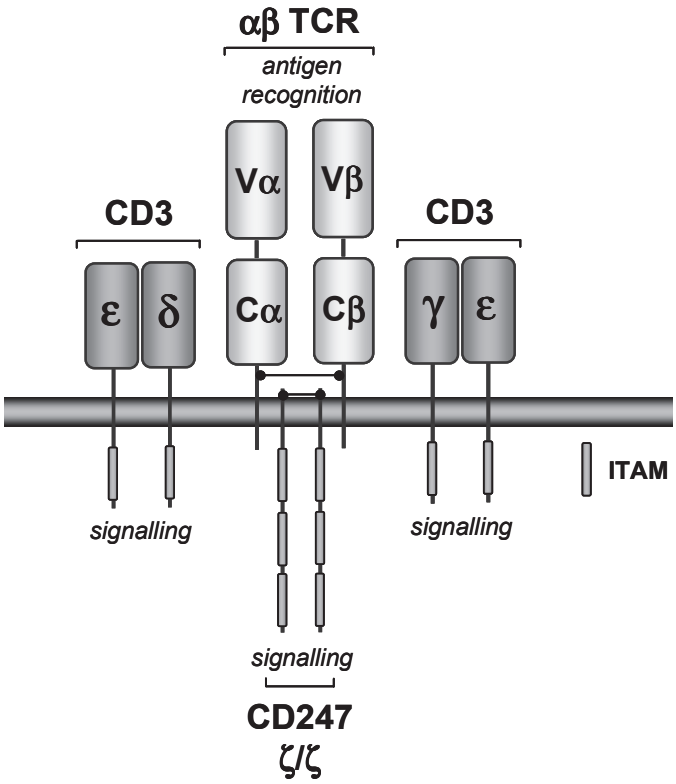


Figure 3. The T-cell receptor complex
The T-cell receptor (TCR) complex is expressed on all mature T-cells. 95% of all peripheral T-cells carry αβ-TCR variants depicted here consisting of extracellular *immunoglobulin superfamily* α- and β-chains with membrane-distal variable (V) and membrane–proximal constant (C) domains forming anti-parallel β-sheets. Antigen peptides bound to major histocompatibility complexes (MHC) are recognised through protein-protein interactions by hypervariable amino acids residues within the variable regions. Signal propagation is carried out by CD3 and CD247 signalling molecules that associate with the TCR heterodimer through electrostatic interactions of negatively and positively charged membrane-integral amino acids of both molecules. CD3 and CD247 dimers possess long intracellular domains carrying *immunoreceptor tyrosine-based activation motifs* (ITAMs) motifs with the conserved sequence YxxI/L$x_{6\text{-}12}$YxxI/L (Y = tyrosine, x any amino acid, I = isoleucine, L = leucine). Phosphorylation of tyrosine residues within the ITAMs by Src family tyrosine kinases, such as LCK (*lymphocyte-specific protein tyrosine kinase*), FYN or LYN represents the initial step in proximal TCR signalling.

Antigen-peptide/MHC recognition and binding is carried out by hypervariable amino acid residues within the distal extracellular variable (V) regions of both α- and β-chains [36]. Due to its very short cytoplasmatic tails, the αβ-TCR can not propagate extracellular signals into the cytoplasm. TCR signal transmission requires the association of the αβ-

TCR with the three dimeric signalling proteins CD3γ/ε, CD3δ/ε and CD247ζ/ζ constitutively expressed on the surface of T-cells and hereinafter collectively referred to as CD3 or CD247 molecules (Fig. 3) [37, 38].

Conserved sequence motifs, called *immunoreceptor tyrosine-based activation motifs* (ITAMs) in the intracellular portions of CD3 and CD247 molecules are crucial elements for signal propagation and serve as substrates for the tyrosine kinase LCK (*lymphocyte-specific protein tyrosine kinase*) and other SRC family kinases, such as FYN or LYN [39]. Phosphorylation of two tyrosines within the ITAM sequence is the earliest event in intracellular TCR signalling upon antigen/MHC-triggering of TCR complexes (see 1.3.6 and Fig. 4). Antigen-binding additionally involves the recognition of MHC complexes by CD4 or CD8 co-receptors on $CD4^+$ and $CD8^+$ T-cells (Fig. 4) [40]. While CD4 co-receptors interact with MHC class II molecules on the surface of APCs, MHC-I complexes found on virtually all nucleated cells are recognised by CD8 molecules. Intracellular domains of the CD4 and CD8 co-receptors are associated with LCK and mediate the initial LCK-dependent ITAM phosphorylation.

1.3.4 T-cell receptor diversity and T-cell development

To ensure specificity against a wide array of unknown pathogen-peptides, each individual mature T-lymphocyte possesses a unique TCR-variant with distinct antigen specificity determined by the sequence and structure of the variant αβ-TCR chains. TCR/antigen/MHC interaction is degenerated, that means that one given antigen is recognised by several TCR variants and one particular TCR variant recognises many potential antigens. Somatic recombination of gene segments encoding for structural elements of the α- and β-chains at early stages of T-cell development creates a unique TCR variant with distinct antigen specificity for each individual T-cell clone. Somatic TCR gene rearrangement is a stochastic process and creates countless TCR variants specific for virtually all possible antigens, including foreign pathogen-antigens [41].

As the first step of T-cell development *lymphoid progenitor cells*, originating from bone-marrow-resident pluripotent haematopoietic stem cells, migrate into the thymus where they differentiate into mature T-cells. In the thymus early $CD4^-/CD8^-$ thymocytes start to express pre-TCR α-chains. If T-cells are capable to express functional TCR β-chains that properly interact with the pre-TCR α–chain, they rapidly expand and begin to express both CD4 and CD8 co-receptors. In two additional selection rounds $CD4^+/CD8^+$ thymocytes

bearing TCR variants with inappropriate affinity to self MHC-I/II (too weak or too strong) or that are auto-reactive to self-antigens are eliminated through apoptosis. Furthermore, interaction of $CD4^+$/$CD8^+$ thymocytes with either MHC-II or MHC-I decides whether a T-cell clone develops into a $CD4^+$ helper T-cell or cytotoxic $CD8^+$ T-cell. Positive and negative selection is carried out by specialised thymic cells that are capable to express a wide range of self-antigens in context of MHC-I or II. T-cells with eligible self-tolerant TCR variants receive survival signals and differentiate into mature T-cells released into the periphery [41].

1.3.5 T-cell receptor antigen-recognition and initiation of TCR signalling

Although the TCR structure is known since many decades, the exact mechanism how antigen-binding by TCR complexes propagates signals across the cell membrane is not fully understood. Current models of antigen/TCR-triggering imply a step-wise mechanism leading to activation of intracellular TCR signalling molecules [42]. In the resting state TCR/CD3/CD247 complexes exist as monomers in close proximity of the TCR-inhibitory membrane-bound tyrosine phosphatases CD45 and CD148 which reverse ITAM phosphorylations by constitutive active LCK. TCR triggering by antigen/MHC complexes induces conformational changes increasing susceptibly of the ITAMs to LCK-catalysed phosphorylation. Furthermore, ligand binding stimulates segregation of the TCR/CD3/CD247 complexes from the inhibitory phosphatases SHP (*SH2-containing phosphatase*) and SHIP (*SH2-containing inositol phosphatase*). TCR-triggering also induces the spatial redistribution of TCR complexes in lipid domains leading to the aggregation of TCR/CD3/CD247 complexes facilitating LCK trans-autophosphorylation of the ITAMs. As a result of these events, microclusters of intracellular TCR signalling molecules are formed promoting ITAM phosphorylation and proximal TCR signalling.

1.3.6 Proximal T-cell receptor signalling

Proximal T-cell receptor signalling involves a complex cascade of multiple steps of activation / deactivation cycles of numerous signalling proteins summarised in Figure 4. Upon antigen/MHC-triggering, phosphorylated tyrosine residues within ITAM sequences serve as docking sites for *Src homology 2* (SH-2) domain containing proteins. Via its two SH2 domains, the ZAP-70 kinase (*ζ-chain associated protein 70*) is recruited to the

CD3/CD247 complexes where it gets phosphorylated and activated by LCK. Subsequently, activated ZAP-70 phosphorylates the adaptor proteins LAT (*linker of activated T-cells*) and SLP76 (*SH2 domain containing leukocyte protein of 76kDa* or LCP-2). Phosphorylated LAT and SLP76 form a membrane-associated adaptor complex recruiting different crucial downstream TCR signalling molecules such as *phospholipase C γ1* (PLCγ1) [43]. PLCγ1 catalyses the cleavage of the membrane lipid PIP_2 (*phosphatidylinositol 4,5-bisphosphate*), thereby promoting the formation of cytosolic IP_3 (*inositol 1,4,5-trisphosphate*) and the membrane lipid *diacylglycerol* (DAG). The binding of IP_3 to its respective receptors induces the release of Ca^{2+} ions from the endoplasmatic reticulum. Increasing cytosolic levels of Ca^{2+} activate and open *calcium release-activated calcium channels* (CRAC) at the plasma membrane leading to influx of extracellular calcium ions. Elevated cytosolic Ca^{2+} levels stimulate the protein phosphatase *calcineurin* that in turn removes inhibitory phosphate groups from the transcription factor NFAT (*nuclear factor of activated T-cells*). De-phosphorylation of NFAT leads to conformational changes and exposure of the *nuclear localisation sequences* (NLS) resulting in the nuclear import of NFAT where it cooperates with other nuclear transcription factors to regulate the expression of TCR target genes [43].

Similar to IP_3, diacylglycerol also act as an important signal transmitter. DAG can bind and stimulate RasGRP1 (*Ras guanyl-releasing protein 1*), a guanine nucleotide exchange factor for the small GTPase Ras. DAG-activated RasGRP1 converts inactive GDP-bound Ras into the active Ras-GTP form, by facilitating the release of GDP and replacement by GTP [44]. Ras is the starting point of the Ras/MAPK (*mitogen activated protein kinase*) signalling cascade involving a series of three downstream kinases ultimately leading to phosphorylation and activation of ERK (*extracellular signal-related kinase*). Activated ERK translocates into the nucleus where it activates transcription factors such as AP-1 (*activator protein 1*) that modulate expression of TCR target genes. DAG also stimulates *protein kinase Cθ* (PKCθ), a serine / threonine kinase that regulates the release of the transcription factor NfκB (*nuclear factor kappa-light-chain-enhancer of activated B cells*) from its inhibitor IκB (*inhibitor of NfκB*). NfκB migrates into the nucleus where it acts as a central transcriptional regulator for TCR effector genes [45]. The IL-2 gene is the prototype of TCR-regulated gene expression as its induction is greatly enhanced upon TCR complex stimulation. The promoter region of the IL-2 gene integrates all major TCR and co-receptor signalling pathways since it has binding sites for NFAT, AP-1 and NfκB [46].

IL-2 is secreted by TCR-activated T-cells and promotes T-cell proliferation and differentiation in an autocrine fashion.

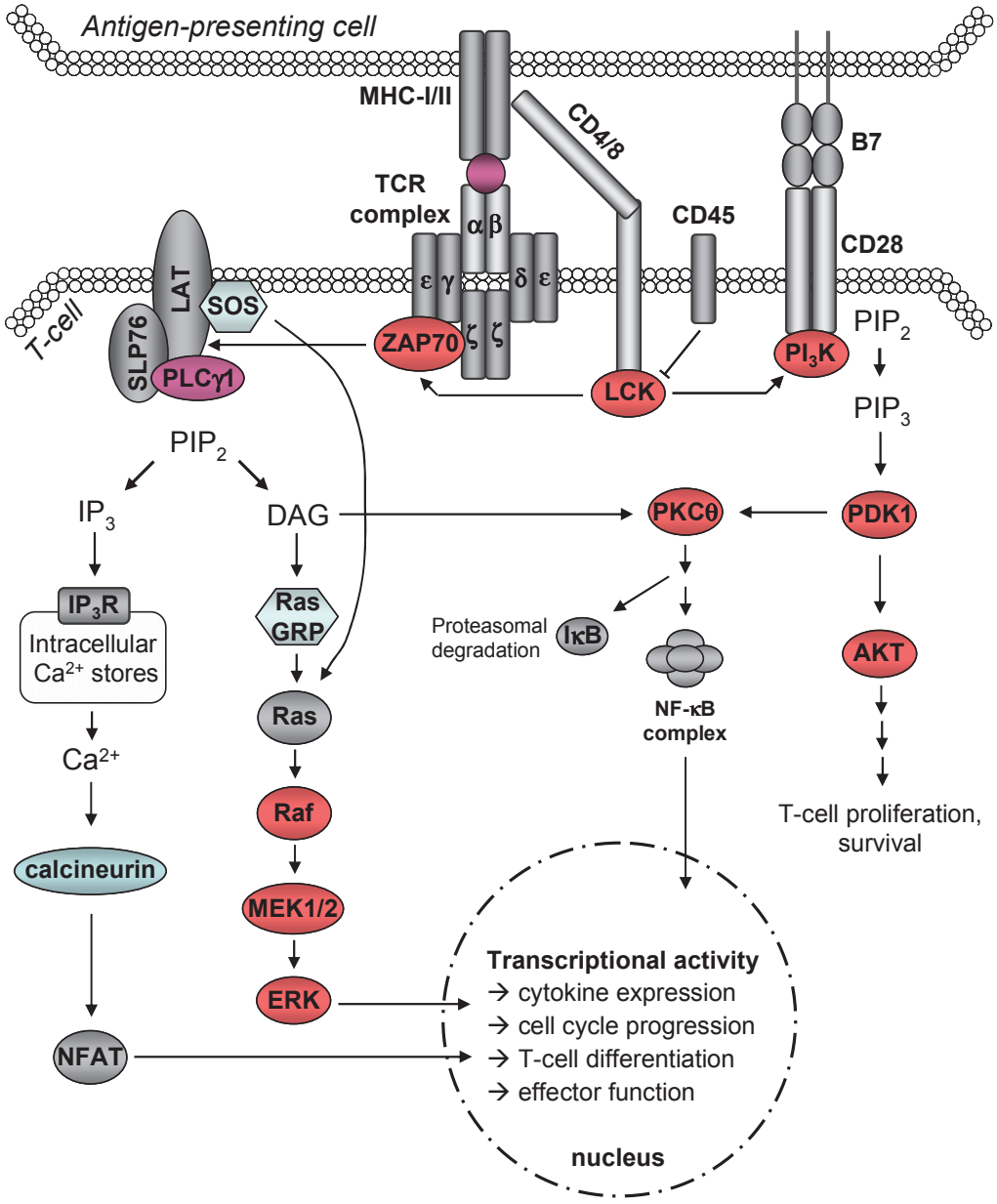


Figure 4. T-cell receptor signalling
Schematic overview of proximal TCR complex signal transduction. Processed pathogen-peptides are presented in the context of major histocompatibility complex class I or II (MHC I or II) by antigen-presenting cells (APCs). Activated APCs express co-stimulatory molecules such as B7 family ligands that co-stimulate CD28 on T-cells. MHC/antigen recognition induces phosphorylation of tyrosine residues within ITAM sequence motifs in the intracellular portions of CD3 and CD247 TCR accessory molecules. Phosphorylated ITAMs serve as docking site for ZAP-70 that is in turn activated / phosphorylated by SRC kinases such as LCK. ZAP-70 activation and interaction with CD3/247 orientates ZAP-70 to phosphorylate the membrane-associated adaptor protein LAT. Phosphorylation of LAT induces the formation of a large signalling complex and represents a branching point in TCR signalling. Together with co-receptor (e.g. CD28) stimulation and signalling, proximal TCR signalling induces transcriptional modulation of numerous TCR effector genes, mediating T-cell activation, proliferation, differentiation and survival. In addition, TCR signalling controls non-transcriptional effector responses, such as cytoskeleton and membrane structure rearrangement.

1.3.7 Co-stimulatory signals in T-cell activation

Complete antigen-dependent T-cell activation and differentiation into effector T-cells requires additional co-stimulatory signals provided by antigen-presenting cells [47]. Activated APCs express the co-stimulatory ligands CD80 (B7.1) and CD86 (B7.2) that stimulate the co-stimulatory receptor CD28 expressed on T-cells (Fig. 4). During antigen-mediated TCR stimulation the interaction of CD80/86 with CD28 provides co-stimulatory signals as it activates the PI_3-kinase/AKT-pathway promoting survival and proliferation of the stimulated T-cell clone (PI_3-kinase*: phosphatidylinositol-4,5-bisphosphate 3-kinase*). CD28 can also directly augment TCR signalling by stimulating Ras/MAPK signalling or enhancing the catalytic activity of LCK. TCR/CD3/CD247 engagement with concomitant co-stimulation of CD28 by the same APC is a mechanism to prevent unintended T-cell activation, for example by self-antigens in absence of an infection. Activated T-cells also express other co-stimulatory receptors that bind ligands of the B-7 protein family that further enhance co-stimulatory signals [48]. Prominent examples are ICOS (*inducible T-cell co-stimulator,* CD278) a member of the CD28-superfamily and CD27, a member of the TNFα receptor family [49]. In addition, secretion of cytokines, such as IL-4, IL-7 or IL-12 by APCs regulates the differentiation of activated T-cells into specific effector T-cells (see 1.3.2).

Antigen-specific TCR/CD3/CD247 activation in the absence of complete co-stimulation can induce a state of T-cell hyporesponsiveness, also referred to as T-cell anergy. T-cell anergy is a state in which T-cells are cell-autonomously impaired in their capacity to proliferate and elicit effector functions upon antigen (re)encounter but remain alive for an extended period of time [50]. Anergic T-cells can be characterised by impaired TCR signalling, increased T-cell inhibitory signalling, reduced IL-2 production, cell-cycle arrest or epigenetic alterations [51]. T-cell anergy is a pivotal mechanism to impose peripheral tolerance by blocking self-reactive T-cells that have escaped thymic selection. Cells presenting antigens in absence of infections do not provide co-stimulatory ligands, thus creating an environment where auto-reactive T-cells are converted into a state of unresponsiveness.

1.3.8 Inhibitory receptors regulate T-cell activation

In order to control T-cell responses, T-cells express inhibitory receptors that can terminate T-cell activation and help to establish immune homeostasis after the infection has been

eliminated [52]. Important inhibitory T-cell regulators are CTLA-4 *(cytotoxic T-lymphocyte-associated protein 4*, CD152), PD-1 (*programmed cell-death 1*) and BTLA (*B- and T-lymphocyte attenuator*). All three molecules are expressed on T-cells after TCR-mediated activation and bind to molecules of the B-7 family found on activated antigen-presenting cells [52].

CTLA-4 is related to the stimulatory CD28 co-receptor as it binds the same B-7 ligands, but with much higher affinity than CD28, thereby suppressing effective CD28 co-stimulation. Through ligand-induced phosphorylation of CTLA4 intracellular *immunoreceptor tyrosine-based inhibition motifs* (ITIMs) CTLA-4 also directly transmits inhibitory signals. Phosphorylated tyrosine residues within the ITIM sequences stimulate the inhibitory phosphatases SHP and SHIP. While SHP removes activating tyrosine phosphorylations of activated TCR signalling molecules (e.g. LCK), SHIP converts PIP_3 into PIP_2 blocking CD28 co-stimulatory signals. Similar to CTLA-4, PD-1 and BTLA negatively regulate T-cell activation by recruiting SHP and SHIP after binding to other ligands of the B-7 family expressed on APCs during infection.

1.3.9 The dynamics of T-cell responses

In acute local infections dendritic cells, macrophages and neutrophils, engulf pathogens and pathogen particles followed by intracellular processing and surface presentation of antigen peptides in the context of MHC molecules. Stimulated antigen-presenting cells migrate into lymph nodes where they activate antigen-specific T-cell clones. Antigen-activated T-cells undergo rapid clonal expansion generating a pool of pathogen-specific effector $CD4^+$ and/or $CD8^+$ T-cells that leave the lymph node and migrate to the site of infection where they encounter their cognate antigen inducing effector T-cell responses (Fig. 5) [53]. After the infection is cleared the majority of effector T-cells undergoes apoptosis due to the absence of TCR signals. A small fraction of T-cells survives and develops into persistent memory T-cells patrolling in tissues and lymph nodes. Memory T-cells increase the TCR-pool reactive to the respective antigen by 100 to 1000-fold thereby greatly accelerating the T-cell response upon antigen reencounter. Furthermore, they are more sensitive to the respective antigen resulting in enhanced effector responses [53].

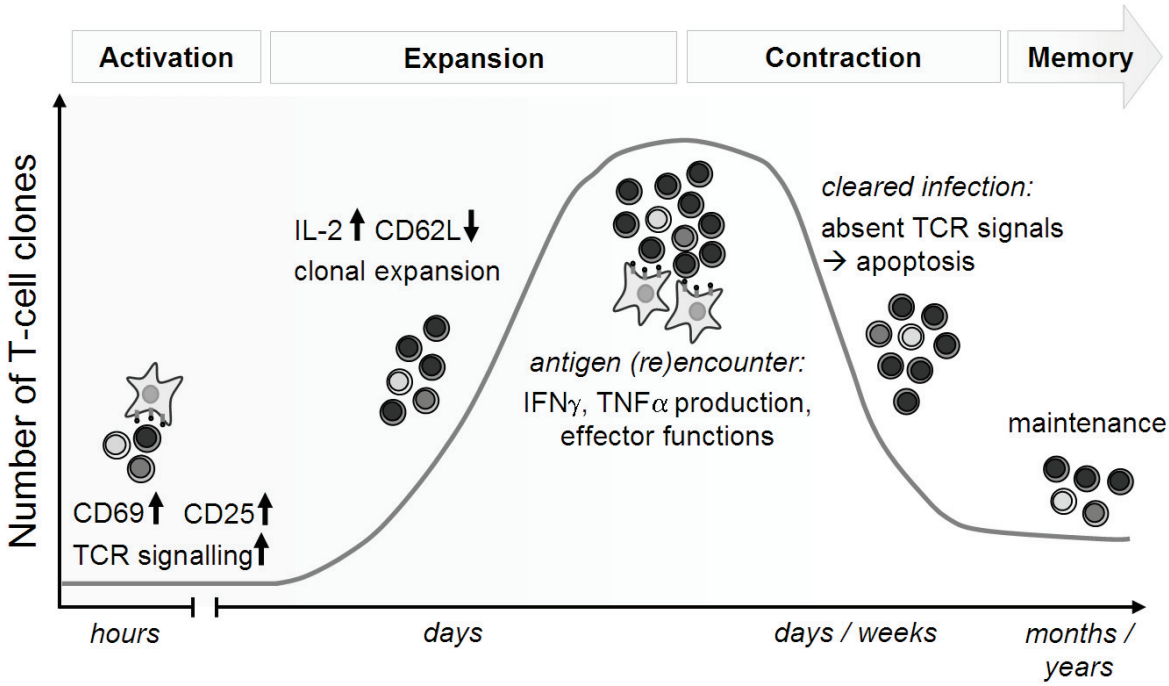


Figure 5. T-cell response dynamics in microbial infections
Schematic overview of T-cell dynamics in bacterial or viral infections. Activated antigen-presenting cells such as macrophages or dendritic cells present peptide fragments of engulfed pathogens to T-cells. Only $CD4^+$ and $CD8^+$ T-cell clones with appropriate T-cell receptor variants are selectively activated. During the activation phase MHC/antigen along with proper co-stimulation induces proximal TCR signalling that leads to T-cell activation that can be tracked by the up-regulation of T-cell activation marker including CD69 and CD25 (IL-2 receptor α-chain). In the expansion phase antigen-stimulated T-cells secrete IL-2 that mediates clonal T-cell proliferation in an autocrine fashion. In addition, naïve T-cells differentiate into effector T-cells with distinct surface marker expression, such as down-regulation of CD62L (L-selectine) or up-regulation of CD44. If effector $CD4^+$ and $CD8^+$ T-cells (re)encounter pathogen antigens they secrete effector cytokines, including IFNγ or TNFα to orchestrate immune responses. Furthermore, effector T-cells perform effector functions, such as killing of infected cells (cytotoxic $CD8^+$ T-cells) or phagocyte activation as well as the activation of B-cells to produce antibodies ($CD4^+$ T-helper cells). After the infectious trigger has been cleared, the absence of TCR signals leads to the apoptosis of the majority of the effector T-cells. A small fraction differentiates into persistent memory $CD4^+$ or $CD8^+$ T-cells mediating fast and effective T-cell immunity upon pathogen reencounter.

1.4 Impaired T-cell immunity in SIRS and sepsis

1.4.1 The role of the T-cells in acute systemic inflammation and sepsis

T-cell subsets have been implicated in the early host response during SIRS and sepsis and play a role in morbidity and mortality, although with conflicting data from different studies [54]. In one study using CD4- or CD8-deficient mouse strains, early survival after polymicrobial sepsis was decreased in mice lacking $CD4^+$ T-cells but not in mice deficient

for $CD8^+$ lymphocytes [55]. Worse outcome of $CD4^+$ T-cell-depleted mice was associated with impaired activation of neutrophils, increased bacterial burden and elevated levels of IL-6. The authors conclude that $CD4^+$ T-cells control the innate septic host response, in particular neutrophil effector functions. On the other hand, another study found that the lack of $CD4^+$ lymphocytes in the same rodent model of polymicrobial sepsis did not affect mortality, bacterial clearance and inflammatory responses [56]. Intriguingly, the same laboratory showed that $CD8^+$-deficient mice have a better early survival and decreased signs of morbidity after subjected to polymicrobial sepsis [57]. Despite the contradictory conclusions, these data underline the importance of T-cell immunity in systemic inflammation and sepsis.

1.4.2 Systemic inflammation and sepsis lead to alterations in T-cell immunity

The nature of immunosuppression in SIRS and sepsis is not fully understood but there is a large body of evidence that compromised adaptive $CD4^+$ and $CD8^+$ T-cell immune responses have a decisive role in the disease-related immune deficiency (Fig. 6). Sepsis-triggered apoptosis of both $CD4^+$ and $CD8^+$ T-cell populations is unarguably one of the key mechanism of T-cell malfunction in systemic inflammation and sepsis [25, 58]. The significance of T-lymphopenia to outcome in sepsis is underlined by the finding that prevention of T-lymphocyte apoptosis improves T-cell immunity and overall survival in experimental models of sepsis [59]. Importantly, the early apoptotic loss of T-cells does not require the presence of actual pathogens, since administration of bacterial compounds such as lipopolysaccharide is sufficient to induce early $CD4^+$ and $CD8^+$ T-cell apoptosis in spleen and other lymph organs [60]. This finding strongly suggests that the initial inflammatory cascade, rather than prolonged bacteraemia, induces the profound T-cell loss.

In addition to the early loss of T-cells, research data also suggest functional disturbances of T-cells as contributing factors for sepsis-related immune-deficiency. A much-noticed study from J.S. Boomer and colleagues could show that $CD4^+$ and $CD8^+$ T-cell responses to *in vitro* stimulation of splenocytes preparations from deceased sepsis patients are impaired on multiple levels [61]. T-cells exhibited a decreased cytokine response, increased expression of the inhibitory receptors PD-1 and CTLA-4 as well as lowered CD28 co-receptor expression. Furthermore, T-cells from the septic group showed a decreased expression of

the IL-7 receptor α-chain, a cell surface receptor that is crucial for T-cell homeostasis and development. The authors concluded that T-cells enter a suppressive state in severe sepsis contributing to pathogenesis of the disease. In line with these findings, similar anergic states of T-cells were described in sterile SIRS after trauma or burns (Fig. 6) [22].

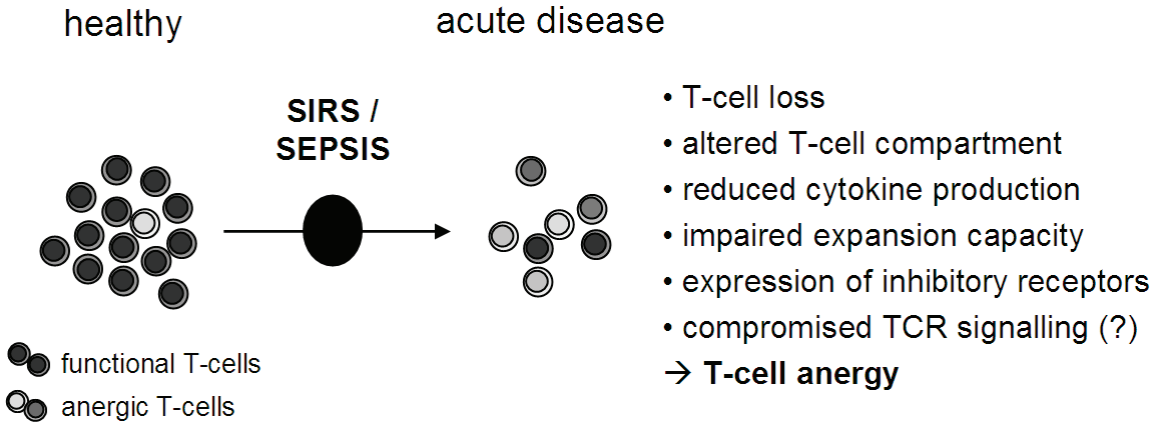


Figure 6. Overview of T-cell dysfunction at acute stages of systemic inflammation and sepsis
Acute episodes of systemic inflammation and sepsis induce a profound systemic loss of naïve $CD4^+$ and $CD8^+$ T-cells. Differential susceptibility of different T-cell subsets to disease-induced apoptosis further leads to an altered composition of the T-cell compartment. In addition to cell loss, T-cells feature an array of functional disorders that further compromise T-cell immunity at acute stages of SIRS and sepsis. However, detailed mechanisms of T-cell dysfunction are not fully understood yet. For example, only a few studies are available that studied proximal T-cell receptor signalling to identify potential mediators of T-cell hyporesponsiveness.

Moreover, impaired effector and memory $CD8^+$ T-cell responses after polymicrobial sepsis lead to compromised anti-microbial responses to secondary infections associated with decreased survival [62-64]. Impaired $CD8^+$ T-cell immunity in these animal studies was linked with decreased T-cell counts, impaired cytokine production and alterations in the T-cell phenotype.

Much attention was paid to study the impact of SIRS and sepsis on the function of distinct $CD4^+$ T-cell subtypes. Since regulatory $CD4^+$ T-cells are known to control and suppress immune responses of T-cells and other immune cells under physiological and pathophysiological conditions, they have been proposed as mediators of immune and T-cell suppression in SIRS and sepsis. Investigations in patients showed that the frequency of regulatory T-cells is increased at early stages of SIRS or sepsis. Importantly, the elevated

frequency results from the more robust resistance to apoptosis rather from the actual increase in total numbers [65]. Furthermore, it has been shown that both SIRS and sepsis can increase the immunomodulatory capacity of regulatory T-cells leading to decreased T-cell (and other immune cells) effector responses [65]. However, adoptive transfer experiments revealed that regulatory T-cells also possess a beneficial role and can improve outcome by dampening and controlling innate immune responses in SIRS and sepsis [66, 67]. In sum, frequencies and function of regulatory T-cells are affected by SIRS and sepsis but their contribution to morbidity and mortality is bivalent and dependent on multiple factors such as type, severity and progression of the disease.

The differentiation of naïve $CD4^+$ T-cells to either Th1 or Th2 (among others) effector T-cells is a key mechanism to orchestrate $CD4^+$ T-cell responses during infections (see 1.3.2). In sepsis the unbalanced shift from Th1 cell-mediated pro-inflammatory responses towards anti-inflammatory Th2 responses is believed to disturb the initial pro-inflammatory cellular response to pathogens [20]. However, this model of T-cell mediated immunosuppression in sepsis is highly controversial. Although altered type 1 and 2 responses were detected in $CD4^+$ T-cells from SIRS and sepsis patients, other studies found a general down-regulation of $CD4^+$ effector cytokines and transcription factors driving Th1 and Th2 differentiation [68, 69]. Other studies found decreased Th1-responses without activation of Th2 immunity [22]. Different results from various studies once more underline the heterogeneous nature of systemic inflammation and sepsis and the difficulties to find universally valid concepts of T-cell functionality in background of these syndromes.

Other less prominent subsets of T-cells including γδ-T-cells or mucosal associated invariant T-cells, have been shown to be affected by SIRS and sepsis. But since the present thesis focuses on 'canonical' naïve and effector αβ-TCR $CD4^+$ and $CD8^+$ T-cells, they are not discussed here.

1.4.3 T-cell suppression in sepsis: molecular mechanisms

Animal and human studies provided detailed insights in mechanisms and triggers of sepsis-induced T-cell apoptosis. Early T-cell loss in sepsis is mediated by both the FAS receptor and mitochondrial pathway [25]. By using transgenic mouse models, FADD (*Fas-associated protein with death domain*) and BID-1 (*BH3 interacting-domain death agonist*) were identified as mediators of sepsis-induced T-cell apoptosis via the FAS receptor

pathway. Expressing a dominant negative mutant of FADD in T-cells resulted in decreased activation of caspase 8 and thus to less T-cell death [70]. The same study found that BCL-2 protein family members BIM and PUMA (p53 *up-regulated modulator of apoptosis*) are critical activators of the mitochondrial (intrinsic) apoptosis pathway in T-cells during sepsis. Furthermore, sepsis-induced cell death by both pathways is executed by the activation of various caspases bringing them into focus of potential therapeutic targets [71]. As a crucial mediator of T-cell apoptosis during sepsis the cytokine TRAIL (*TNF-related apoptosis-inducing ligand*) was described in several animal studies [72, 73] and is reviewed in [74]. TRAIL is up-regulated during inflammatory processes and through binding to its respective receptors it induces apoptosis via activation of caspases-3, -6, and -7. Importantly, early T-cell apoptosis during sepsis is not caused by *activation-induced cell death* (AICD) since T-cell death is not dependent on T-cell activation, TCR engagement and proliferation [75].

While principle mechanisms of sepsis-induced T-cell apoptosis have been elucidated, molecular mechanisms of how sepsis alters the functionality of T-cells are much less understood. Some researchers hypothesise that the same mechanisms observed in T-cell anergy also play important roles in sepsis-induced T-cell suppression. Anergic T-cells can be characterised by increased expression of T-cell inhibitory receptors blocking T-cell activation and TCR signalling. In line with that, several studies with human septic patients found increased expression of PD-1 [61, 76, 77] CTLA-4 [61] and BTLA [78] on T-cells. The concept of SIRS- and sepsis-induced T-cell anergy is supported by the finding that T-cells from patients exhibit impaired IL-2 production and thereby exhibit defects in proliferation, a hallmark of T-cell anergy [79].

Antigen-specific T-cell receptor triggering is the key mechanism of T-cell activation. However, since most T-cell studies focus on immunological approaches, astonishingly little is known about potential sepsis-triggered alterations of TCR activation and signalling. To my knowledge, no detailed analysis of how SIRS and sepsis affect TCR-dependent T-cell activation and TCR signalling has been carried out yet. Only a few available studies provide very limited insights into disease-induced alterations of TCR signal transduction. A small study from Lopez-Collazo and colleagues found decreased mRNA expression levels of αβ-TCR, CD3 and LAT in lymphocytes derived from 17 human sepsis patients suggesting impaired TCR-dependent signalling and T-cell activation [80]. However, the investigators did not perform functional TCR analyses nor did they analyse expression profiles on protein levels. A more functional orientated TCR signalling study using a

rodent model of gram(-) bacterial sepsis found defective Ca^{2+} signalling linked with decreased proliferation upon TCR activation early after sepsis onset [81]. Another study using a mouse model of thermal injury-induced SIRS, linked defective Ca^{2+} signalling with decreased activation of the TCR-downstream signalling molecules ERK and p38 after TCR stimulation [82]. A remarkable animal study from Carson and colleagues found decreased phosphorylation of ERK and JNK *(c-Jun N-terminal kinase*) in naïve $CD4^+$ T-cells from septic mice after *ex vivo* TCR simulation [83]. Additionally, the same study showed altered mRNA expression profiles of cytokines, co-stimulatory and inhibitory receptors as well as T-cell differentiation factors in resting and TCR-stimulated T-cells from the septic group. Most interestingly, the investigators linked disturbed T-cell effector function and differentiation with histone methylations in promoter regions of cytokines and differentiation regulators.

1.4.4 Protracted T-cell suppression in systemic inflammation and sepsis

In addition to alterations at early stages of systemic inflammation and sepsis, persistent malfunction of T-cell responses is also believed to contribute to protracted immunosuppression after SIRS and sepsis, affecting the long-time survival of patients.

Early apoptosis of $CD4^+$ and $CD8^+$ T-lymphocytes is a hallmark of acute SIRS and sepsis and affects early host responses and outcome. But T-lymphopenia is transient as total splenic $CD4^+$ and $CD8^+$ T-cell numbers recovered to normal levels within several weeks post septic insult as shown in an animal study using a rodent model for polymicrobial sepsis [84]. However, even though T-cells numbers have recovered, T-cell responses to infections are impaired months after the initial septic insult [62, 63] possibly explained by the loss of T-cell precursors accompanied by narrowed T-cell receptor diversity after homeostatic proliferation. In line with these findings polymicrobial sepsis increases the susceptibility to chronic infections and exacerbates $CD8^+$ T-cell exhaustion at post-acute stages associated with increased inhibitory receptor expression and disturbed antigen-induced cytokine production. One study linked higher susceptibility to secondary bacterial infections and impaired T-cell effector responses observed weeks after sepsis onset with an increase of $CD4^+/CD25^+$ regulatory T-cells [85]. Controversially, there is some evidence that acute impaired T-cell responses to secondary infections have the ability to recover at post-acute stages of sepsis [86].

Despite the few available studies mentioned here, very little is known about persistent alterations in T-cell immunity at post-acute stages of SIRS and sepsis. There is a lack of studies that provide detailed immunological and biochemical insights into cellular T-cell function at post-acute stages of SIRS and sepsis. A fact that results from focusing on acute disease stages in the majority of the available research studies. The characterisation of T-cell function at post-acute stages of SIRS and sepsis would improve the understanding of protracted disease-related immunosuppression and could provide novel therapeutic approaches to treat the increasing number of immune-compromised post-septic patients.

2 Objectives

Protracted immunosuppression after Systemic Inflammatory Response Syndromes (SIRS), predominantly sepsis, is a major health concern as patients who survived an acute episode of the disease exhibit increased risks for infections months and years after the initial insult. The immune suppressive state after SIRS and sepsis contributes to the reduced health-related quality of life and is associated with late morbidity and mortality.

T-cells represent a central cellular component of the immune system and are involved in immune reactions against virtually all types of pathogens. Research over the past decade revealed that impaired T-cell immunity contributes to early morbidity and mortality in acute episodes of SIRS and sepsis. However, very little is known about T-cell function at post-acute and late stages of the disease and its potential contribution to protracted immunosuppression after SIRS or sepsis.

The aim of the present thesis was to perform an in-depth functional analysis of T-cell function at post-acute stages of systemic inflammation and sepsis using clinical relevant murine models. To achieve this aim following main objectives were addressed in the present thesis.

- To meet the heterogeneity of the disease, four murine models of systemic inflammation and sepsis are employed and characterised. The use of murine models of sterile SIRS and polymicrobial sepsis facilitates to understand whether the initial excessive inflammatory host response alone or the actual presence of an infection leads to persistent alterations in T-cell immunity.

- By the means of various functional assays the capacity of T-cells to respond to T-cell receptor stimulation is investigated at post-acute stages of the disease. T-cell responses are examined on multiple levels including activation marker up-regulation, proliferation capacity and multiple intracellular TCR signalling cascades.

- By using secondary infections models in background of SIRS and sepsis, functional T-cell analyses are extended to the level of antigen-specific effector T-cells. Furthermore, these models facilitate studying whether potential impaired T-cell responses are mediated by inherent T-cell (cell-autonomous) alterations or T-cell extrinsic (environmental) factors.

3 Material and Methods

3.1 Material

Material, Reagents and peptides

LPS L2880 /Lot #102M4017V)	(*Sigma-Aldrich*)
CPG-ODN 1826 (TCCATGACGTTCCTGACGTT)	(*Sigma-Aldrich*)
Meropenem Hospira 500mg	(*Hospira Inc.*)
Lithium-heparin blood tubes	(*Sarstedt*)
FUJI DRI-CHEM SLIDE LDH-P III	(*FujiFilm*)
FUJI DRI-CHEM SLIDE GPT/ALT-P III	(*FujiFilm*)
FUJI DRI-CHEM SLIDE GOT/AST-P III	(*FujiFilm*)
CD8a (Ly-2) MicroBeads, mouse	(*Miltenyi Biotec*)
CD4 (L3T4) MicroBeads, mouse	(*Miltenyi Biotec*)
CD4/CD8 T-cell activation/Expansion Kit, mouse	(Miltenyi Biotec)
Streptavidin	(*JacksonImmunoResearch Laboratories Inc.*)
FURA 2 / AM	(*Enzo Life Science*)
Pluronic F-127	(*Invitrogen, Molecular Probes, Inc.*)
Ionomycin	(*Enzo Life Science*)
2-mercapto-ethanol	(Carl *Roth GmbH*)
PBS	(*Biochrome AG*)
CFSE Amine dye	(*Enzo Life Science*)
Oxidizing Reagent Plus	(*PerkinElmer Inc.*)
Enhanced Luminol Reagent Plus	(*PerkinElmer Inc.*)
Cytofix/Cytoperm Solution	(*BD Pharmingen™*)
10x PermWash buffer	(*BD Pharmingen™*)
VitaLyse™ Lysing Buffer	(*BioE*)
Golgi Plug™ Protein Transport Inhibitor	(*BD Pharmingen™*)
LCMV peptide GP33-41 (GP33) sequence: KAVYNFATM	(*Bio-Synthesis, Inc.*)
LCMV peptide GP61-80 (GP61) sequence: GLKGPDIYKGVYQFKSVEFD	(*Bio-Synthesis, Inc.*)

Antibodies

Table 1: List of antibodies for western blot analyses

Antibodies western blot	clone	species	dilution	diluted in	company
P44/42 MAPK (Erk1/2)	137F5	rabbit mAb	1:1000	TBS-T + 1% BSA	Cell Signaling Technology
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	D13.14.4E	rabbit mAb	1:2000	TBS-T + 1% BSA	Cell Signaling Technology
ZAP-70	1E7.2	mouse mAb	1:500	TBS-T + 1% BSA	Santa Cruz Biotechnology Inc
Phospho-ZAP-70 (Tyr319)/Syk (Tyr352)	polyclonal	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling Technology
PLCy1	1249	rabbit mAb	1:500	TBS-T + 1% BSA	Santa Cruz Biotechnology Inc
Phospho-PLCy1 (Tyr783)	polyclonal	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling Technology
PanAkt	11E7	rabbit mAb	1:2000	TBS-T + 1% BSA	Cell Signaling Technology

Phospho-Akt (Ser473)	D9E	rabbit mAb	1:2000	TBS-T + 1% BSA	Cell Signaling Technology
LAT	polyclonal	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling Technology
Phospho-LAT (Tyr195)	polyclonal	rabbit	1:1000	TBS-T + 1% BSA	Cell Signaling Technology
Anti-Rabbit IgG (H+L), Peroxidase labelled	polyclonal	goat	1:20000 (stock 1mg/mL)	TBS-T + 1% BSA	KPL, Kirkegaard & Perry Laboratories, Inc.
Anti-Mouse IgG (H+L), Peroxidase labelled	polyclonal	goat	1:10000 (stock 0.5mg/mL)	TBS-T + 1% BSA	KPL, Kirkegaard & Perry Laboratories, Inc.

Table 2: List of antibodies for flow cytometry

Antibodies flow cytometry	clone	fluorophore	dilution	diluted in	company
anti-mouse CD3e	145-2C11	FITC	1:10	FACS buffer I	ImmunoTools
anti-mouse CD4	YTS 191.1.2	PE	1:10	FACS buffer I	ImmunoTools
anti-mouse CD4	GK1.5	PerCP-eFluor710	1:800	FACS buffer II	eBioscience Inc.
anti-mouse CD8a	YTS169.4	APC	1:25	FACS buffer I	ImmunoTools
anti-mouse CD8a	53-6.7	APC-eFluor780	1:200	FACS buffer II	eBioscience Inc.
anti-mouse CD25	7D4	APC	1:10	FACS buffer I	Miltenyi Biotec GmbH
anti-mouse CD25	PC61.5	APC	1:150	FACS buffer II	eBioscience Inc.
anti-mouse CD44	KM81	PE	1:25	FACS buffer I	ImmunoTools
anti-mouse CD62L	MEL-14	FITC	1:25	FACS buffer I	ImmunoTools
anti-mouse CD69	H1.2F3	FITC	1:50	FACS buffer I	eBioscience Inc.
anti-mouse CD69	H1.2F3	PE	1:200	FACS buffer II	eBioscience Inc.
anti-mouse CD154	MR1	PE	1:50	FACS buffer I	eBioscience Inc.
anti-mouse Thy1.1	HIS51	PerCP-Cy5.5	1:1000	FACS buffer II	eBioscience Inc.
anti-mouse Thy1.2	53-2.1	FITC	1:1000	FACS buffer II	eBioscience Inc.
anti-mouse IFNg	XMG1.2	APC	1:200	1x PermWash	eBioscience Inc.
anti-mouse TNFa	MP6-XT22	PE	1:200	1x PermWash	eBioscience Inc.

Table 3: List of antibodies and reagents for T-cell stimulation

Antibodies/reagents T-cell stimulation	clone	species	company
Biotin Hamster Anti-Mouse CD3ε	145-2C11	hamster	BD Pharmingen™
Purified Hamster Anti-Mouse CD3ε	145-2C11	hamster	BD Pharmingen™
Biotin Hamster Anti-Mouse CD28	37.51	hamster	BD Pharmingen™
Goat Anti-Armenian Hamster IgG (H+L)	polyclonal	goat	Jackson ImmunoResearch Laboratories Inc.
CD4/CD8 T-cell activation/Expansion Kit, mouse			Miltenyi Biotec GmbH

Buffers

RP0: RPMI 1640 (*Biochrome AG*)
25mM Hepes
penicillin / streptomycin

RP3: RPMI 1640 (*Biochrome AG*)
3% FCS (*Biowest LLC*)
penicillin / streptomycin

RP3 w/o phenol red: RPMI 1640 without phenol red (*Biochrome AG*)
3% FCS (*Biowest LLC*)

RP10:	RPMI 1640 (*Biochrome AG*) 10% FCS (*Biowest LLC*) penicillin / streptomycin
wash buffer:	PBS pH 7.4 (*Biochrome AG*) 0.5% Bovine Serum Albumine (BSA) (*PAA Laboratories*) 2mM EDTA (*AppliChem GmbH*)
FACS buffer I:	PBS pH 7.4 (*Biochrome AG*) 0.5 - 1% Bovine Serum Albumine (BSA) (*PAA Laboratories*) 2mM EDTA (*AppliChem GmbH*)
FACS buffer II:	PBS pH 7.4 (*Biochrome AG*) 1% FCS (*Biowest LLC*) 15mM sodium azide
ACK buffer	10mM $KHCO_3$ 150mM NH_4Cl 0.1mM EDTA
lysis buffer:	50mM Hepes pH 7.5 140mM NaCl 5mM $MgCl_2$ 1mM EGTA pH 7.5 1% NP-40 0.1% lauryl maltoside Add prior use: protease inhibitor mix at appropriate concentration 3.4nM microcystin, 100µM sodium vanadate, 50mM 2-gylcerol phosphate
2x sample buffer:	124mM Tris pH 6.8 20% glycerol 1.2% SDS 10% 2-mercapto-ethanol 0.02% bromophenol blue
5% stacking gel:	for 10mL 6.3mL dist. water 2.6mL 0.5M Tris pH 6.8 1mL acrylamide (Rotiphorese® 37.5%) (Carl *Roth GmbH*) 50µL 20% SDS 50µL 20% ammonium persulfate 10µL TEMED
10% separation gel:	for 30mL 18.1mL dist. water 5.6mL 2M Tris pH 8.6 6mL ProSieve™ 50 Gel Solution (*Lonza Group Ltd*) 150µL 20% SDS 150µL ammonium persulphate 12µL TEMED

10x PAGE buffer:	250mM Tris 2M glycine 35mM SDS Adjust to pH 8.3; dilute 1:10 with dist. water prior use
10x transfer buffer:	250M Tris 2M glycine 10% methanol, add prior use Adjust to pH 10; dilute 1:10 with dist. water prior use
10x TBS-Tween:	100mM Tris 1M NaCl 1% Tween 20 Adjust to pH 7.6; dilute 1:10 with dist. water prior use
Stripping buffer:	100mM 2-mercapto-ethanol 62.5mM Tris 2% SDS Adjust to pH 6.7

3.2 Methods

3.2.1 Laboratory mice

All described experiments were performed with 8 – 16 weeks old wild-type C57BL/6 laboratory mice. For some experiments performed at the *University of Iowa* TCR-transgenic $CD8^+$ T-cells (P14 T-cells) were obtained from C57BL/6 Thy1.1/1.1 or Thy1.1/1.2 P14 TCR-transgenic mice. Every single experiment was performed with sex-matched mice. Mice were kept in specialized animal facilities with educated stuff in legal compliance of the respective countries at the *Jena University Hospital*, Germany or at the *University of Iowa, Carver College of Medicine*, USA. Animals were maintained under artificial day-night cycles, constant 23°C room temperature, 30 – 60% humidity and received a standard mouse diet and water *ad libitum*. All experiments done at the *Friedrich-Schiller University Jena*, Germany were performed in accordance with the German legislation on protection of animals and with permission of the regional animal welfare committee of Thuringia. Experiments done at the *University of Iowa*, USA were performed in accordance with the University of Iowa Institutional Animal Care and Use Committee protocols.

3.2.2 Induction of Systemic Inflammatory Response Syndrome and sepsis

Four murine experimental models of infection-free SIRS and polymicrobial peritoneal sepsis were employed. SIRS: (a) lipopolysaccharide (LPS) / endotoxemia model and (b) CpG oligonucleotide (CpG-DNA) model. Sepsis: (c) *Peritoneal Contamination and Infection* (PCI) *model* and (d) C*ecal Ligation and Puncture* (CLP) *model*.

In order to trigger LPS-induced SIRS, 9 – 11 mg/kg body weight (b.w.) LPS (in 500µL 0.9% NaCl solution) were injected intraperitoneally (i.p.). To induce CpG-DNA-triggered SIRS, 4.5mg/kg b.w. CpG-DNA (in 500µL 0.9% NaCl solution) were administered i.p.. CpG-DNA treatment was repeated three times at day 2, 4 and 6 after initial treatment. PCI-sepsis was induced by i.p. injection of 3µL/g b.w. of a processed human stool suspension. Human faeces was donated by healthy male volunteers, processed and microbiologically characterised at the *Jena University Hospital*, Germany. For a detailed description of sampling and processing of the human stool see reference [87]. Faeces aliquots were kindly provided by PD Dr. R. Claus. Six hours after the insult, the broad-spectrum antibiotic *Meropenem* (25mg/kg b.w.) was injected subcutaneously. Antibiotic treatment was repeated once daily for two days. For inducing a polymicrobial sepsis with the CLP model the abdomen of anesthetised mice was shaved and disinfected. The cecum was exposed and identified via an abdominal incision. The distal one-third of the cecum was ligated with silk sutures (4-0) followed by puncturing the ligated portion with a 25-gauge needle. After a small amount of faeces content was extruded into the peritoneum the cecum was returned into the abdomen. The incision was closed by suturing the peritoneum and conglutination of the skin using *Vetbond* tissue adhesive. 0.9% saline solution was injected subcutaneously for resuscitation. *Bupivacaine* was administered at the incision site and *flunixin meglumine* was administered twice for postoperative analgesia. All animals were clinically monitored (clinical appearance, weights) after induction of SIRS / sepsis.

3.2.3 Complete blood count and clinical chemistry

Ten days post SIRS / sepsis blood was obtained by puncturing the facial vein with a sterile needle and collected in anticoagulant lithium-heparin coated blood tubes. Complete blood count was performed by automated veterinary haematology (Poch-100iv-Diff; *Sysmex*). For experiments performed at the *University of Iowa*, USA a defined volume (50 or 100µL) blood was obtained via retro-orbital bleeding and collected in 15mL falcon tubes.

In order to prevent coagulation, 500µL ice-cold RP10 were added to the blood. To lyse erythrocytes, 1mL VitaLyse were added and incubated for 5min at room temperature followed by stopping the reaction with 9mL ice-cold RP10. After centrifugation (5min, 500g, 4°C) the supernatant was discarded and cell pellets were washed with 5mL RP10 followed by centrifugation. After the supernatant was discarded, cells were resuspended in 500µL RP10. Total numbers of leukocytes were determined using a Neubauer counting chamber. To estimate frequencies and numbers of lymphocytes, large cell populations and T-cells, processed blood samples were subjected to flow cytometry (see below).
Blood plasma was separated from blood cells by centrifugation (2000g, 15min, 4°C). The plasma was immediately frozen in liquid nitrogen and stored at -80°C. Plasma levels of lactate dehydrogenase (LDH), aspartate aminotransferase (GOT) and alanine transaminase (GPT) were measured using the clinical chemistry analyser Fuji Dri-Chem 3500i (*FujiFilm*).

3.2.4 Total numbers of splenic T-cells and purification of splenic $CD4^+$ and $CD8^+$ T-cells

Ten days post SIRS / sepsis mice were sacrificed and spleens were harvested. A single cell suspension from pooled or single spleens was prepared by using a 70µM cell strainer. The cell suspension was washed with wash buffer and centrifuged (800g, 10min, 4°C). The supernatant was removed and erythrocyte lysis was performed by resuspending the cell pellet in 1-2mL ice-cold ACK buffer. After 3min incubation red blood cell lysis was stopped by adding excessive amount of wash buffer followed by one washing cycle. Total splenocyte numbers were counted with a Neubauer counting chamber using an appropriate cell dilution. In order to determine total numbers of T-cells and $CD4^+$/$CD8^+$ T-cell ratios in spleen, approximately $1x10^6$ whole splenocytes were transferred into 1.5mL Eppendorf tubes and subjected to flow cytometry (CD3/CD4/CD8 staining, see 3.2.6). $CD4^+$ and $CD8^+$ T-cells were purified from processed splenocyte suspensions via magnetic bead cell separation using the autoMACSPro (*Miltenyi Biotec*) cell separator and CD4 and CD8α MicroBeads. For separation all manufactures instructions were followed. Purity of cell separations was determined via flow cytometry (% CD3(+)) and routinely exceeded 90%.

3.2.5 T-cell cultivation and ex vivo T-cell activation assays

In order to study T-cell receptor- (TCR) mediated T-cell activation *ex vivo* at post-acute stages of SIRS and sepsis, T-cell activation assays were employed using monoclonal anti-CD3ε and/or anti-CD28 antibodies. TCR complex and/or CD28 co-receptor triggering with anti-CD3ε and anti-CD28 antibodies is commonly used as an experimental model for stimulation of polyclonal naïve T-cells. The interaction of anti-CD3ε with the two CD3ε-chains of the TCR complex leads to clustering and conformational changes resulting in intracellular signalling cascades mediating T-cell effector functions, such as cytokine production, clonal expansion, cytotoxicity and cell-cell interactions. However, in addition to TCR/CD3 triggering, complete and physiological activation of T-cells requires stimulation of the co-receptor CD28 *ex vivo* and *in vivo*. *Ex vivo* co-stimulation of T-cells can be triggered by clustering of CD28 co-receptors using anti-CD28 antibodies. Immobilisation of anti-CD3ε on dish surfaces and beads or cross-linking of biotinylated anti-CD3ε antibodies with streptavidin induces different qualities of T-cell responses. For example, streptavidin cross-linked anti-CD3ε/biotin antibodies induce robust proximal TCR signalling but fail to elicit a productive TCR response, such as cytokine expression and proliferation. In order take these findings in account, a panel of different TCR stimuli were employed covering a wide range of physiological productive and unproductive triggers: (i) 1.7µg/mL anti-CD28/biotin + 5µg/mL streptavidin in solution, (ii) 1.7µg/mL anti-CD3ε/biotin + 5µg/mL streptavidin in solution, (iii) 1.7µg/mL anti-CD3ε/biotin + 1.7µg/mL anti-CD28/biotin without streptavidin in solution. For dish surface immobilisation of anti-CD3ε/biotin (iv), 5µg/mL anti-CD3ε/biotin (in PBS) were incubated in the respective wells for at 2-4 h, at 37°C and washed with PBS before plating the T-cells in the presence of 1.7µg/mL anti-CD28. Beads coupled with anti-CD3ε and anti-CD28 (v) were used according to the manufactures protocol (T-cell expansion kit, *Miltenyi*). Stimulated $CD4^+$ and $CD8^+$ T-cells were cultured in RP3 medium for 18h (CD25, CD69 expression) or in RP10 medium supplemented with 0.00035% 2-mercapto-ethanol for 48h (proliferation capacity) at a density of $1x10^6$ cells/mL in 24-well cell culture plates in an incubator (37°C, 5% CO_2). CD25 (*IL-2 receptor α-chain*) and CD69 (*C-lectin binding domain type II transmembrane glycoprotein*) were used as markers for T-cell activation. CD69 and CD25 are not expressed on resting naïve $CD4^+$ and $CD8^+$ T-cells, though $CD4^+$/$FoxP3^+$ regulatory T-cells exhibit constitutive expression of CD25. Upon TCR stimulation *in vitro* and *in vivo*, CD25 is up-regulated and associates with

constitutively expressed β- and common γ-chains to form the trimeric IL-2 receptor required for IL-2-dependent T-cell expansion [88]. CD69, also referred to as *early lymphocyte activation marker*, is rapidly expressed on activated T-cells and it is assumed that CD69 acts as a co-stimulatory receptor for T-cell activation and expansion, although no physiological ligand has been found yet [89].

3.2.6 Flow cytometry

Stimulated T-cells (18h) and cells from processed whole splenocyte suspensions were transferred into 1.5mL Eppendorf tubes and washed with 500µL FACS buffer I followed by centrifugation (800g, 7min, 4°C). After removing the supernatant cells were stained for 20-30min on ice in the dark with a total volume of 50µL FACS buffer I containing the respective flow cytometry antibodies in appropriate fluorochrome combinations and concentrations (table 2). Cells were washed with 1mL FACS buffer I, centrifuged and resuspended in 300µL PBS. Data were acquired using a FACS Calibur (BD Pharmingen^{TM}) and analysed with FlowJo software (TreeStar Inc.)

3.2.7 T-cell receptor signalling analysis via western blot

Purified $CD4^+$ and $CD8^+$ T-cells were resuspended with RP0 buffer in 1.5mL Eppendorf tubes at a density of 3 x 10^6 cells/mL and rested prior stimulation for at least 15min at 37°C. T-cells were stimulated with 1.5µg/mL anti-CD3ε/biotin + 1.5µg/mL anti-CD28/biotin for 1min, 5min, 30min, and 4h at 37°C in a water bath. Long-time stimulation (18h and 24h) was performed in appropriate cell culture dishes in an incubator (37°C, 5% CO_2). Both stimulation antibodies were mixed prior adding to cells. After stimulation cells were centrifuged for 20s. The supernatant was aspirated off quickly and the cell pellet was lysed by adding 250µL ice-cold lysis buffer followed by vortexing. The cell lysates were incubated at room temperature for 10min and subsequently on ice for 10min. In order to clear the lysates from cell debris, samples were centrifuged for 15min at full speed at 4°C. 200µL of cleared lysates were transferred into vials containing 200µL 2x sample buffer and boiled at 95°C for 5min.

To separate proteins for western blotting analysis a *sodium dodecyl sulphate polyacrylamide gel electrophoresis* (SDS-PAGE) was performed using a 10% gradient separation gel (pH 8.6) and a 5% stacking gel (pH 6.8) (see 3.1). Protein samples and prestained protein standard ladder were applied on two gels with the same composition

with a Hamilton glas pipette. Gels were run in 1x PAGE buffer with 45mA and max. 400V for approximately 1:45h using the *PROTEAN® II xi Cell* (*Bio-Rad Laboratories, Inc.*) electrophoresis apparatus. A wet / tank blotting system (*Trans-Blot® Cell*, Bio-*Rad Laboratories, Inc.*) was used to transfer the proteins from the gel to a *polyvinylidene fluoride* (PVDF) membrane. *Whatman* filter papers and PVDF blotting membranes were cut in the same size like the gels. PVDF membranes were activated with methanol, washed with dist. water and equilibrated in 1x transfer buffer together with the Whatman filter papers for 30min. A blotting sandwich (3x Whatman filter papers, PVDF membrane, gel and 3x Whatman filter papers) was assembled and transferred to the XCell box. The protein transfer was run at 0.75mA per gel for 100min. Subsequently, the membranes were blocked with 1x TBS-T + 1% BSA for 30min and cut into pieces in order to detect several proteins with different protein sizes at the same time. Membranes were exposed to primary antibody solutions over night at 4°C under continuously shaking.

Phosphorylated protein levels of PLCγ1 (Y783, 155kDa), ZAP70 (70kDa), LAT (Y195, 36 / 37 kDa), Erk1/2 (Thr202/Tyr204, 42/44kDa) and Akt (S473, 60kDa) were detected using phospho-specific antibodies (see 3.1). After incubation with primary antibody, membranes were washed three times for 10min with 1x TBS-T followed by incubation with the respective secondary antibodies (see 3.1) for 30 – 45min at room temperature. Membranes were washed three times for 10min with 1x TBS-T. For protein detection, membranes were soaked with *Oxidizing Reagent Plus* and enhanced *Luminol Reagent Plus* for 1 min. The horseradish peroxidase-catalysed chemiluminescence reaction was detected with the FujiFilm LAS-3000 (*FujiFilm*) imaging system. In order to remove primary and secondary antibodies, membranes were incubated with stripping buffer for 30min at 50°C. After membrane stripping, membranes were rinsed with dist. water and washed with 1x TBS-T followed by a new protein detection cycle.

3.2.8 Live imaging of Ca^{2+} release upon TCR triggering

The release of calcium ions from intracellular Ca^{2+} stores upon TCR stimulation is a crucial step for physiological T-cell responses. The fluorescent dye *Fura-2-acetoxymethyl ester* (Fura-2-AM) is commonly used to study Ca^{2+} release in biological systems. Fura-2-AM is cell membrane permeable and is converted into impermeable Fura-2 by cleaving the acetooxymethyl ester group by esterases within living cells. Fura-2 binds calcium ions in the cytoplasm with a high affinity, thereby changing its fluorescent properties. Fura-2 is

excited at 340 and 380nm and fluorescent emission is detected at 510nm. Binding of Ca^{2+} ions increases 510nm emission of Fura-2 upon 340nM excitation, while 510nm emission at 380nM excitation declines. Using the ratio of 510nM fluorescence upon 340nm and 380nM excitation facilitates perpetual quantification of the amount of Ca^{2+} ions released into cytoplasm.

Purified $CD4^+$ and $CD8^+$ T-cells (2 x 10^6 cells per stimulation) were transferred into 50mL Falcon tubes and washed with 5mL RP3 w/o phenol red. After centrifugation (800g, 10min, RT) the supernatant was removed and cells were stained at a density of 5 x 10^6 cells/mL with the respective amount of RP3 w/o phenol red containing 10µM Fura-2-AM and 0.02% *Pluronic F-127*. Cells were incubated for 45min at 37°C in the dark. After Fura-2-AM loading cells were washed twice with 5mL RP3 w/o phenol red and resuspended for stimulation at a density of 1 x 10^6 cells/mL RP3 w/o phenol red. 2mL (2 x 10^6 cells) were transferred into a quartz cuvette and placed into the spectrofluorometer (Jasco FP 6500, *Jasco Inc.* or RF-5301PC spectrofluorophotometer, *Shimadzu*). The spectrofluorometers possess heated cuvette cells with magnetic stirrers in order to ensure optimal stimulation conditions (37°C and quick distribution of stimuli in cell suspension). Prior starting stimulation kinetics cells were equilibrated for 5min within the cuvette at 37°C. Following kinetics were used to measure Ca^{2+} responses of T-cells: 100sec basal reading, 150sec anti-CD3ε + anti-CD28 stimulation (1µg/mL hamster anti-CD3ε and anti-CD28), 200sec crosslinker stimulation (2.5 µg/mL anti-hamster IgG) and 150sec ionomycin (1µg/ mL ionomycin). Ionomycin was added as a positive control and used for normalisation in data analysis since its forms pores in biological membranes leading to the profound influx of Ca^{2+} ions independent of TCR triggering. Fura-2 was excited simultaneously at 340nm and 380nm and fluorescence intensity was measured at 510nm. For data imaging and analysis following equilibration was used for normalisation: $y = [f(x) - f(x)_{min}] / [f(x)_{max} - f(x)_{min}]$; f(x) = 510nm emission at time point x, $f(x)_{min}$ = average 510nm emission at basal reading, $f(x)_{max}$ = average 510nm emission after ionomycin treatment from 580-600s.

3.2.9 T-cell proliferation analysis

In order to analyse the proliferation capacity of T-cells after SIRS / sepsis, isolated pure $CD4^+$ and $CD8^+$ T-cells were loaded with the fluorescent dye *carboxyfluorescein succinimidyl ester* (CFSE). CFSE is cell membrane permeable and is retained within cell

due to covalently coupling of the succinimidyl group with amine moieties. The stable linkage of CFSE within cells facilitates to monitor cell division due to the progressive halving of CFSE fluorescence in each daughter cell. The characteristic CFSE fluorescent pattern enables quantification of (a) number of cell divisions per cell and (b) the frequency of cells that undergo at least on cell division cycle. CFSE fluorescence profiles are analysed via flow cytometry and appropriate softwares.

For CFSE loading purified $CD4^+$ and $CD8^+$ T-cells (1 x 10^6 per stimulation) were transferred into 15mL Falcon tubes, washed with 5mL PBS and centrifuged (800g, 10min, RT). The supernatant was aspirated and cell pellets were resuspended in 1mL PBS containing 1-2µM CFSE. Cells were incubated for 5min at 37°C in the dark. The reaction was stopped by adding 9mL PBS followed by centrifugation. After the supernatant was removed cells were resuspended in RP10 supplemented with 0.00035% 2-meracpto-ethanol and stimulated *ex vivo* with a panel of different anti-CD3ε and/or anti-CD28 combinations in solution or immobilised on dish surfaces or beads (see 3.2.5). 48h post stimulation cells were harvested and CFSE fluorescence was measured via flow cytometry using a FACS Calibur (BD Pharmingen™) and analysed with FlowJo software (TreeStar Inc.).

3.2.10 *Lymphocytic choriomeningitis virus* infection, and *ex vivo* LCMV-peptide stimulation

In order to expand and generate LCMV-peptide-specific effector $CD4^+$ and $CD8^+$ T-cells in background of SIRS and sepsis, mice were infected with the Armstrong strain of *Lymphocytic choriomeningitis virus* (LCMV-Arm). LCMV-Arm was kindly provided by V.P. Badovinac (*University of Iowa*). Ten days post LPS- and CpG-treatment and CLP surgery, 200µL 0.9% saline containing 2 x 10^5 plaque forming units (PFU) LCMV-Arm were injected into mice i.p.. Eight days later mice were sacrificed and spleens were harvested. A single cell suspension from individual spleens was prepared using a mesh. The single cell suspension was filtered with filter paper, washed with RP10 and spun down (5min, 800g, 4°C). The supernatants were discarded and erythrocytes were lysed with 1mL VitaLyse buffer. After 3min incubation at room temperature erythrocyte lysis was stopped with 9mL RP10 followed by centrifugation. The supernatants were removed and pellets were resuspended in 7mL RP10. 100µL of splenocyte suspension were transferred into a 96-well plate for each separate peptide stimulation concentration.

Titrating concentrations of synthetic LCMV-peptides GP61 and GP33 were used to stimulate LCMV-specific $CD4^+$ and $CD8^+$ T-cells, respectively. Splenocytes were separately stimulated with 0; 0.0256 x 10^{-3}; 0.128 x 10^{-3}; 0.64 x 10^{-3}; 3.2 x 10^{-3}; 0.016; 0.08; 0.4; 2; 10 and 50µg/mL GP61 or with 0; 0.01; 0.025; 0.05; 0.1; 0.25; 0.5; 1; 2; 5; 10 and 200nM GP33 for 5h. Peptide concentrations (2 x in RP10, 100µL per stimulation) were prepared by a serial dilution of a 10µg/mL GP61 stock solution or a 1mM GP33 stock solution. 100µL of respective peptide solution was added to 100µL splenocyte suspension in a 96-well plate. *Golgi Plug™ Protein Transport Inhibitor* (BD Pharmingen™) was added (1:1000) to accumulate produced cytokines within the cell and inhibit secretion via the Golgi apparatus pathway. Cytokine response was measured via flow cytometry (see 3.2.12)

3.2.11 *Listeria monocytogenes* infection and *in vivo* GP33-peptide stimulation

TCR-transgenic Thy1.1/1.2 P14 or Thy1.1/1.1 P14 $CD8^+$ T cells were obtained from spleen or peripheral blood of naïve transgenic P14 mice. The majority of peripheral T-cells from these mice are $CD8^+$ and possess T-cell receptor molecules specific for the LCMV-peptide GP33. The frequency of $CD8^+$ T-cells within white blood cells or splenocytes was determined prior adoptive transfer via flow cytometry. According to frequency of $CD8^+$ T-cells in the blood or spleen, cell preparations of blood or spleen were prepared containing 5,000 $CD8^+$ T-cells (P14 T-cells). Cells were transferred to new vial and 0.9% saline was added to fill up to 200µL. Cells were adoptively transferred into the retro-orbital vein of recipient mice using a syringe with a fine needle. Mice received P14 T-cells one day prior and ten days post LPS, CpG treatment or CLP surgery.

Ten days post SIRS / sepsis induction mice were intravenously infected with 1 x 10^7 colony forming units (CFU) *attenuated Listeria monocytogenes* expressing an Ova/GP33 fusion protein (LM-GP33). LM-GPP33 was kindly provided by V.P. Badovinac (*University of Iowa*). In order to prepare a LM-GP33 solution with 1 x 10^7 (CFU), a 1mL stock solution of LM-GP33 was thawn and transferred into 50mL Falcon tubes containing 9mL *Tryptic Soy Broth* (TSB) and streptomycin (50µg/mL). Bacteria were grown in an incubator under continuously shaking at 37°C until reaching an optical density of 0.06. The optical density was measured using an appropriate photometer at 600nm. As a blank standard TSB (+ streptomycin) without bacteria was used. An optical density of 0.06 is

equal to a bacteria concentration of 5 x 10^7 CFU/mL. 5mL bacteria suspension were transferred into fresh falcon tubes and spun down. The supernatant was removed and bacteria were resuspended in 5mL 0.9% NaCl solution. 200µL of bacteria/NaCl solution (1 x 10^7 CFU) were injected in each mouse i.v.. In order to verify the infection dose used in each experiment, the bacterial suspension was plated and grown on culture dishes containing TSB broth + streptomycin for 24h in an incubator (37°C, 5% CO2). The number of colonies was counted and did not differ significantly from 5 x 10^7 CFU/mL. Seven days post attenuated LM-GP33 infection 5µg GP33-peptide in 200µL 0.9% saline solution were injected into the retro-orbital vein of peptide receiving mice. One mouse in each experiment did not receive GP33-peptides and served as a control to facilitate an appropriate gating strategy in flow cytometry data analysis. Two hours post peptide injection mice were sacrificed and individual spleens were harvested. Spleens were quickly processed as described in 3.2.10. Flow cytometry and intracellular cytokine staining were performed as described in 3.2.12.

3.2.12 Intracellular cytokine staining via flow cytometry

The present section describes the flow cytometry procedure of experiments involving secondary infection models. 100µL processed blood samples (see 3.2.3) or 100µL of e*x vivo* or *in vivo* peptide-stimulated splenocytes single cell suspensions were washed with 150µL FACS buffer II (in 96-well plates) and spun down (3min, 800g, 4°C). Prior cell fixation and permeabilisation for intracellular cytokine staining, cell surface molecules (CD4, CD8, CD25, CD69, Thy1.1 and/or Thy1.2) were stained. Cell pellets were resuspended in 75µL FACS buffer II containing flow cytometry antibodies at appropriate concentrations and fluorophores (see 3.1). To minimise unspecific antibody binding on the surface of present phagocytes, Fc-blocking reagent was added to the staining solution. Cells were stained for 20-30min at 4°C in the dark, washed with 150µL FACS buffer and spun down. For fixation, cells were resuspended in 100µL ice-cold *Cytofix/Cytoperm Solution* (*BD Pharmingen*^{TM}) for 10 – 15min at 4°C in the dark. Cells were washed twice with 150µL 1x *PermWash buffer* (*BD Pharmingen*^{TM}). Fixed and permeabilised cells were stained with 75µL 1x *PermWash buffer* containing flow cytometry antibodies at appropriate concentrations and fluorophores (see 3.1) for 20-30min at 4°C in the dark. After washing with 200µL FACS buffer cells were centrifuged and pellets were

resuspended in 300µL FACS buffer II. Data were acquired using a FACS Canto II (*BD Pharmingen™*) analysed with FlowJo software (*TreeStar Inc*.).

3.2.13 Statistical analyses

GraphPad Prism5 (*GraphPad Software Inc.*) was used for statistical data analysis. A Mann-Whitney U test (two-tailed, confidence interval 95%) was used to determine significances between two experimental groups. A Kruskal-Wallis one-way analysis with Dunns post test was performed to determine significances between more than two experimental groups. Statistical survival was performed with SPSS Statistics (IBM). n.s. non significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

4 Results

4.1 Murine models of systemic inflammation and sepsis

4.1.1 Mortality and morbidity

Systemic Inflammatory Response Syndromes, including sepsis are very heterogeneous diseases characterised by a complex interplay of cells, processes and molecules that contribute to disease-related morbidity and mortality. Disease progression and outcome are highly variable depending on multiple factors including the nature and origin of the disease, comorbidities of the host as well as genetic and non-genetic predispositions. To account for the heterogeneity of the disease, four different murine models of systemic inflammation and sepsis were employed (Fig. 7A). Using two models of sterile SIRS (LPS- and CpG-DNA-induced SIRS) and two models of polymicrobial sepsis (PCI and CLP sepsis models) moreover enables to discriminate effects induced by the inflammatory host response from effects caused by the actual presence of viable pathogens. LPS and CpG-DNA (unmethylated CpG-rich oligonucleotides) are bacterial compounds and induce a profound systemic inflammation via activation of TLR4 (LPS) and TLR9 (CpG-DNA) expressed on neutrophils, macrophages and B-cells. By inducing microbial infections in the abdominal area, the PCI and CLP models trigger a marked peritoneal sepsis with polybacterial origin.

To characterise the pathophysiology of the utilized disease models animals were clinically monitored for ten days post insult, determining weight loss, clinical appearance and mortality. LPS / endotoxemia and peritoneal sepsis (PCI and CLP) induced a severe illness characterised by profound weights loss, lethargy, ruffled fur, swollen and clotted eyes within the first 48h (Fig. 7B). Animals suffering from septic peritonitis additionally exhibited signs of diarrhoea. On the contrary, mice treated with CpG oligonucleotides did not show symptoms of severe sickness except mild weight loss and lethargy within 24 hours. After acute illness surviving mice from all experimental groups quickly gained weight and recovered exhibiting no symptoms of sickness ten days post SIRS / sepsis (Fig. 7B). Animals from the PCI group showed an attenuated increase in body weight, not reaching initial weight at day ten post sepsis.

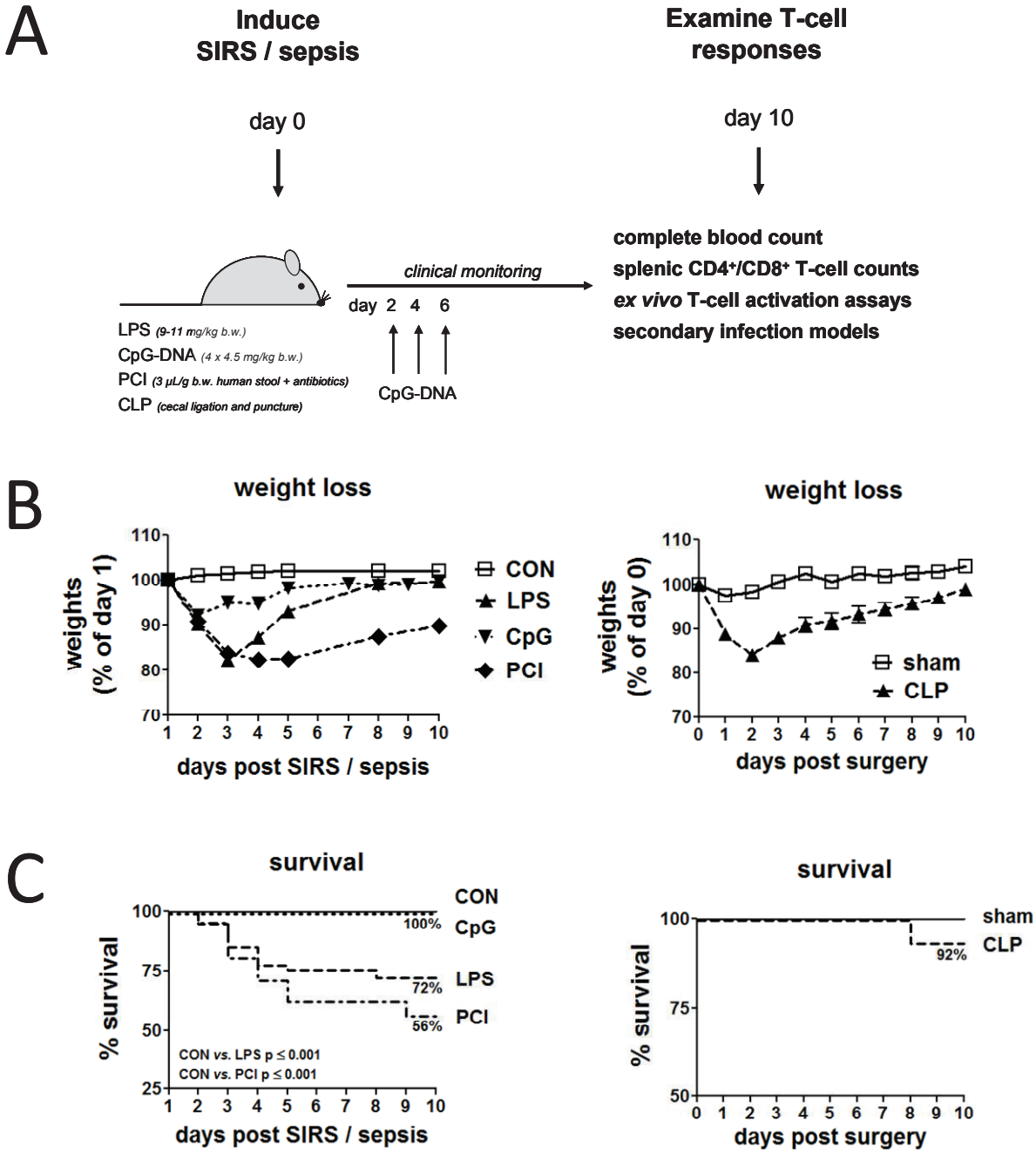


Figure 7. Experimental design and clinical features of murine SIRS and sepsis models
(A) Experimental design. A sterile Systemic Inflammatory Response Syndrome (SIRS) was induced by the intraperitoneal injection (i.p.) of 9 – 11 mg/kg b.w. lipopolysaccharide (LPS) or i.p. injection of 4 x 4.5 mg/kg b.w. CpG oligonucleotides (CpG-DNA) 2d, 4d and 6d post initial treatment. Polymicrobial peritoneal sepsis was induced by i.p. injection of 3 µL/g b.w. processed human stool (PCI) followed by antibiotic treatment (meropenem, 25mg/kg subcutaneously) 6h, 24h and 48h post insult. Surgical ligation and puncture of the cecum (CLP) was employed as a second murine polymicrobial sepsis model. **(B)** Time course of body weights (survivors) and **(C)** survival rates with at least 26 animals per group. CLP-sepsis experiments were performed in a different laboratory and one representative experiment (N=8) is depicted. Animals undergoing sham surgery represent the control group (sham) in these experiments. For survival analysis Log Rank (Mantel-Cox) was used to determine significances.

Most importantly, considerable inter-model differences were observed in SIRS- or sepsis-induced mortality (Fig. 7C). Peritoneal sepsis triggered by the intraperitoneal injection of human faeces (PCI model) exhibited a mortality rate of about 40% within the acute stage of the disease (four days post insult) with a total mortality of 46% at day ten post insult. Similar disease dynamics were observed for LPS-induced SIRS but with less overall mortality. Within four days after disease onset 25% of all mice died. A total mortality of 28% was observed at day ten. According to the mild clinical appearance, CpG-DNA-induced systemic inflammation did not induce disease-related mortality. Since the present study focused on the long-time impact of SIRS / sepsis in surviving mice, sublethal CLP surgery was performed to induce a marked septic insult with clear symptoms of severe sickness but low mortality (<10%). The employed CLP-surgery exhibited a total mortality rate of 8% within ten days post disease onset.

4.1.2 Organ / liver damage after systemic inflammation and sepsis

Acute episodes of systemic inflammation and sepsis are characterised by tissue and organ damage. Elevated blood plasma levels of enzymes normally expressed within intact cells are commonly used as markers for cell / tissue damage in research and diagnosis. *Lactate dehydrogenase* (LDH) is ubiquitously expressed within all cells, while plasma levels of LDH are low under normal conditions. Death of cells results in the systemic release of LDH into blood where it can be detected as a marker for systemic cell/tissue damage in humans and rodents. More specifically, the presence of the liver enzymes *glutamate pyruvate transaminase* (GPT) and *glutamate oxaloacetate transaminase* (GOT) in blood plasma is an indicator for liver damage / dysfunction.

The plasma levels of LDH, GPT and GOT at day ten post insult were determined by quantification of the specific catalytic activity via colorimetric analyses using an automated clinical chemistry analyser (Fig. 8A). As expected, no elevated levels of all three markers (LPS model) or only slightly increased levels of LDH, but not GOT and GPT (PCI model), were observed ten days after the insult, underlining the recovery of surviving mice from the acute insult. Intriguingly, animals from the CpG-group still showed elevated plasma levels of all three tested organ/liver damage markers ten days after starting the treatment, although these animals did not show any signs of severe illness. However, this finding shows that injection of CpG-DNA indeed caused a systemic inflammation associated with tissue / organ damage.

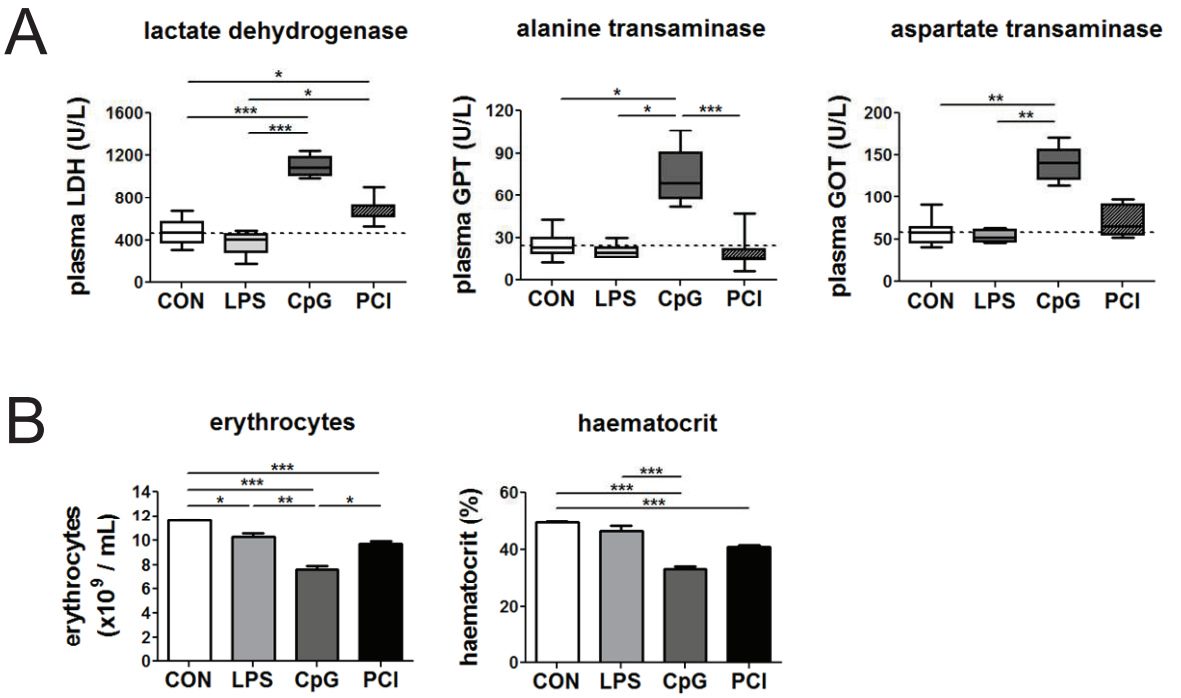


Figure 8. Organ/tissue damage and erythrocyte compartment at post-acute SIRS / sepsis
(A) Plasma levels of lactate dehydrogenase (LDH), alanine transaminase (GPT) and aspartate transaminase (GOT) were measured via an automated clinical chemistry analyser in survivor animals at day ten post SIRS / sepsis insult. Data are presented as Box Whisker plots (vertical bar: median, whiskers: min and max) and include at least 5 animals per group. **(B)** Erythrocyte counts and heamatocrit in blood from survivor animals at day ten post SIRS / sepsis insult were determined via automated haemocytometry. Data are presented as means + standard error of mean (SEM) including at least 13 animals per experimental group. A Kruskal-Wallis one-way analysis with Dunns post test was performed to determine significances (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

4.1.3 The red blood cell compartment after systemic inflammation and sepsis

It has been shown that the early loss of red blood cells together with lowered heamatocrit and haemoglobin levels are common features of septic patients and most likely contribute to worse outcome in sepsis [90-92]. In order to investigate potential alterations of the red blood cell compartment at post-acute stages of SIRS and sepsis, ten days post LPS- and CpG-treatment or polymicrobial sepsis (PCI model) peripheral blood was withdrawn from the facial vein and immediately analysed by automated veterinary haematology. Animals from all three experimental groups still exhibited decreased total numbers of erythrocytes and lowered heamatocrit values to varying degrees (Fig. 8B). CpG-induced systemic inflammation had a stronger impact on the red blood cell compartment than LPS / endotoxemia and septic peritonitis. The observed qualitative and quantitative differences of

the erythrocyte compartment underline the distinct biological outcome of the employed murine models of SIRS and sepsis. Since experiments using the CLP sepsis model were carried out in a different laboratory, quantification of organ / liver damage (Fig. 8A) and red blood cell counts (Fig. 8B) were not determined due to the lack of appropriate technical equipment.

4.2 The impact of systemic inflammation and sepsis on T-cell immunity at post-acute disease stages

4.2.1 Systemic inflammation and sepsis lead to persistent leukopenia and/or loss of naïve T-cells

The early loss of leukocytes, in particular lymphocytes, in peripheral blood is a common hallmark of SIRS and sepsis and is believed to contribute to immune paralysis after SIRS / sepsis (see introduction). However, the majority of studies in humans and rodents focused on early stages of the disease and much less is known about potential recovery of total leukocyte and lymphocyte numbers at post-acute stages. To investigate whether SIRS- and sepsis-triggered leukopenia and lymphopenia is a phenomenon still present at post-acute disease stages, total leukocyte and lymphocyte counts were analysed ten days post SIRS and polymicrobial sepsis (Fig. 9A and B). Pronounced leukopenia was observed in both employed models of sterile systemic inflammation (Fig. 9A). Decreased total leukocytes counts are primary caused by a marked drop of lymphocytes (Fig. 9B) while total numbers of large cell populations (primary granulocytes in mice) are unaffected (Fig. 9C). Conversely, both murine models of peritoneal sepsis did not lead to protracted leukopenia (Fig. 9A) but induced strong lymphopenia still present at post-acute stages of sepsis (Fig. 9B). Total leukocytes numbers in both sepsis models are compensated by the profound numerical increase of large cell populations in peripheral blood (Fig. 9C).

These data strongly suggest that the SIRS- and sepsis-triggered loss of lymphocytes in peripheral blood is persistent and most likely contributes to impaired adaptive immunity as a long-time consequence of systemic inflammation and sepsis. Moreover, the leukocyte compartment exhibits a changed composition after sepsis with potential impacts on immune responses at post-acute stages of sepsis.

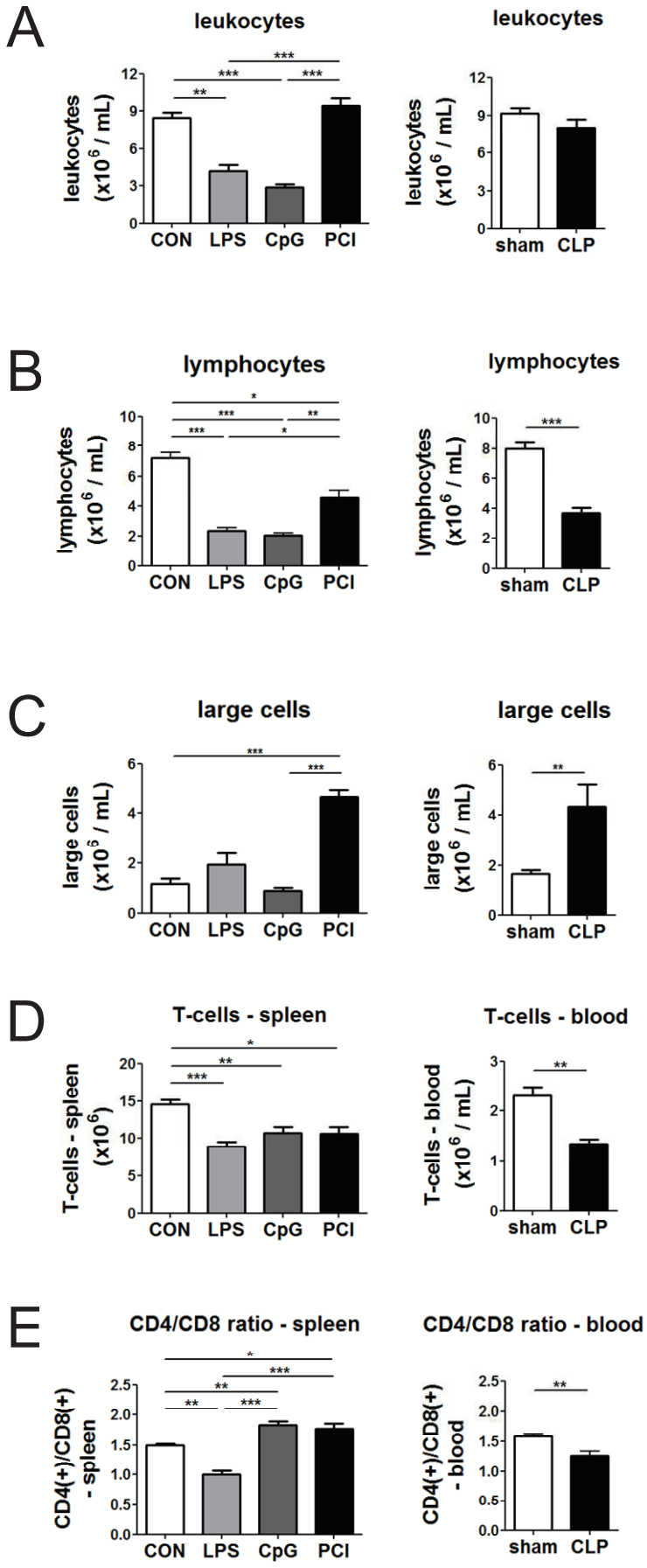


Figure 9. Blood cell counts and splenic T-cell numbers at post-acute SIRS / sepsis

Ten days post SIRS / sepsis insult the total number of **(A)** leukocytes, **(B)** lymphocytes and **(C)** large cells (granulocytes) in peripheral blood were determined by automated heamocytometry from at least 9 survivor animals. In experiments with CLP sepsis leukocyte numbers were determined by cell counting of processed blood samples. Lymphocyte and large cell counts were assessed via flow cytometry by gating on lymphocytes and large cell populations (N=8, representative of four independent experiments). **(D)** Total T-cell counts and **(E)** $CD4^+/CD8^+$ ratios in spleen (at least 9 animals/group) or blood (CLP experiments, N=8, representative of four independent experiments) were determined with flow cytometry by gating on CD4(+) and CD8(+) cells. A Kruskal-Wallis one-way analysis with Dunns post test was performed to determine significances in experiments with more than two experimental groups. A Wilcoxon-Mann-Whitney U test was performed to determine significances in experiments with two experimental groups (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

However, lymphopenia observed in peripheral blood must not necessarily reflect a systemic total loss of lymphocytes, since the majority of B- and T-cells reside in secondary lymph organs, in particular the spleen. Additionally, an inflammatory response induces the recruitment of peripheral blood immune cells to the infected tissues and lymph organs. To take these considerations in account, the total number of $CD4^+$ and $CD8^+$ T-cells was determined via flow cytometry in the spleen ten days post SIRS and sepsis. Significant persistent loss of splenic T-cells was found in all experimental groups (Fig. 9D) conforming that the early systemic drop of T-cell cellularity is a phenomenon also present in later stages of SIRS and sepsis. Since experiments using the CLP sepsis model involved secondary infections in living animals beyond day ten post insult (see Fig. 13 and 16 for experimental design), total numbers of $CD4^+$ and $CD8^+$ T-cells in CLP animals were measured from peripheral blood. In line with the findings from the PCI septic peritonitis model, animals from the CLP group exhibited a profound loss of $CD4^+$ and $CD8^+$ T-cells ten days post disease onset (Fig. 9D).

SIRS- and sepsis-induced apoptosis did not affect T-cell populations indiscriminately (Fig. 9E). LPS / endotoxemia primarily affected $CD4^+$ T-cell numbers leading to decreased $CD4^+/CD8^+$ T-cell ratios, whereas SIRS / sepsis induced by CpG or PCI resulted in slightly increased $CD4^+/CD8^+$ T-cell ratios. Similar to LPS treatment, CLP-induced T-cell loss in peripheral blood preferably affected $CD4^+$ T-cells as illustrated in decreased $CD4^+/CD8^+$ ratios.

4.2.2 T-cell responses to *ex vivo* TCR stimulation are not disturbed at post-acute stages of systemic inflammation and sepsis

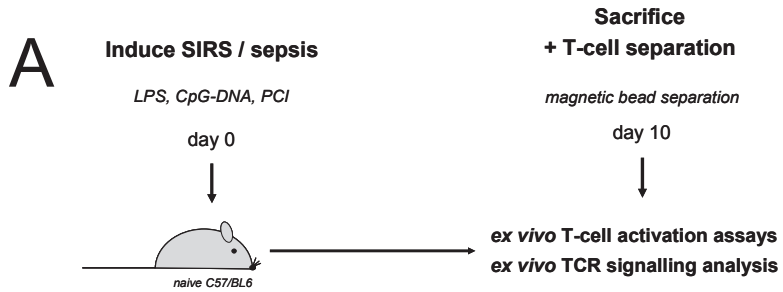
All described data (see above) illustrate that the employed murine models of systemic inflammation and polymicrobial sepsis fulfil all clinical criteria of SIRS and sepsis and therefore, are suitable protocols to study the impact of the diseases on T-cell function. As shown in Fig. 9, SIRS and sepsis resulted in the profound persistent loss of peripheral T-cells and thereby, most likely affects T-cell immunity at late stages of the disease. In addition to T-cell loss, a cellular malfunction of T-cells has been proposed contributing to compromised adaptive immunity at post-acute stages of sepsis but no detailed studies have been carried out yet.

In order to investigate TCR-mediated (adaptive) T-cell responses at post-acute stages of SIRS and sepsis, $CD4^+$ and $CD8^+$ T-cells were isolated from spleens from post-septic mice

and challenged with a panel of TCR/CD3 and/or CD28 co-receptor triggers *ex vivo* (Fig. 10A). Monoclonal anti-CD3ε and/or anti-CD28 antibodies were used in different combinations and types of presentation to cover a wide range of physiological productive and unproductive TCR-triggers (see section 3.2.5). The cell surface expression of CD25 (IL-2 receptor α-chain) and CD69 is commonly used as a marker for TCR-mediated T-cell activation. Here, the induction of CD25 and CD69 was determined via flow cytometry 18h post *ex vivo* TCR stimulation of purified $CD4^+$ and $CD8^+$ T-cells.

As expected, no induction of expression of CD25 (Fig. 10B) or CD69 (Fig. 10C) was detected in all experimental groups without stimulation or upon activation with anti-CD28 alone. Only weak T-cell activation was observed after challenge with anti-CD3ε in solution in the absence of CD28 co-stimulation. Anti-CD3ε in combination with anti-CD28 co-stimulation induced an intermediate CD25 and CD69 response in the control group, while immobilisation and clustering of anti-CD3ε on the dish surface in combination with anti-CD28 stimulation triggered a very strong T-cell activation as 80-90% of all T-cells expressed CD25 or CD69. T-cell stimulation with beads coupled with anti-CD3ε and anti-CD28 induced a modest response in CD25 expression but resulted in very pronounced CD69 accumulation. In sum, the panel of *ex vivo* TCR triggers employed here covers a wide array of T-cell responses ranging from weak to very strong in terms of CD25 and CD69 T-cell activation marker expression. Therefore, this assay is a suitable protocol to detect even slight inherent T-cell alterations in TCR responses after SIRS and sepsis.

Splenic $CD4^+$ and $CD8^+$ T-cells purified from post-acute SIRS (LPS and CpG) or septic animals (PCI model) did not show impaired functional responses to *ex vivo* TCR stimulation with either TCR trigger indicating that SIRS and polymicrobial sepsis did not lead to inherent defects in T-cell activation. It is worth noting that T-cells from all SIRS / sepsis settings showed a clear trend (although not statistically significant in all groups) of increased CD25 and CD69 expression profiles upon stimulation with anti-CD3ε together with anti-CD28 in solution. This finding might indicate a 'primed state' of T-cells after SIRS / sepsis rather than desensitised T-cell activation.


A
Induce SIRS / sepsis
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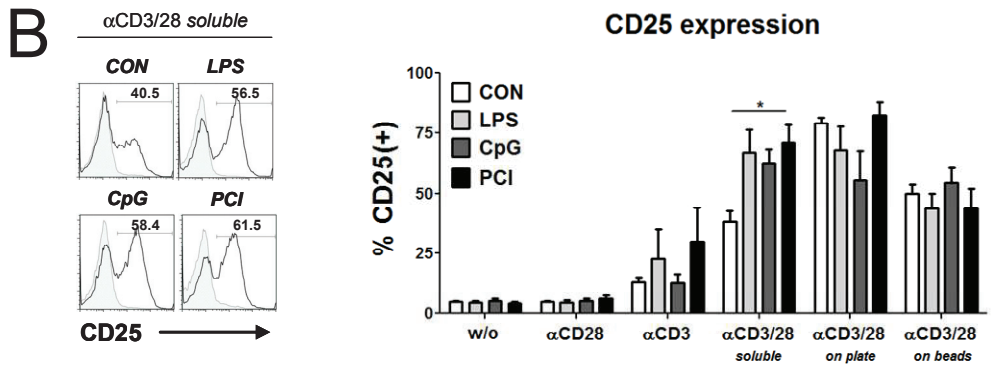
+ T-cell separation
magnetic bead separation
day 10
ex vivo T-cell activation assays
ex vivo TCR signalling analysis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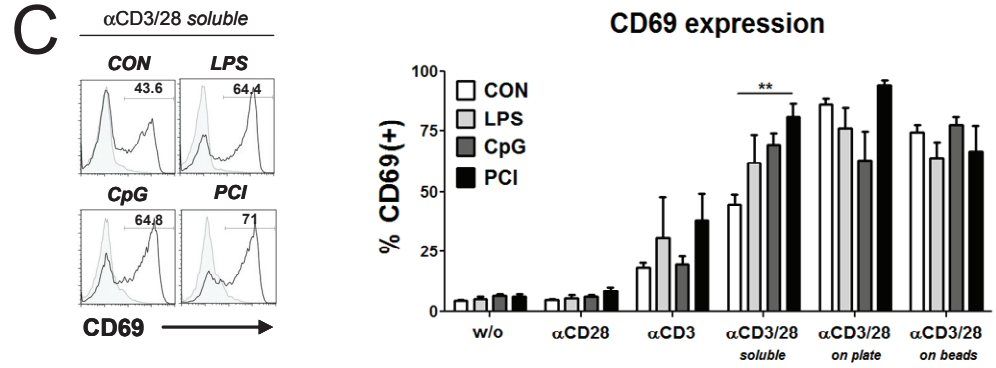
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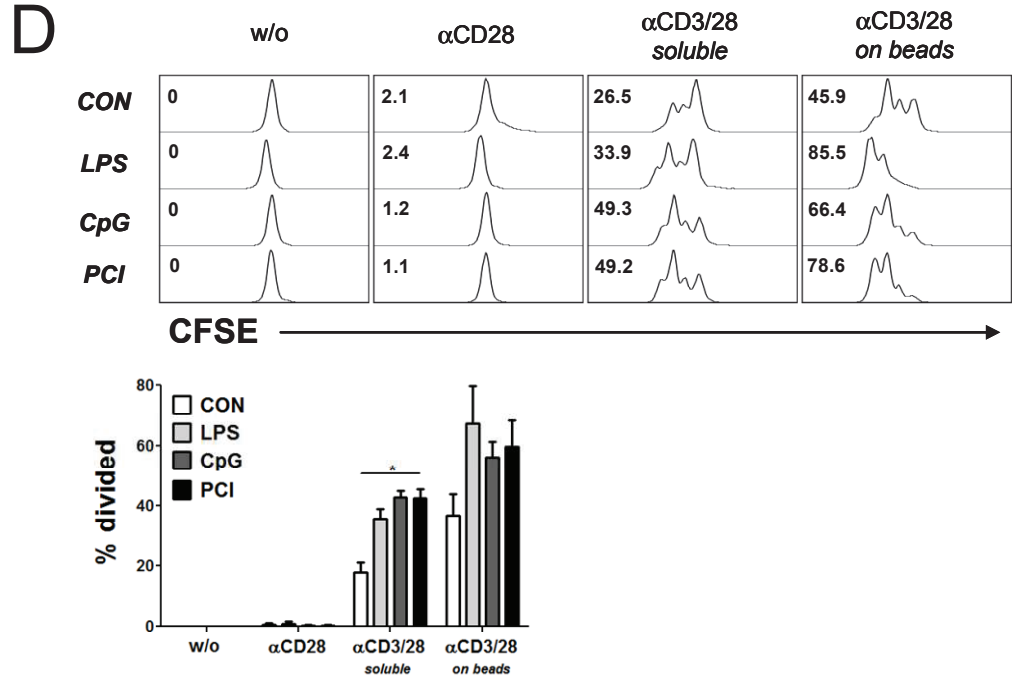
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1.1
49.2
78.6
CFSE
% divided
CON
LPS
CpG
PCI
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αCD28
αCD3/28 soluble
αCD3/28 on beads

Figure 10. T-cell responses to *ex vivo* TCR stimulation at post-acute SIRS / sepsis
(A) Experimental design. Ten days post SIRS / sepsis insult animals were sacrificed and spleens were harvested. Splenic $CD4^+$ and $CD8^+$ T-cells were purified via magnetic bead separation and *ex vivo* stimulated with a panel of anti-CD3ε and/or anti-CD28 TCR stimuli. 18h later **(B)** CD25 and **(C)** CD69 expression was determined via flow cytometry by gating on CD25 or CD69 positive cells (at least four independent experiments each including several animals). Representative fluorescence histograms are shown on the left side of the panel with stimulated (empty curves) *vs.* non-stimulated (shaded grey). **(D)** Purified $CD4^+$ and $CD8^+$ T-cells were labelled with CFSE and stimulated with the indicated TCR stimuli for 48h. T-cell proliferation was assessed via CFSE fluorescence dilution with flow cytometry and quantified as described in Material and Methods (at least 3 mice / group). Representative CFSE patterns are shown. Data are presented as means + SEM. A Kruskal-Wallis one-way analysis with Dunns post test was performed to determine significances (* $p \leq 0.05$, ** $p \leq 0.01$).

4.2.3 The expansion capacity of T-cells is not impaired at post-acute stages of systemic inflammation and sepsis

T-cell activation, determined by the means of CD25 and CD69 surface expression upon TCR stimulation indicated no inherently impaired T-cell activation after SIRS and sepsis. The capacity of T-cells to proliferate after TCR and co-receptor stimulation is a key feature of adaptive T-cell responses (Fig. 5). Vigorous T-cell expansion requires three independent signals: (i) TCR engagement and signalling, (ii) co-stimulatory signals and (iii) cytokine stimulation, in particular IL-2. Enduring SIRS- or sepsis-induced T-cell intrinsic defects in one of these steps must consequently lead to impaired expansion upon T-cell stimulation. In order to study the proliferation capacity of T-cells ten days post SIRS / sepsis, purified splenic T-cells were loaded with CFSE and challenged with a panel of anti-CD3ε and/or anti-CD28 TCR triggers *ex vivo*. 48h later, the CFSE fluorescence pattern was determined via flow cytometry to quantify the proliferative T-cell response after TCR/co-receptor stimulation (Fig. 10D).

TCR stimulation with anti-CD3ε in combination with anti-CD28 or coupled on beads induced a strong proliferative response of $CD4^+$ and $CD8^+$ T-cells, while CD28 co-receptor stimulation alone was not sufficient to trigger T-cell proliferation. In line with non-defective T-cell activation (Fig. 10B and C), splenic T-cells from SIRS and septic animals did not exhibit defects in TCR-induced expansion. Interestingly, tendentially more pronounced T-cell proliferation upon soluble anti-CD3ε/CD28 stimulation was observed in all SIRS / sepsis groups confirming the findings from the activation marker expression (Fig. 10B and C).

4.2.4 T-cell receptor signalling upon *ex vivo* TCR-challenge is not altered at post-acute stages of systemic inflammation and sepsis

All data described above indicate that TCR/co-receptor-mediated T-cell responses are not impaired on *per cell* base at post-acute stages of SIRS and sepsis. In order to confirm these findings on a molecular base, the activation pattern of crucial intracellular TCR and CD28 signalling molecules was analysed upon *ex vivo* TCR/CD28 triggering with anti-CD3ε + anti-CD28. The activation / phosphorylation kinetics of ZAP70, LAT, ERK and AKT were assessed via western blot analyses (Fig. 11). Additionally, the TCR-triggered release of Ca^{2+} ions, as a central molecule in TCR signalling, was determined via live ratio-metric Fura-2 fluorescence measurements (Fig. 12).

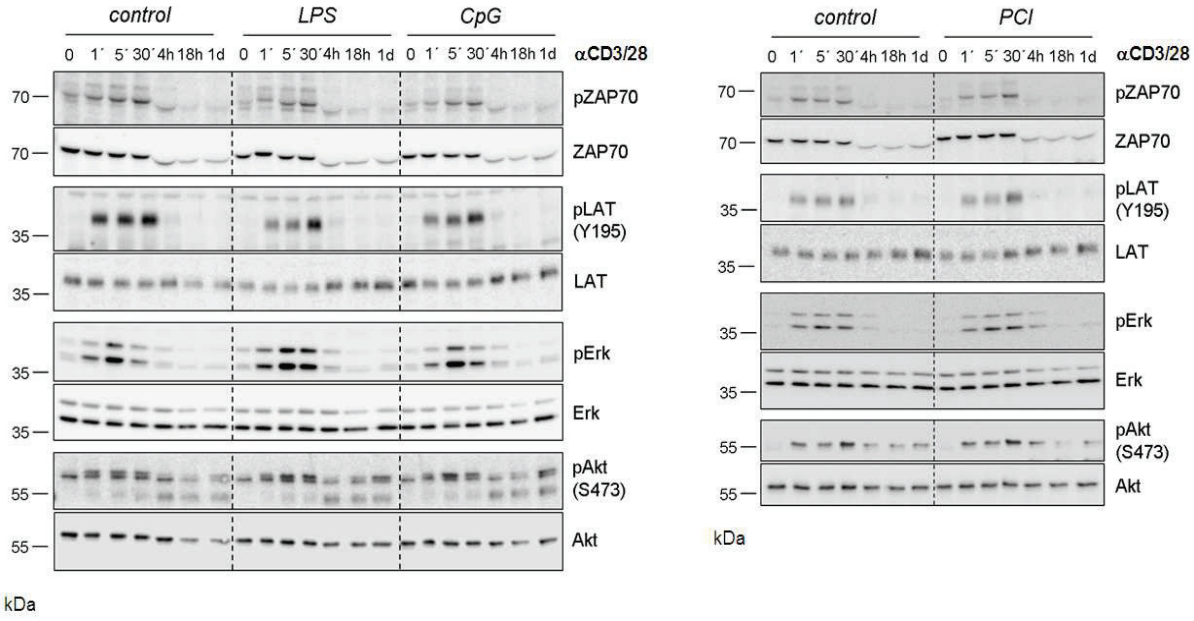


Figure 11. Proximal TCR signalling upon *ex vivo* TCR stimulation at post-acute SIRS / sepsis
Ten days post SIRS / sepsis insult animals were sacrificed and spleens were harvested. $CD4^+$ and $CD8^+$ T-cells were purified via magnetic bead separation and stimulated *ex vivo* with anti-CD3ε and anti-CD28. Stimulated cells were lysed at the indicated time points and cell lysates were subjected to standard western blot analyses detecting phosphorylated and total protein levels of ZAP-70, LAT, ERK and AKT. Depicted western blots are representative for several independent similar experiments. Protein marker sizes in kilo-dalton (kDa) are shown on the left side of the panel.

Upon anti-CD3ε/CD28 stimulation all studied TCR signalling molecules were rapidly phosphorylated and thus activated, as revealed by using phospho-protein-specific antibodies in western blot analyses (Fig. 11). Total protein levels, detected by protein-specific antibodies, served as loading controls and showed no changed expression pattern

over the course of the experiment. None of the experimental SIRS or sepsis groups exhibited impaired or altered phosphorylation kinetics, suggesting that SIRS and sepsis did not lead to disturbed or changed proximal TCR/CD28 signalling at post-acute stages of the disease. These data confirm the findings of non-disturbed T-cell responses described above. On the other hand, TCR/co-receptor signalling studies did not provide any evidence for stronger T-cell responses upon stimulation with non-immobilised anti-CD3ε/28 as phosphorylation dynamics were indistinguishable from the control groups.

In addition to activating post-translational phosphorylations of TCR/CD28 signalling molecules, the release of Ca^{2+} ions from intracellular calcium stores into cytoplasm represents a crucial step in T-cell activation and in the modulation of numerous T-cell effector responses. TCR/CD28-induced cytosolic Ca^{2+} accumulation in T-cells after SIRS and sepsis was analysed using the ratio-metric fluorescent dye Fura-2. The ratio of 510nM fluorescence emission upon 340nm and 380nM excitation is plotted over the time and facilitates live quantification of cytosolic Ca^{2+} release in living T-cells (Fig. 12).

As illustrated in Fig. 12, TCR/CD28 complex stimulation with hamster anti-CD3ε/28 induced a weak and slow accumulation of Ca^{2+}, while cross-linking of anti-CD3ε/CD28 with anti-hamster IgG triggered the rapid and profound release of Ca^{2+} ions into cytoplasm. Ionomycin as an unspecific pore forming agent induces a maximal cellular influx of Ca^{2+} irrespective of TCR signalling and was used as a positive control and for normalisation. In line with the TCR/CD28 signalling molecule phosphorylation analyses, SIRS and sepsis did not induce persistent alterations of TCR-triggered Ca^{2+} release. Temporal kinetics and the extent of cytosolic calcium accumulation in T-cells from SIRS and septic animals were indistinguishable from the control groups. In sum, biochemical analyses of intracellular T-cell receptor signal transduction do not provide any indication of impaired T-cell function on *per cell* base at post-acute stages of SIRS and sepsis.

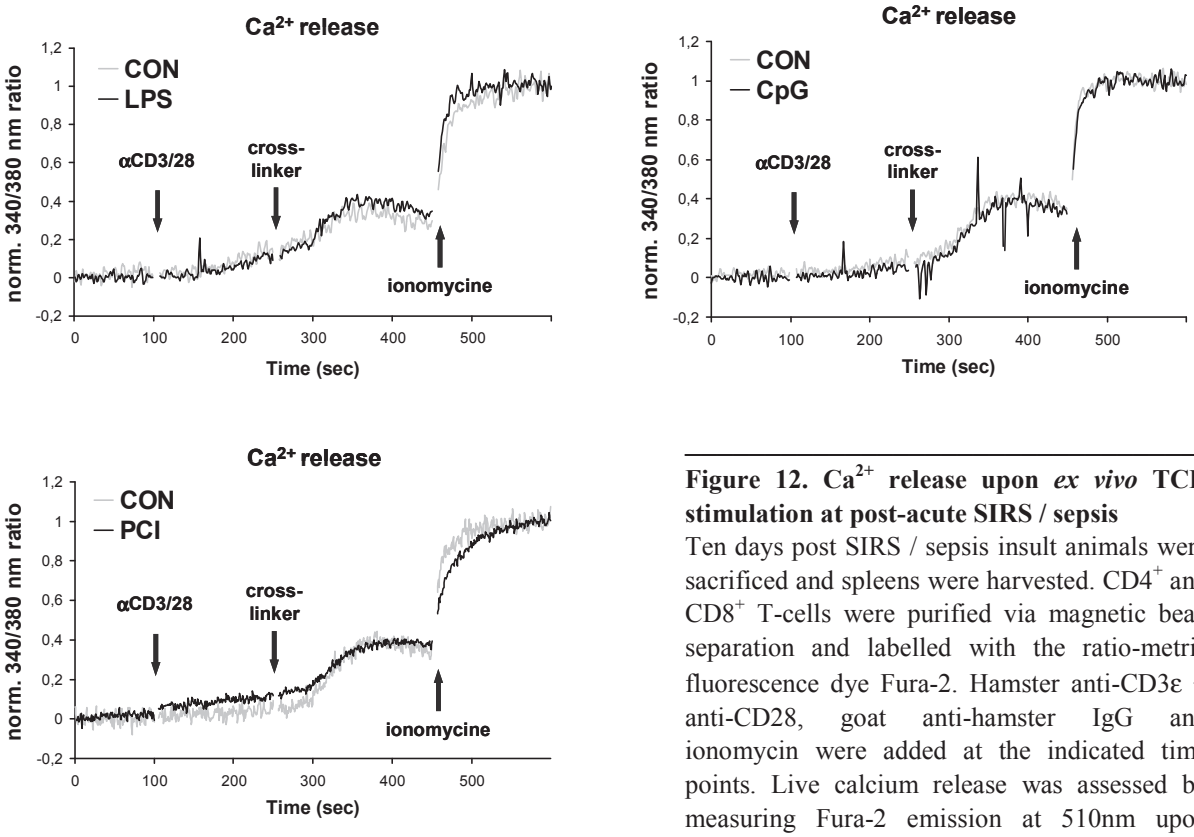


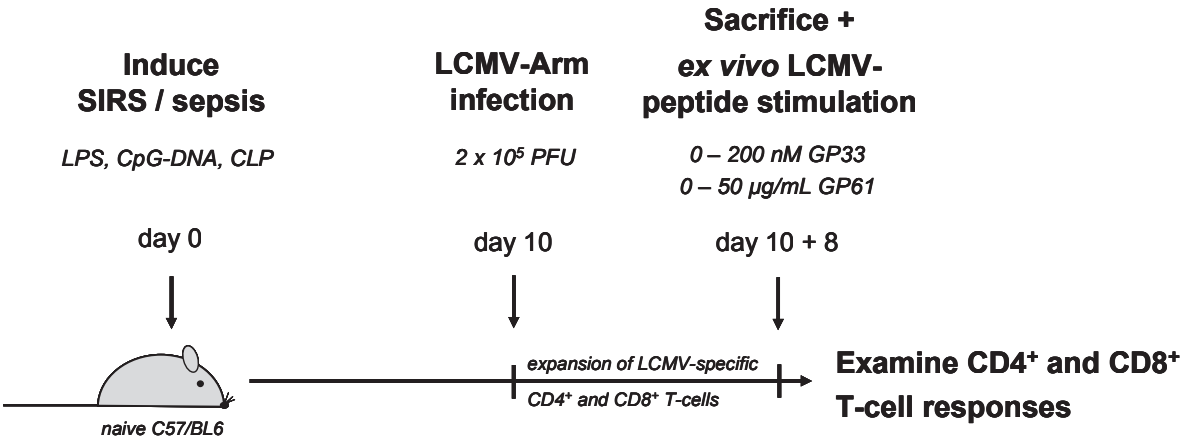
Figure 12. Ca^{2+} release upon *ex vivo* TCR stimulation at post-acute SIRS / sepsis
Ten days post SIRS / sepsis insult animals were sacrificed and spleens were harvested. $CD4^+$ and $CD8^+$ T-cells were purified via magnetic bead separation and labelled with the ratio-metric fluorescence dye Fura-2. Hamster anti-CD3ε + anti-CD28, goat anti-hamster IgG and ionomycin were added at the indicated time points. Live calcium release was assessed by measuring Fura-2 emission at 510nm upon excitation at 340nm and 380nm.
Calcium transients are visualised by plotting normalised 340/380nm ratios as described in Material and Methods. Experiments are representative of several independent similar experiments.

4.2.5 Systemic inflammation and sepsis induce persistent defects in antigen-dose responses of T-cells *ex vivo* in background of secondary virus infections

Ex vivo TCR complex and CD28 co-receptor stimulation of pure naïve $CD4^+$ and $CD8^+$ T-cells with monoclonal antibodies is a suitable experimental approach to study fundamental polyclonal adaptive T-cell responses on *per cell* base as it excludes extrinsic factors that modulate T-cell activation *in vivo* (e.g. antigen presentation or bystander cytokine signals). However, this approach fails to recapitulate T-cell activation in its entire complexity occurring under *in vivo* situations (Fig. 5). For example, in infectious diseases, only T-cell clones are activated that bear TCR variants specific for the invading pathogens. Pathogen-induced T-cell stimulation results in the generation of a large pool of effector T-cell clones that fight the infectious triggers. This clonal antigen-specific T-cell response is regulated by antigen-presenting cells and other immune cells that induce activation and T-cell effector responses. To take these considerations in account, secondary infection models

were used to extend T-cell studies since they involve all steps of *in vivo* T-cell activation and facilitate *ex vivo* and *in vivo* analyses of clonal antigen-specific responses of effector $CD4^+$ and $CD8^+$ T-cells in background of systemic inflammation and sepsis. To study antigen-specific effector T-cell responses at late stages of SIRS and sepsis, mice were infected with the Armstrong strain of *Lymphocytic choriomeningitis virus* (LCMV-Arm) ten days post SIRS and sepsis. Systemic infection with LCMV-Arm induces a profound activation of $CD8^+$ and $CD4^+$ T-cells *in vivo* characterised by the accumulation of virus-specific T-cell clones including $CD4^+$ and $CD8^+$ T-cells specific for the LCMV-peptides GP61 and GP33, respectively.

The experimental design employed in the present study represents a 'two-hit experiment' (Fig. 13). Ten days post SIRS (LPS and CpG models) and polymicrobial sepsis (CLP model) mice were infected with LCMV-Arm to induce an antiviral response and expand LCMV-specific $CD4^+$ and $CD8^+$ effector T-cells. Eight days after LCVM-infection mice were sacrificed and whole splenocyte homogenates were challenged *ex vivo* with titrating concentrations of LCMV peptides GP61 or GP33 to examine clonal antigen-specific $CD4^+$ and $CD8^+$ effector T-cell responses in a dose-dependent manner.



LCMV-Arm: *Lymphocytic choriomeningitis virus* – Armstrong strain

Figure 13. Experimental design of 'two-hit' LCMV-infection model
Ten days post SIRS / sepsis insult mice were infected with the Armstrong strain of *Lymphocytic choriomeningitis virus* (LCMV-Arm) (2 x 10^5 PFU, intraperitoneal). Eight days post secondary LCMV-Arm infection mice were sacrificed and spleen homogenates were stimulated with titrating concentrations of either LCMV-GP33 or LCMV-GP61 for 5h in the presence of a secretion inhibitor. $CD4^+$ and $CD8^+$ T-cell responses were assessed via flow cytometry.

The fraction of T-cells responding to LCMV-peptide stimulation was assessed by production of the central T-cell effector cytokine IFNγ via intracellular cytokine staining with flow cytometry (Fig. 14 and Fig. 15). At saturating peptide concentration the maximal fraction of splenic peptide-specific $CD4^+$ and $CD8^+$ effector T-cells was activated to produce IFNγ. Accordingly, e*x vivo* stimulation with decreasing antigen levels resulted in progressively lower frequencies of activated T-cells (IFNγ(+)) following sigmoidal dose-response kinetics (Fig. 14A and 15A). Dose-response curves were calculated with an appropriate software and used to assess the functional avidity (or antigen sensitivity) of effector T-cells to cognate antigens. Antigen sensitivity was determined and quantified by the means of the peptide concentration that triggers half maximal IFNγ production and are depicted as EC_{50}-values.

As shown in Fig. 14A, CpG-SIRS and CLP-induced sepsis induced protracted attenuated antigen-dose responses of effector $CD4^+$ T-cells as judged by right shifts of dose response curves. In line with that, the EC_{50} values for IFNγ production were significantly increased in these experimental groups. Contrary, no impaired functional avidity of $CD4^+$ effector T-cells was detected in mice from the LPS / endotoxemia group. Dose response kinetics and EC_{50} values were indistinguishable from the control group (Fig. 14A). The same results were observed for effector $CD8^+$ T-cells (Fig. 15A). $CD8^+$ T-cells from the CpG and CLP groups exhibited an attenuated fractional IFNγ production upon antigen stimulation, although less pronounced than $CD4^+$ T-cells. In line with the findings from effector $CD4^+$ T-cells, no increased EC_{50} values for IFNγ production were found in the LPS group.

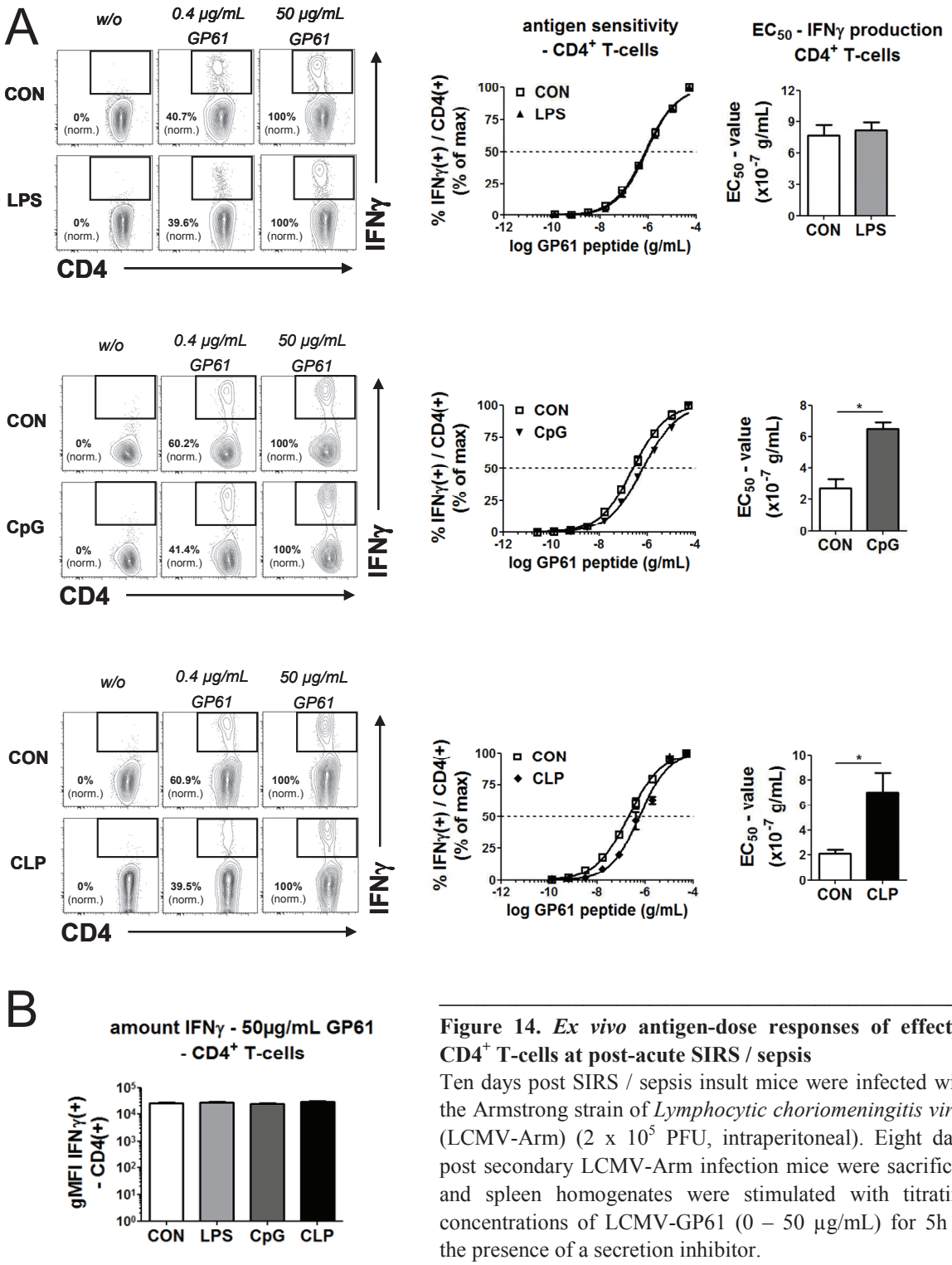


Figure 14. *Ex vivo* antigen-dose responses of effector CD4$^+$ T-cells at post-acute SIRS / sepsis

Ten days post SIRS / sepsis insult mice were infected with the Armstrong strain of *Lymphocytic choriomeningitis virus* (LCMV-Arm) (2 x 10^5 PFU, intraperitoneal). Eight days post secondary LCMV-Arm infection mice were sacrificed and spleen homogenates were stimulated with titrating concentrations of LCMV-GP61 (0 – 50 µg/mL) for 5h in the presence of a secretion inhibitor.

(A) IFNγ production in CD4$^+$ T-cells was assessed via intracellular cytokine staining with flow cytometry. Fractional IFNγ induction of CD4$^+$ in splenocytes was normalised setting IFNγ induction after 50µg/mL LCMV-GP61 to 100%. GraphPad Prism software was used to fit a sigmoidal dose-response curve and for calculation of EC_{50} values for fractional IFNγ production. Representative flow cytometry profiles are depicted on the left side of the panel. **(B)** Geometric mean fluorescence index (MFI) of IFNγ(+) in CD4(+) T-cells upon stimulation with LCMV-GP61. Data are presented as means + SEM with at least 3 animals per group and are representative of 2 – 4 independent experiments. A Kruskal-Wallis one-way analysis with Dunns post test was performed to determine significances in experiments with more than two experimental groups. A Wilcoxon-Mann-Whitney U test was performed to determine significances in experiments with two experimental groups (* p≤0.05).

Importantly, antigen-triggered synthesis of IFNγ in LCMV-specific effector $CD4^+$ and $CD8^+$ T-cells was not impaired on *per cell* base. The total cellular amount of synthesized IFNγ was quantified by the geometric mean fluorescence index (MFI) in activated T-cells (IFNγ(+)). None of the experimental groups featured defects in IFNγ synthesis after stimulation with saturating antigen concentrations (Fig. 14B and 15B). Interestingly, $CD8^+$ effector T-cells from all experimental groups exhibited trends or significantly increased levels of IFNγ production (Fig. 15B), once more indicating a 'primed T-cell state' at post-acute stages of SIRS and sepsis (see Fig. 10). In line with that, the cellular production of TNFα, another crucial $CD8^+$ T-cell effector cytokine, was also not impaired on a cellular base (Fig. 15C).

In sum, T-cell analyses using a secondary viral infection model indicate that systemic inflammation and polymicrobial sepsis can potentially lead to protracted decreased antigen-dose responses (antigen sensitivity) of effector $CD4^+$ and $CD8^+$ T-cells, though heterogeneous inter-model outcomes were observed in the SIRS settings. On the other hand, cytokine responses of $CD4^+$ and $CD8^+$ T-cells were not affected on a cellular level suggesting that attenuated T-cell responses were caused by disease-induced T-cell extrinsic defects, such as impaired antigen presentation or co-stimulation.

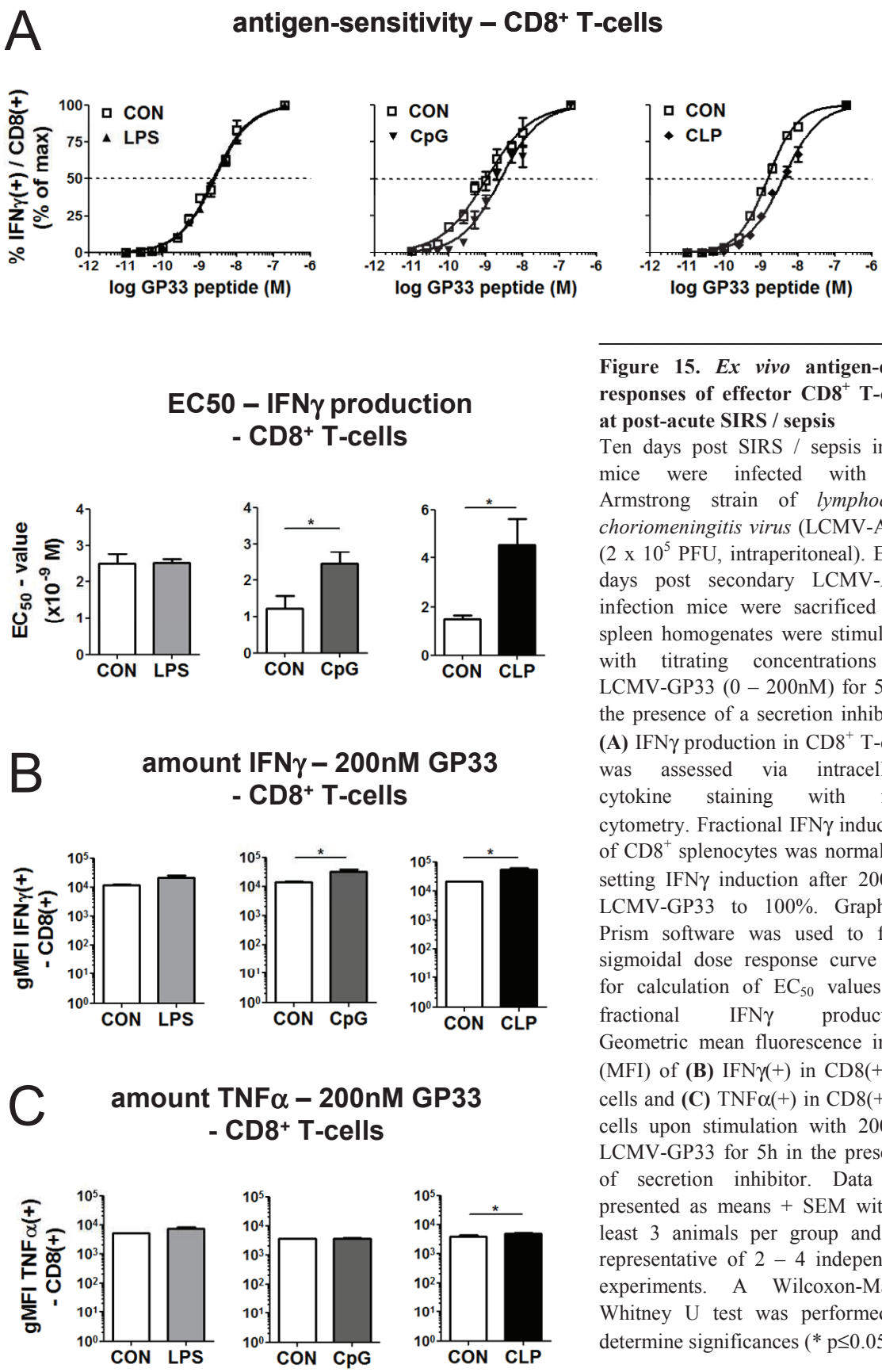


Figure 15. *Ex vivo* antigen-dose responses of effector CD8$^+$ T-cells at post-acute SIRS / sepsis

Ten days post SIRS / sepsis insult mice were infected with the Armstrong strain of *lymphocytic choriomeningitis virus* (LCMV-Arm) (2 x 10^5 PFU, intraperitoneal). Eight days post secondary LCMV-Arm infection mice were sacrificed and spleen homogenates were stimulated with titrating concentrations of LCMV-GP33 (0 – 200nM) for 5h in the presence of a secretion inhibitor. **(A)** IFNγ production in CD8$^+$ T-cells was assessed via intracellular cytokine staining with flow cytometry. Fractional IFNγ induction of CD8$^+$ splenocytes was normalised setting IFNγ induction after 200nM LCMV-GP33 to 100%. GraphPad Prism software was used to fit a sigmoidal dose response curve and for calculation of EC_{50} values for fractional IFNγ production. Geometric mean fluorescence index (MFI) of **(B)** IFNγ(+) in CD8(+) T-cells and **(C)** TNFα(+) in CD8(+) T-cells upon stimulation with 200nM LCMV-GP33 for 5h in the presence of secretion inhibitor. Data are presented as means + SEM with at least 3 animals per group and are representative of 2 – 4 independent experiments. A Wilcoxon-Mann-Whitney U test was performed to determine significances (* p≤0.05).

4.2.6 Systemic inflammation and sepsis do not induce persistent defects of *in vivo* effector CD8$^+$ T-cell responses

Data from the LCMV-infection model experiments indicate disturbed antigen-dependent T-cell responses at post-acute stages of SIRS and sepsis mediated by environmental cues rather than T-cell autonomous defects. In order to study these findings in more detail, a novel experimental approach was employed that allows functional *in vivo* T-cell studies with simultaneous discrimination of defects mediated by T-cell extrinsic factors from T-cell inherent alterations (Fig. 16A). TCR-transgenic P14 CD8$^+$ T-cells, specific for the LCMV-peptide GP33 were used to study clonal T-cell activation and effector responses upon *in vivo* injection of GP33. P14 T-cells were adoptively transferred in the same mice at physiological low numbers prior ('pre-SIRS') and ten days post ('post-SIRS') induction of SIRS / sepsis. Since 'post-SIRS' T-cells are transferred late after the SIRS / sepsis insult, potential functional defects in this population would strongly suggest persistent T-cell extrinsic alterations that modulate T-cell responses. On the other hand, functional disturbances that occur solely in the 'pre-SIRS' P14 T-cell population would strongly suggest inherent defects induced by the diseases. Expression of different allele variants of the T-cell-specific surface molecule Thy1 (Thy1.1/1.1 and Thy1.1/1.2, respectively) facilitates discrimination of 'pre-SIRS' and 'post-SIRS' P14 T-cells populations from endogenous T-cells (Fig. 16B).

In order to accumulate GP33-specific P14 T-cells prior *in vivo* antigen-challenge, ten days post disease onset mice were infected with non-virulent *attenuated Listeria monocytogenes* expressing LCMV-derived GP33 (att. LM-GP33). Seven days post att. LM-GP33 infection P14 T-cells were challenged *in vivo* by intravenous injection of their cognate antigen GP33. Two hours later, mice were sacrificed, spleens harvested and prepared for flow cytometry. Flow cytometric staining for Thy1.1 and Thy1.2 was used to distinguish and to gate on 'pre-SIRS' and 'post-SIRS' P14-T-cell populations (Fig. 16B). To examine *in vivo* P14 T-cell responses, activation marker up-regulation (CD25 and CD69) and effector cytokine production (IFNγ and TNFα) were immediately measured via flow cytometry from whole splenocyte homogenates without additional *ex vivo* incubation or stimulation steps. As judged by the expression profile of CD69 and CD25, *in vivo* GP33-antigen challenge resulted in rapid activation of both P14 effector T-cell populations (Fig. 16C and D). As expected, non-peptide-receiving mice exhibited no signs of T-cell activation

underlining the eligibility of the employed *in vivo* T-cell assay (see Fig16C – F, shaded grey histograms).

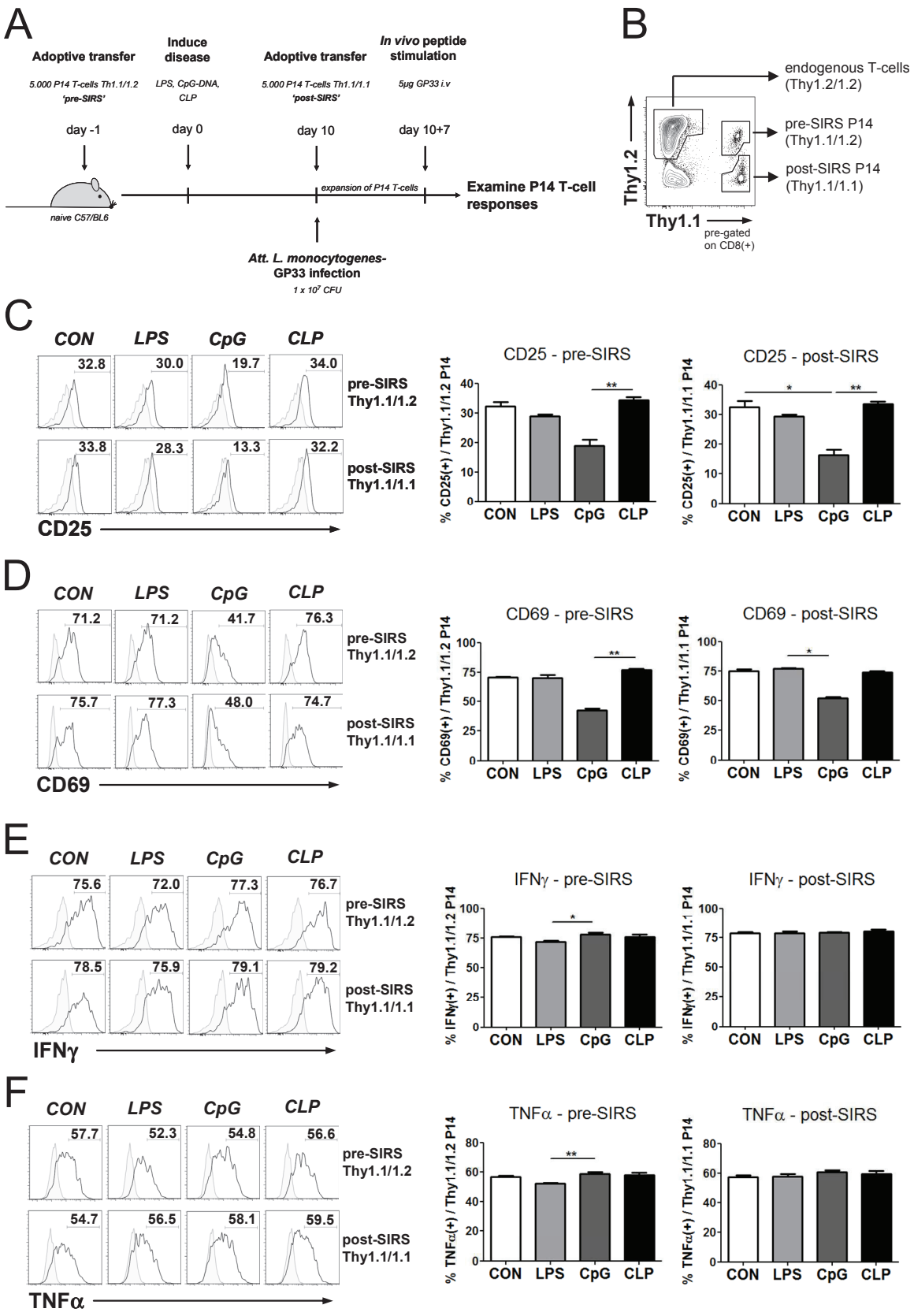


Figure 16. *In vivo* CD8$^+$ T-cell responses to antigen stimulation at post-acute SIRS / sepsis
(A) Experimental design. 5,000 TCR-transgenic LCMV-GP33-specific P14 T-cells were adoptively transferred one day before ('pre-SIRS', Thy1.1/1.2) and ten days post ('post-SIRS', Thy1.1/1.1) induction of SIRS / sepsis. Ten days post SIRS / sepsis insult mice were infected with *attenuated Listeria monocytogenes* expressing LCMV-GP33 (1 x 10^7 PFU, intravenously) to accumulate P14 T-cells. Seven days post secondary infection P14 T-cells were challenged *in vivo* by intravenous injection of 5µg LCMV-GP33. Two hours later, mice were sacrificed and spleens were harvested followed by flow cytometry staining. **(B)** 'pre-SIRS', 'post-SIRS' P14-T-cells and endogenous T-cells were distinguished via flow cytometry by their differential expression of Thy1 alleles. Representative Thy1.1/Thy1.2 2D-plot is shown. **(C)** CD25, **(D)** CD69, **(E)** IFNγ and **(F)** TNFα induction after *in vivo* LCMV-GP33 stimulation was determined via surface staining (CD25, CD69) and intracellular cytokine staining (IFNγ, TNFα) with flow cytometry with pre-gating on CD8(+) and Thy1.1(+)/Thy1.2(+) ('pre-SIRS) or Thy1.1(+)Thy1.2(-) ('post-SIRS'). Representative fluorescence profiles are depicted on the left side of the panel with non-stimulated (shaded grey) *vs.* peptide-stimulated (empty curves). Data are presented as means + SEM with at least 4 animals per group and are representative of 2 independent similar experiments. A Kruskal-Wallis one-way analysis with Dunns post test was performed to determine significances. (* $p \leq 0.05$, ** $p \leq 0.01$).

Importantly, antigen-triggered P14 T-cell activation was not altered in animals from the LPS-SIRS and CLP-sepsis groups, indicating no inherent or extrinsic defects in T-cell activation. On the other hand, CpG-treated animals exhibited strong trends of lower fractional induction of CD69 and CD25 expression indicating impaired or attenuated T-cell activation after CpG-induced SIRS. Since in this experimental group both P14 T-cell populations were affected indiscriminately, one can conclude that defective T-cell activation was induced by T-cell extrinsic (environmental) alterations rather than disease-induced T-cell autonomous disturbances. If SIRS / sepsis would have induced inherent cellular alterations in P14 T-cells, impaired CD25 and CD69 would have been noticeable exclusively in the 'pre-SIRS' P14 T-cells, as this cell population was present during disease onset.

Intriguingly, fractional production of INFγ and TNFα in both P14-T-cell populations was not impaired in all SIRS / sepsis settings (Fig. 16E and F), although decreased activation marker expression indicated impaired T-cell activation in the CpG-group. Moreover, none of the experimental groups exhibited alterations in antigen-induced total production of IFNγ or TNFα on *per cell* base as indicated by the unaltered MFIs in cytokine-producing P14 CD8$^+$ T-cells (data not shown). In sum, these data illustrate that SIRS and sepsis do not lead to substantial enduring defects in TCR-mediated T-cell responses *in vivo* and largely underline the findings described in this thesis. Moreover, non-affected cytokine production in both P14 T-cells populations clearly argues against profound environmental defects in T-cell activation. However, attenuated activation marker expression upon

antigen challenge in the CpG-group, suggests alterations in T-cell activation mediated by extrinsic cues, a notion that was raised by the findings of impaired antigen-dose responses described in 4.2.5.

4.2.7 Remarks

All experiments presented in this thesis were performed by the author alone or with technical support from colleagues. All depicted results were obtained from experiments with substantial contribution from the author of the thesis. However, the CFSE-proliferation experiment shown in Fig. 10D was performed by Dr. R. Requardt. This particular experiment was chosen for presentation since it contains all four experimental groups in the same assay facilitating a clear and structured data presentation. The author of the present thesis performed similar CFSE-proliferation experiments each including one SIRS- or sepsis-group yielding essentially the same results.

5 Discussion

Systemic Inflammatory Response Syndromes, predominantly sepsis and its subsets, are major health concerns with very high prevalence on intensive care units and patients who underwent surgical interventions. In fact, systemic inflammation and sepsis are the leading causes of death on ICUs worldwide, with steadily rising incidence due to a rising number of surgeries, increasing antibiotic resistances and ageing population.

In addition to the malevolent acute pro-inflammatory cascade, SIRS and sepsis are accompanied by a series of anti-inflammatory processes. The de-regulated excessive inflammatory responses are the cause of acute and protracted immune suppressive states that contribute to early mortality and are associated with elevated mortality at post-acute stages of the diseases. Since advances in critical care medicine and improved medical guidelines result in continuously increasing numbers of patients surviving the acute insult [93], persistent immunosuppression becomes a main health issue drawing attention of both physicians and researchers. Malfunction of the T-cell compartment is known to contribute to impaired immunity at acute stages but very little is known about the functional state of T-lymphocytes at post-acute stages of SIRS and sepsis and its potential contribution to late morbidity and mortality. The present thesis represents a significant contribution helping to close this gap of knowledge.

5.1 Murine models of systemic inflammation and sepsis

5.1.1 Features of employed experimental murine models of systemic inflammation and sepsis

Systemic Inflammatory Response Syndromes are very heterogeneous clinical diseases with highly diverse progression, affected organs/systems, severity and outcome depending on multiple factors such as septic trigger, site of infection/inflammation and the use of appropriate treatment protocols. The complexity of septic responses is even more increased due to host determinants including age, gender, genetic factors and comorbidities [94]. Over the past two decades several murine models of the diseases were established to study fundamental mechanisms of SIRS and sepsis. Available cell culture systems, albeit preferable under ethical aspects, fail to reproduce the systemic nature of sepsis in its full

complexity involving numerous cells types, mediators and tissue networks. However, the clinical relevance of the commonly employed animal disease models is consistently addressed and heavily disputed in clinical sepsis research [95, 96]. Some arguments raised in these articles can not be denied easily. Although most models have high resemblance to the pathophysiology in human patients, the characterisation of key regulators and processes failed to translate into effective therapeutic interventions. In fact, in Europe no specific drug is approved to treat patients with acute episodes of sepsis.

To account for the heterogeneity of the disease and the drawbacks of individual rodent animal models, four different models of SIRS and sepsis were employed bearing distinct clinical features, such as trigger mechanism, severity and outcome. As documented in Fig. 7 – 9 and discussed in more detail below, all four experimental models feature high resemblance to the heterogeneous clinical manifestation in humans with inter-model differences in morbidity, mortality and outcome. The broad spectrum of disease models increases the clinical relevance of this study and allows translating the findings to human patients. Importantly, the employment of sterile SIRS models and models of bacterial-triggered sepsis facilitates the investigation of clinical consequences induced by the inflammatory host response alone or effects triggered by the actual presence of an infection. All four experimental models induce an acute episode of SIRS or sepsis originating from the abdominal area, a very frequent site of infection in human patients [3, 4].

An infection-free Systemic Inflammatory Response Syndrome was induced by the intraperitoneal injection of free microbial components, here lipopolysaccharide and CpG-DNA. Both compounds have been shown to induce a profound SIRS that is mediated by macrophages and other immune cells upon binding to TLR4 (LPS) or TLR9 (CpG-DNA) [97-100]. LPS as a compound of the cell wall of gram-negative bacteria was chosen to mimic systemic bacterial gram-negative infections as these infections are the trigger of half of all sepsis cases [3, 4, 101]. Unmethylated CpG-rich DNA motifs are highly underrepresented in vertebrate genomes but are prevalent in bacterial DNA and thus, induce a strong inflammatory response in the host organism [98]. CpG-DNA injection was used to broaden the spectrum of acute systemic inflammatory responses, allowing studying common or diverse effects of different TLR-induced SIRS insults on T-cell immunity. LPS and CpG-DNA have been clearly associated with the inflammatory response in septic patients and represent key molecules in the pathogenesis of many septic cases [98-100]. By definition [1], administration of LPS and CpG-DNA does not meet the criteria for clinical

sepsis since the insult does not originate from a real infection. Taking this notion in account, the present thesis collectively terms both models as (sterile) SIRS, although LPS and CpG-DNA are bacterial compounds, unarguably leading to an infectious (systemic) immune response similar to the clinical manifestation in sepsis.

The second strategy mimicked a peritoneal polybacterial sepsis triggered by i.p. injection of human stool (PCI model) and ligation and perforation of the cecum (CLP model). In contrast to the SIRS models, polybacterial sepsis simultaneously triggers multiple pattern recognition receptors, thereby modulating the nature of the acute inflammatory response and potentially resulting in different outcomes. Both models are based on systemic bacterial infections and share clinical features observed in human septic patients. For example PCI- and CLP-triggered sepsis induces acute organ damage / failure, predominantly in the liver and the renal system [87, 102-104], organs also frequently affected in human patients. Moreover, both PCI and CLP induce a strong acute inflammatory response mediated by factors, such as IL-6 and IL-10 also relevant in humans [87, 105-107].

5.1.2 PCI *vs.* CLP

Functional assays involving polyclonal *ex vivo* T-cell stimulation with monoclonal anti-CD3ε/28 antibodies included PCI-treated animals while the secondary infection model experiments included animals that underwent CLP surgery. Different sepsis models were employed, since T-cell assays were performed in laboratories in two different countries. The laboratory at the *University of Iowa*, USA did no have the capability to employ the PCI sepsis model.

Optimally, the same disease models should be used in the same study to allow direct comparison of the data as different models can potentially result in different outcomes. A significant difference of both employed models lies in survival, with PCI exhibiting mortality rates of 44%, while CLP sepsis was low-lethal with a mortality rate of 8%. On the other hand, the PCI and CLP models feature certain similar characteristics. First, both models involve an infection in the abdominal area. Second, the infectious pathogens, primary bacteria, arise from the intestinal microbiota of mice (CLP) or human (PCI). The composition of the gut flora may be different, although it has been shown that there is a great similarity at the level of division (evolutionary lineage) in both species [108]. Third, both PCI and CLP are characterised by the profound release of inflammatory mediators

(e.g. IL-6 or IL-10) at the acute stage of disease associated with organ / tissue damage. Moreover, both models result in the persistent loss of lymphocytes to almost the same degree that is accompanied by an increase of total phagocyte counts in peripheral blood suggesting an analogous apoptotic pattern in both models (Fig. 9). Accordingly, episodes of PCI- and CLP-induced sepsis lead to decreased T-cells counts at day ten post insult, although $CD4^+$ and $CD8^+$ T-cell subsets are differently affected, as judged by different CD4/CD8 ratios (Fig. 9D - E). In sum, although precise mechanisms in both models are distinct, major aspects are analogous thereby facilitating drawing eligible and comparable conclusions from both models.

5.1.3 Morbidity and mortality of murine models of systemic inflammation and sepsis

The broad spectrum of the four employed murine disease models is underlined by inter-model differences in morbidity and mortality (Fig. 7). Treatment with LPS or human faeces (PCI model) induced an acute severe illness characterised by profound weight loss, lethargy, ruffled fur and clotted eyes and was associated with an mortality of 28% and 44%, respectively. The dynamics of morbidity and survival in these models is very close to the situation observed in human patients suffering from sepsis and severe sepsis and thereby reflect suitable protocols to study the long-time consequences of SIRS and sepsis on various biological parameters, e.g. function of T-cells.

Patients in ICUs suffering from an acute episode of sepsis are treated with antibiotics to eliminate the infectious trigger of the disease. Rapid and appropriate administration of antibacterial treatment has been shown to be the primary determinant of disease progression, morbidity and outcome in human patients [109, 110]. This notion is recapitulated in the PCI sepsis model through daily injection of antibiotics during the acute stage of the disease. Similar to human patients, almost all animals would die without antibacterial therapy. As a matter of fact, the PCI model is the only murine model of polymicrobial sepsis that involves antibiotic treatments as a standard procedure thereby increasing the clinical relevance of this disease model.

Although severe sepsis and septic shock are associated with high mortality (>30%), a considerably large number of patients with SIRS and sepsis exhibit mortality rates of 10% [111] and 15% [2], respectively. A fact that is not considered in most clinical studies as human studies often exclusively include cases of severe sepsis and/or tissue samples from

deceased patients. In line with that, many animals studies employ sepsis models with very high mortality rates (often more than 50%), that do not properly reflect the situation in human patients. In fact, cases of SIRS and non-severe sepsis represent the great majority of insults. To take these considerations in account, sublethal murine models of SIRS and polymicrobial sepsis were additionally used in the present study. Injection of CpG-DNA induced a Systemic Inflammatory Response Syndrome without mortality and without signs of severe sickness. The actual potency of CpG-DNA to induce SIRS associated with organ damage and excessive secretion of cytokines (e.g. IFNγ, IL-6, IL-10) also found in humans patients has been well documented [97, 98] and was additionally confirmed in the present thesis (see below).

CLP surgery was used to induce a polymicrobial sepsis with deliberately low mortality rates (about 10%) to study the long-time impact of sepsis on T-cell immunity. The CLP model of peritoneal sepsis is considered as the 'gold-standard' in sepsis research and is the most employed murine model of polymicrobial sepsis [112]. Despite the relatively low mortality, animals suffering from CLP-induced sepsis showed signs of severe sickness at acute stages of the disease including profound weight loss, diarrhoea and lethargy (Fig. 7).

5.1.4 Haematological changes at post-acute stages of systemic inflammation and sepsis

Acute and post-acute alterations in the haematological system including leukocytes, lymphocytes, erythrocytes and platelets are hallmarks in patients with Systemic Inflammatory Response Syndromes. In sepsis and its subsets, virtually all patients exhibit malevolent haematological changes regarding total cellular counts and function, associated with the fatal outcome of the disease [90, 113]. All employed murine models of SIRS and sepsis recapitulated the profound alterations in the haematological compartment, once more underlining the clinical resemblance and relevance of these disease models.

A marked total loss of lymphocytes (Fig. 9B) was detected in all models at day ten post disease onset, suggesting that lymphocyte apoptosis is a common feature in all Systemic Inflammatory Response Syndromes, disregarding of the nature and origin of the disease. Lymphopenia occurred early within the acute stage of the disease since decreased lymphocyte numbers were also detected within the first 4 days post initial insult ([63], data not shown). Importantly, the present study shows that lymphopenia is a phenomenon also

present at post-acute stages, a fact that was poorly investigated in many SIRS and sepsis studies.

Interestingly, protracted lymphopenia is not necessarily accompanied by a total loss of leukocytes in blood. Both peritoneal sepsis settings did not exhibit leukopenia at day ten post insult, while both SIRS-disease models induced a profound decrease in total leukocyte numbers (Fig. 9A). In the LPS and CpG-DNA groups leukopenia was primary caused by the loss of lymphocytes as the frequency of large cell populations (primary granulocytes in mice) did not change significantly (Fig. 9C). In peritoneal sepsis, the loss of lymphocytes in peripheral blood was numerical compensated by the simultaneous marked increase of large cell populations resulting in non-altered overall leukocyte numbers (Fig. 9C). These data clearly suggest that the infectious nature of the septic insult has differential effects on granulocytes counts in blood than a sterile TLR-mediated systemic inflammatory host response. Moreover, increased numbers of neutrophil or macrophage / monocytes populations were also observed in the spleen at post-acute stages of all four employed SIRS / sepsis models (data not shown). These results are supported by a recent study investigating the correlation between the number of immature granulocytes in septic *vs.* SIRS human patients in acute and post-acute stages [114]. In this study an infectious septic insult resulted in increased frequencies of immature granulocytes compared to SIRS patients without infections. One can speculate that this notion affects immune responses at late stages after sepsis on multiple levels. For example, phagocyte populations modulate T-cell immunity as they are involved in antigen presentation and T-cell activation. However, the contribution of these cells on T-cell functionally during septic insults is not well understood and thus represents an objective for future studies based on the intriguing findings discussed here. In first line, the identification and characterisation of this population would facilitate investigation of functional patterns of these cells associated with T-cell function.

In addition to altered leukocyte cellularity and composition, acute and protracted anaemia is a very common feature in patients with severe sepsis and septic shock [90, 92]. Consistent with the situation in human patients, the employed murine animal models induced a decrease in erythrocyte levels accompanied by lowered heamatocrit values in all tested disease models, although with inter-model differences (Fig. 8B). The protracted nature of anaemia indicates persistent damages in hemopoietic tissues or in the kidneys that stimulate erythropoiesis by secretion of erythropoietin under anaemic conditions.

Due to the post-acute time of investigation, profound disease-induced organ damage could not be detected in the LPS and PCI groups (Fig. 8A). However, together with the normal clinical appearance these findings underline the recovery of the animals from the acute insult. Interestingly, animals from the CpG group exhibited signs of organ and liver damage at day ten post insult, even though they did not exhibit symptoms of severe sickness. Acute signs of tissue damage argue against the post-acute nature of the CpG-setting, a finding that can be explained by the experimental setting (Fig. 7A). The CpG-DNA SIRS model involved the repeated administration of CpG-DNA 2, 4 and 6 days after the initial treatment leading to continuously high levels of CpG-DNA associated with a persistent systemic inflammation accompanied by prolonged organ / liver damage. Technically, animals from the CpG-SIRS group only recovered 4 days rather than ten days from the final CpG-DNA administration. Nevertheless, this protocol was chosen to unify the experimental SIRS and sepsis settings with day ten defined as ten days post initial treatment / surgery. In order to confirm the post-acute nature of the CpG-protocol, one could determine the levels of key cytokines, such as IL-1β, IL-6, IL-10 or IFNγ that are released during the acute stage and are present at low levels at later stages. However, a number of assays employed in the present study involved T-cell analyses well beyond day ten post insult at unarguably post-acute CpG-SIRS time points (Fig. 13 - 16).

5.2 The impact of systemic inflammation and sepsis on T-cell numbers at post-acute stages of the disease

5.2.1 Lymphopenia and T-cell loss at post-acute stages of systemic inflammation and sepsis

Lymphopenia, in particular T-cell apoptosis, is a hallmark of acute systemic inflammation and sepsis affecting adaptive immune responses at acute disease stages. Much less is known about T-cell counts at later stages of SIRS and sepsis. The data presented here clearly show that surviving animals at post-acute stages of SIRS and sepsis exhibit systemically decreased total numbers of lymphocytes (Fig. 9B) including T-cells (Fig. 9D). Since this finding occurred in all experimental groups, one can draw the conclusion that T-cell loss is a consequence of the septic host response and does not rely on the presence of intact pathogens. Moreover, the protracted loss of T-lymphocytes does not dependent on the nature/origin of systemic inflammation suggesting that profound activation of either

TLR4 or TLR9 in SIRS can ultimately lead to lymphocyte and T-cell apoptosis. Importantly, T-cell death is a secondary event caused by the excessive activation of other immune cells rather than direct effects of LPS and CpG-DNA on T-cells, since T-cells do not express TLR4 and TLR9 in biological significant quantities. This notion and the findings discussed here, strongly support the concept that the rigorous uncontrolled release of cytokines by activated innate immune cells ('cytokine storm') is the root of (protracted) T-cell loss. Interestingly, the degree of persistent lymphocyte and T-cell loss does not correlate with the morbidity and mortality of the disease models, a notion that is from great medical interest for patients with non-severe SIRS or sepsis, a cohort that is underrepresented in many clinical research studies.

Decreased T-cell numbers unarguably affects adaptive immune responses at post-acute stages of SIRS / sepsis and represents a factor that contributes to disease-related protracted immunosuppression. As a consequence of enduring systemic loss of T-cells, the pool of T-cells reactive to potential pathogen antigens is markedly reduced and thereby, the dynamics of adaptive T-cell responses are severely impaired. Moreover, the loss of naïve T-cells is accompanied by a decreased diversity of TCR variants, additionally reducing the spectrum of antigen-specific adaptive T-cell responses. The clinical significance of this finding is underlined by the fact that the degree of $CD4^+$ and $CD8^+$ T-cell apoptosis positively correlates with increased mortality in sepsis as shown in a retrospective analysis of human patients [115]. Of note, this correlation could not be observed in the present study since post-septic animals were kept under sterile, pathogen-free conditions. These findings suggest that the therapeutic blockade of T-cell apoptosis in episodes SIRS and sepsis would have beneficial effects far beyond acute disease stages and thus represents a very promising approach to prevent disease-related immune deficiencies. In line with that, pre-clinical studies have been successfully carried out to inhibit T-cell loss in sepsis using recombinant IL-7 or IL-15. In these studies prevention of T-cell loss was clearly associated with improved outcome [116]. However, induction of T-cell apoptosis occurs very early after disease onset providing only a small therapeutic window to effectively prevent T-cell loss. For example, IL-7 or IL-15 therapy was only effective when the compounds were administered within few hours after sepsis induction [116]. This notion questions the applicability in the daily clinical practice where the start of appropriate sepsis treatment is often delayed due to difficulties in diagnosis, delayed admission to ICUs and the priority of live-saving measures. However, in future improved and faster diagnosis and treatment

protocols may allow rapid targeted therapeutic interventions at early stages of SIRS and sepsis.

5.2.2 Differential susceptibility of $CD4^+$ and $CD8^+$ T-cells to SIRS- and sepsis-induced apoptosis

Since T-cell loss occurred in all tested experimental animals models, it is tempting to speculate that the molecular base of T-cell apoptosis is the same in all systemic inflammatory syndromes. On the other hand, differential loss of $CD4^+$ or $CD8^+$ T-cells in the employed animal disease models argues against one single trigger mechanism. While LPS-SIRS and CLP-sepsis preferentially induced profound persistent loss of $CD4^+$ helper T-cells, the CpG-DNA and PCI groups exhibited a more pronounced loss of $CD8^+$ cytotoxic T-cells resulting in increased, albeit modest, CD4/CD8 ratios (Fig. 9E). These controversial findings are also recapitulated in studies with human sepsis patients. Some studies found preferential loss of $CD4^+$ T-cells [25, 117] whereas other studies did not observe this effect [61, 115]. Data from the present study and from human cohorts, did not allow drawing a generally eligible conclusion about the susceptibility of certain T-cell subsets in sepsis. This notion underlines the heterogeneity of the disease and the importance to employ a broad spectrum of disease models in experimental studies. Moreover, the present data suggest distinct mechanisms of T-ell apoptosis dependent on the nature of the septic insult. TLR4-mediated systemic inflammatory host responses (LPS model) have a much different outcome in regard of CD4/CD8 ratios than TLR9-resticted immune responses triggered by CpG-DNA, a finding that provides some initial insights in differential apoptotic regulation during SIRS and sepsis. Based on the discussed observations, one can further conclude that persistent loss of T-cells accompanied by altered CD4/CD8 ratios are not dependent on morbidity and mortality of SIRS and sepsis as there was no correlation between CD4/CD8 ratios and clinical outcomes of the employed murine disease models.

However, the impact of altered CD4/CD8 T-cell ratios on overall T-cell immunity after SIRS and sepsis is unknown and was not addressed yet in human or animal studies. In classical models $CD4^+$ T-helper cells are predominantly involved in humoral and cellular responses against bacterial infections by activation of B-cells and macrophages, respectively. $CD8^+$ cytotoxic T-cells on the other hand, play essential roles in fighting viral infections and cancer cells by killing virus-infected cells or tumour cells. Under this light it

would be interesting whether the protracted increased fractional loss of $CD4^+$ T-cells in LPS-SIRS and CLP-sepsis ultimately leads to higher susceptibility to bacterial infections and *vice versa,* whether preferential loss of $CD8^+$ T-cells in CpG-SIRS and PCI-sepsis models is associated with elevated risk for viral infections and development of cancer. If this correlation corresponds well with the situation in human patients, measurement of $CD4^+/CD8^+$ T-cell ratios/counts could be prognostic markers for individual patients to assess their risk to develop particular infectious or non-infectious diseases. Consequently, this would help to prevent and treat diseases in septic patients thereby increasing the long-time survival after sepsis.

Importantly, T-cell counts and ratios can differ in different tissues in the same organism due to orchestrated adaptive immune responses at the sites of infection. With other words, $CD4^+/CD8^+$ T-cell ratios and counts in blood must not necessarily reflect the situation in particular tissues (e.g. lymph nodes) during infection and even more so during episodes of systemic inflammation and sepsis. Therefore, the investigation of the differential susceptibility of T-cell subtypes to sepsis-induced cell death and its consequences for overall T-cell immunity requires the determination of T-cell numbers in different tissue such as, blood, secondary lymph nodes or organs in proximity of the infection site.

5.3 The impact of systemic inflammation and sepsis on T-cell function at post-acute stages of the disease

5.3.1 T-cell function after systemic inflammation and sepsis is not impaired on a cellular base

During acute stages of systemic inflammation and sepsis T-cells have been shown to enter a stage of hyporesponsiveness characterised by a compromised proliferation capacity, altered/impaired cytokine expression as well as defective effector responses (see Fig. 6). Protracted T-cell anergy is further believed to contribute to sepsis-acquired immunosuppression beyond acute stages of the disease although there is lack of experimental data addressing this hypothesis. To investigate T-cell function at post-acute disease stages an experimental setting was employed that involved purification of splenic $CD4^+$ and $CD8^+$ T-cells ten days post insult, followed by *ex vivo* T-cell receptor stimulation under controlled experimental conditions. This approach facilitates to study potential intrinsic, cell autonomous alterations in T-cell function, as it excludes T-cell

extrinsic factors that modulate T-cell activity, e.g. antigen presentation or bystander cytokine signals. The data presented and discussed here, strongly argue against a state of T-cell hyporesponsiveness at post-acute stages of SIRS and sepsis. None of the employed assays provided evidence for impaired T-cell responses on *per cell* base. The scientific significance of the results is increased by the fact that cellular antigen-receptor-dependent function of naïve $CD4^+$ and $CD8^+$ was investigated on multiple levels including activation marker up-regulation, proliferation and intracellular signal transduction of various signalling molecules.

TCR complex and co-receptor stimulation of pure splenic T-cells from control and SIRS / sepsis animals using anti-CD3ε and/or anti-CD28 antibodies did not yield disturbed T-cell activation as judged by the expression profiles of the T-cell activation marker CD25 and CD69 (Fig. 10B and C), molecules that are commonly used to assess T-cell activation upon TCR or mitogen stimulation [88, 89]. The strength of the experimental design lies in the involvement of different TCR stimuli covering a wide range of productive and unproductive triggers allowing detection of even slight alterations in T-cell activation patterns potentially bypassed by strong stimuli.

Since CD25 and CD69 expression are dependent on proper Ca^{2+} release [88] and Ras/ERK signalling [118], respectively, one can further conclude that SIRS and sepsis do not induce persistent defects in crucial TCR/co-receptor signalling cascades. This interpretation was largely confirmed by detailed TCR signal transduction analyses covering multiple levels and branches of TCR and CD28 co-receptor signalling (Fig. 11 and 12). The activation of major signalling molecules involved in proximal TCR and CD28 co-receptor signal transduction, namely ZAP-70, LAT, ERK and AKT as well as TCR-induced Ca^{2+} release was indistinguishable from the control group in all SIRS / sepsis settings. In line with these findings, TCR-triggered IL-2 synthesis integrating TCR and CD28 co-receptor signalling cascades, was not impaired as published data from colleagues of the author show [119].

According to the finding of non-defective T-cell activation and TCR signalling, SIRS and sepsis did not induce protracted defects in TCR/co-receptor-mediated T-cell proliferation (Fig. 10D). This finding is from great significance, since proper T-cell expansion is a prerequisite to generate a large pool of effector T-cells eliciting adaptive immunity against invading pathogens (Fig. 5). One can further conclude that SIRS and sepsis did not affect fundamental aspects of T-cell responses since T-cell proliferation requires the integration of multiple signalling events, such as TCR and co-stimulatory signals, cytokine production (e.g. IL-2) as well as autocrine cytokine stimulation/signalling. It is important to note that

TCR signalling analyses did not provide molecular hints for a 'primed' T-cell state after SIRS and sepsis that was suggested by strong trends of more pronounced activation marker expression and proliferation upon non-immobilised anti-CD3ε/CD28 antibody stimulation. From a clinical point of view these result are highly interesting as they clearly show that Systemic Inflammatory Response Syndromes, including sepsis, do not induce protracted inherent functional defects in T-cells. This notion questions whether cellular T-cell dysfunction contributes to SIRS- or sepsis-related protracted immunosuppression. As a consequence, immune-stimulatory therapies targeting T-cell function is no appropriate approach to treat patients with disease-acquired immune suppressive states in the long run. Under this light, clinical sepsis research should focus on other aspects of adaptive immunity, e.g. T-cell apoptosis (see above) or B-cell function.

5.3.2 Limitations of e*x vivo* T-cell stimulation assays

Ex vivo stimulation of pure $CD4^+$ and $CD8^+$ T-cells using monoclonal anti-CD3ε and/or anti-CD28 antibodies are robust assays to study principle aspects of TCR-mediated T-cell function on cellular and biochemical levels. However, this experimental setting has certain drawbacks and limitations that must be considered in order to draw reasonable conclusion from these experiments. TCR/CD3 complex stimulation via anti-CD3ε antibodies is very artificial as its clusters CD3 molecules to large complexes thereby inducing strong proximal TCR signalling. This mode of activation does not involve MHC/antigen-peptide binding by the αβ-TCR-chains with simultaneous stimulation of CD4 or CD8 co-receptors. Similar to CD3 stimulation, strong CD28 co-receptor clustering is artificially induced by monoclonal anti-CD28 antibodies rather than physiological ligand/receptor interaction during APC-mediated T-cell (co)-activation. Moreover, under *in vivo* conditions APCs and other cells provide bystander cytokine signals (e.g. IL-4 or IL-13) that modulate T-cell activation, differentiation and effector responses. This notion can not be recapitulated in *ex vivo* experiments with pure T-cells. Most importantly, anti-CD3ε/28 stimulation potentially overcomes sepsis-induced TCR hyporesponsiveness states as documented for other disease backgrounds [120-122]. Taking these considerations in account, secondary infections models were additionally employed in the present study to confirm the previously described results and to extend the T-cell analyses by multiple levels.

5.3.3 Secondary infection models

Infection models are frequently used to study principle mechanisms of innate or adaptive immunity. In particular, they are appropriate approaches to investigate antigen-specific T-cell responses under physiological relevant conditions as they involve all fundamental steps of adaptive T-cell immunity occurring *in vivo* (Fig. 5). First, infection with viable viruses or bacteria induces an activation of phagocytes engulfing pathogen particles followed by peptide processing and MHC-mediated presentation to $CD4^+$ and $CD8^+$ T-cells. Second, T-cell activation requires recognition and binding to the respective antigen with simultaneous interaction with the APC that provides physiological co-stimulatory signals. Subsequently, clonal expansion generates a pool of pathogen-specific T-cells that elicit effector responses upon antigen (re)encounter. Moreover, secondary infections, in particular nosocomial infection, are from great clinical significance in immune-compromised human sepsis patients after the acute insult as they contribute to post-acute morbidity and mortality. Secondary infection models facilitate to mimic infectious diseases and therefore present suitable approaches for the present study.

The Armstrong strain of *Lymphocytic choriomeningitis virus* (LCMV-Arm) was used as a secondary infection model to study antigen-specific $CD4^+$ and $CD8^+$ T-cell responses at post-acute stages of SIRS (LPS, CpG) and sepsis (CLP) (Fig. 13). LCMV-Arm infection induces a non-virulent systemic viral disease characterised by a profound generation of LCMV-specific effector $CD4^+$ and $CD8^+$ T-cells that mediate virus clearance [123, 124]. *Ex vivo* stimulation of whole splenocyte homogenates with titrating levels of virus peptides was used to assess antigen-specific T-cell responses in a dose-dependent manner. The advantage of this approach over polyclonal anti-CD3ε/CD28 stimulation lies in the physiological fashion of TCR and co-receptor activation not bypassing any potential defects in the TCR machinery. T-cell stimulation with LCMV-peptides requires peptide presentation by APCs in complexes with MHC-II (GP61) or MHC-I (GP33) as well as proper co-stimulatory signals provided by the same cells.

For *in vivo* T-cell activation assays (Fig. 16) a novel approach was developed that facilitated studying T-cell activation patterns *in vivo* with simultaneous discrimination between potential T-cell intrinsic *vs.* extrinsic alterations at late stages of SIRS and sepsis in one single mouse (Fig. 16A). GP33 peptide-specific P14 T-cell populations were adoptively transferred prior SIRS / sepsis onset (‘pre-SIRS’) and ten days post insult (‘post-SIRS’). After modest accumulation of both P14 populations with non-virulent infection with the attenuated strain of *Listeria monocytogenes* expressing the GP33

antigen, T-cells were challenged *in vivo* by injection of GP33 followed by flow cytometric analyses.

5.3.4 T-cell function after secondary infections in background of systemic inflammation and sepsis

In vivo and *ex vivo* T-cell studies using secondary infection models largely confirm the findings of non-disturbed T-cell function on the level of individual T-cells at post-acute stages of SIRS and sepsis. Upon antigen stimulation of $CD4^+$ and $CD8^+$ effector T-cells the total amount of synthesised IFNγ and TNFα was not decreased on *per cell* base in activated cells (Fig. 14B, 15B, C and 16E, F). Pathogen-specific effector T-cells arise in infectious diseases and are the key players fighting the infectious trigger. The present data extend the previously discussed findings from polyclonal naïve T-cells to the population of antigen-specific effector T-cell clones, thereby increasing the biological relevance of these findings. Importantly, *per cell* production of IFNγ and TNFα in activated $CD4^+$ and $CD8^+$ T-cells is not only unaffected at peptide saturation during *ex vivo* stimulation but also at intermediate and low antigen doses (data not shown). These data further show that the cellular function of $CD4^+$ and $CD8^+$ T-cells at post-acute disease stages is not associated with the differential susceptibility of these T-cell subsets to SIRS- or sepsis-induced apoptosis. In this context it is worth to mention that T-cell hyporesponsiveness in acute SIRS / sepsis affects both naïve $CD4^+$ and $CD8^+$ T-cell populations [61] regardless their vulnerability to disease-induced apoptosis.

Both secondary infection models were further employed to assess the long-time impact of SIRS and sepsis on environmental factors that modulate T-cell immunity. However, the data documented in Fig. 14 – 16 are not fully conclusive. In the LCMV-experiments T-cells from the CpG and CLP groups exhibited attenuated fractional induction of IFNγ upon *ex vivo* stimulation with titrating doses of antigen. Since T-cells are not impaired on *per cell* base, one can conclude that T-cell extrinsic factors (e.g. antigen presentation) mediate disturbed T-cell dose responses. Interestingly, $CD4^+$ and $CD8^+$ T-cells from animals from the LPS-SIRS group did not show persistent impaired antigen-dose responses in these experiments. This notion suggests that outcomes in the present assays can not be simply generalised for all SIRS insults, once more underlining the heterogeneous nature of these syndromes.

However, the finding of decreased antigen sensitivity could not be recapitulated for effector $CD8^+$ P14 T-cells in the *in vivo* T-cell assays (Fig. 16 E and F). Both 'pre-SIRS' and 'post-SIRS' P14 T-cell populations from all experimental groups did not exhibit defects in cytokine production as judged by non-disturbed IFNγ and TNFα production. This finding strongly argues against substantial protracted disease-induced environmental alterations leading to decreased $CD8^+$ T-cell responses. Environmental defects involved in T-cell activation would have resulted in impaired T-cell cytokine responses in 'post-SIRS' P14 T-cells that were transferred late after the septic insult. In line with all other data discussed here, *in vivo* T-cell activation assays do also not provide evidence for functional disturbed cellular T-cell effector responses after SIRS and sepsis as judged by non-altered IFNγ and TNFα production in the 'pre-SIRS' T-cell populations.

Importantly, *in vivo* administration of 5µg LCMV-GP33 induced a very profound IFNγ response, potentially close to saturation and thus potentially overcomes functional defects. Using ten-fold lower GP33-peptide doses in the same experimental setting induced a much weaker P14 T-cell response (30 – 40% IFNγ(+)) but also did not reveal impaired IFNγ or TNFα responses (data not shown).

Intriguingly, the CpG-SIRS group showed an attenuated CD25 and CD69 up-regulation in both P14 T-cell populations indicating impaired T-cell activation mediated by T-cell extrinsic factors (Fig. 16C – D). But experimental T-cell activation marker expression does not inevitably correlate with effector function, e.g. proliferation [125] and thus, the biological significance might not be crucial. It is important to note that impaired CD25 and CD69 induction does not result from T-cell inherent disturbances since both 'pre-SIRS' and 'post-SIRS' show indistinguishable CD25 and CD69 expression patterns upon antigen challenge. Defects that were solely caused by T-cell intrinsic defects would have been only be apparent in P14 T-cell transferred prior SIRS / sepsis onset.

The controversial results from both 'two-hit' infection models could probably be explained by the rather weak protracted impact of SIRS and sepsis on T-cell extrinsic factors as judged by an only modest increase (~ two-fold) of EC_{50} values for *ex vivo* antigen-triggered IFNγ production in $CD8^+$ T-cells that was not even evident in both SIRS settings (Fig. 15A). The biological relevance is at least questionable as attenuated effector function of $CD8^+$ T-cells was not recapitulated in the *in vivo* assays that represent the superior experiments to assess extrinsic alterations potentially affecting T-cell immunity *in vivo*. However, it would have been interesting to perform the same experimental *in vivo*

approach using TCR-transgenic $CD4^+$ T-cells specific for LCMV-GP61, since the long-time impact of SIRS and sepsis on antigen-dose responses was more pronounced in $CD4^+$ effector T-cells exhibiting three- to four-fold higher EC_{50}-values for IFNγ production but the respective transgenic mice were not available.

5.3.5 Secondary infections models - limitations

Secondary infection models are useful approaches to study clonal T-cell responses as they bear similarity to infectious diseases occurring under *in vivo* conditions. However, they feature some limitations and drawbacks that must be considered when drawing conclusions from these experiments. Both 'two-hit' secondary infection settings induced the antigen-dependent activation and expansion of naïve T-cell clones at post-acute stages of SIRS and sepsis. It is necessary to ask whether inherent disease-induced T-cell defects are still present after the potentially affected T-cell clones have underwent multiple activation and division cycles during clonal expansion. It is indisputable that the investigated T-cell pool is not same population that was affected by the SIRS or sepsis insult. However, epigenetic alterations in T-cells after sepsis, that could be transferred to daughter cells, have been described (see 1.4.3 and [83]).

Moreover, it could be possible that an experimental secondary infection potentially creates an inflammatory environment that overcomes T-cell inherent defects. To take this consideration in account, low-dose non-virulent bacterial and viral infectious triggers were employed in the discussed experiments, albeit the profound T-cell accumulation indicates a significant inflammatory response. However, *ex vivo* and *in vivo* antigen challenge of $CD4^+$ and $CD8^+$ T-cells was performed when viral or bacteria infections were completely eliminated suggesting that no bystander T-cell activation (e.g. pro-inflammatory cytokines) influenced antigen-dependent T-cell responses. This notion is supported by the fact that non-peptide-challenged effector T-cells in both *ex vivo* and *in vivo* assays did not exhibit signs of activation, e.g. activation marker or cytokine expression.

5.3.6 Impaired antigen presentation to T-cells after systemic inflammation and sepsis?

While all discussed data clearly indicate that SIRS and sepsis do not induce protracted cellular T-cell defects, one cannot fully exclude T-cell extrinsic alterations that potentially impair T-cell responses at post-acute stages of SIRS and sepsis. Proper MHC-I- or II-

restricted antigen presentation is one of the most essential prerequisite for *in vivo* T-cell stimulation by antigen-presenting cells. MHC-I is expressed on virtually all nucleated cells and activates $CD8^+$ T-cells, whereas MHC-II, involved in $CD4^+$ T-cell activation, is found on 'professional' antigen-presenting cells, such as dendritic cells, monocytes / macrophages or B-cells.

In literature, there are multiple human and rodent sepsis studies describing defective antigen presentation due to decreased levels of MHC. In episodes of human sepsis decreased expression of HLA-DR (human homolog of MHC-II) has been found in monocytes [126-128], dendritic cells [128] and B-cells [129]. Importantly, HLA-DR levels on monocytes and dendritic cells remain low at post-acute stages of sepsis unarguably affecting adaptive immune responses at late disease stages [128]. Moreover, reduced MHC-II expression on APCs is recapitulated in the murine CLP sepsis model [130], also shown for sublethal CLP-surgery similar to the CLP model employed in the present study [131]. These findings support the concept of protracted sepsis-induced defects in antigen presentation that impact $CD4^+$ T-cell immunity, although no study was performed directly linking defective antigen presentation with T-cell activation. This notion underlines the importance to recapitulate the employed *in vivo* T-cell activation assay with transgenic $CD4^+$ T-cells. For MHC-I expression, no detailed studies have been carried out, so the impact of sepsis on antigen presentation to $CD8^+$ T-cells remains unclear.

Investigation of antigen presentation linked with T-cell activation is from great interest and essential for understanding T-cell immunity at post-acute stages of SIRS / sepsis in its entire complexity. For example, the discussed *in vivo* assay (Fig. 16) could be extended by additional assessment of MHC-I expression in splenic cells and its correlation with antigen-specific T-cell activation. Another conceivable experiment could be the modification of the *ex vivo* dose response experiments (Fig. 13) by adding APCs from 'post-septic' animals to purified LCMV-specific T-cells from healthy mice followed by peptide stimulation. If T-cells exhibit impaired antigen-dose responses this would strongly indicate protracted disease-induced functional defects of APCs. The use of genetically modified mice bearing T-cells almost exclusively specific for one particular antigen would also be a very useful experimental approach. One could perform direct *ex vivo* or *in vivo* clonal antigen T-cell stimulation without previous infection-induced accumulation of T-cell clones. For example OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/Crl) or OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/Crl) mice, with T-cells specific for chicken ovalbumin peptides

(Ova) are commonly employed mouse models in research studies investigating antigen-specific T-cell responses.

5.4 Summary and outlook

5.4.1 Systemic inflammation and sepsis do not induce enduring defects in T-cell function

The present study represents an in-depth analysis of multiple aspects of T-cell immunity at post-acute disease stages using various clinical relevant rodent models of SIRS and polymicrobial sepsis. All data unarguably show that Systemic Inflammatory Response Syndromes, including sepsis do not induce lasting functional defects on the level of individual T-cells. Cell biological and biochemical approaches could not reveal any disturbances in TCR-mediated T-cell activation, proliferation, cytokine production as well as TCR/co-receptor signalling. Accordingly, immunological-orientated studies using secondary infection models additionally indicate that antigen responses of effector $CD4^+$ and $CD8^+$ T-cells are not impaired on *per cell* base at late stages of SIRS and sepsis. The strength of the present study lies in the employment of four different murine models of SIRS and sepsis with various clinical features meeting the large heterogeneity of the syndromes. All models exhibited virtually the same outcome in respect of T-cell function enabling to adapt the main conclusions of the thesis for all episodes of systemic inflammation and bacterial sepsis. On the other hand, the present thesis could find strong evidence for persistently defective T-cell immunity due to an enduring systemic loss of T-cells. Protracted lymphopenia occurred in both sterile SIRS models as well as in both polybacterial sepsis models suggesting that the inflammatory host response is the cause of this phenomenon and does not rely on the presence of viable pathogens.

In conclusion from all discussed data one can propose following model for cellular adaptive T-cell immunity at post-acute stages of SIRS and sepsis (Fig. 17). Antigen-specific T-cell responses are not disturbed on a cellular level and thus T-cells are capable to mount immunological effector functions in response to infections. Cellular malfunction of T-cells does not contribute to protracted immune suppressive states in patients after SIRS and sepsis. However, overall T-cell immunity at post-acute disease stages is compromised due to the persistent loss of naïve T-cells reducing the pool of T-cell clones reactive against invading pathogens. Moreover, T-cell extrinsic factors might play a role in

defective T-cell immunity after SIRS / sepsis, although the experimental data discussed here are limited and not fully conclusive. This model shifts the focus from T-cell immune-stimulatory therapies in sepsis to other aspects of adaptive T-cell immunity. In first line, the present study suggests that prevention of early T-cell loss is sufficient to maintain proper T-cell immunity at post-acute stages of systemic inflammation and sepsis.

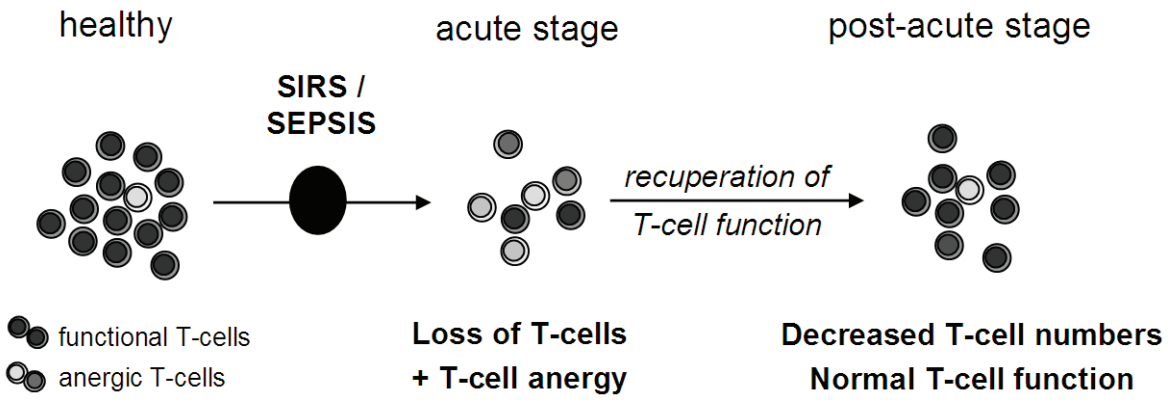


Figure 17. T-cell immunity after SIRS and sepsis
Model of T-cell immunity after systemic inflammation and sepsis. An acute episode of SIRS and sepsis results in a profound systemic loss of T-cells and induces a state of T-cell hyporesponsiveness that contributes to acute disease-induced immunosuppression associated with early mortality. At later stages of SIRS and sepsis T-cells do not feature cellular functional defects but total T-cell numbers are persistently low for a protracted period of time. T-cells at post-acute disease stages exhibit normal TCR-induced T-cell activation (e.g. activation marker up-regulation, TCR signalling) and effector responses (e.g. proliferation, cytokine production, differentiation).

5.4.2 Clinical Relevance of murine data for human patients

When adopting this model and the data from this thesis to human patient cohorts, one must consider that some immunological aspects are different in mice and humans. For example, the composition of the leukocyte compartments is significantly different in mice, exhibiting much higher frequencies of T- and B-lymphocytes in blood while in humans granulocytes represent the main population blood [132]. Furthermore, differences of T-cell biology in both species have been described for development, antigen-dependent activation and signalling as well as for differentiation into effector cells [132]. However, functional inter-species differences are more relevant when studying certain T-cell subtypes or specific molecules and processes involved in T-cell immunity. The study here investigated very fundamental aspects of T-cell functionality that share great similarity in both mice and humans.

Experimental approaches and preliminary data from the present study were the fundament for a human pilot study conducted in the research group of the author. The aim of this study was to investigate the cellular function of peripheral T-cells in a cohort of critically ill human patients at post-acute stages of severe sepsis. The data from human patients largely confirm the key finding of non-disturbed cellular T-cell functions at late stages of sepsis discussed in this thesis (manuscript in preparation).

5.4.3 Other aspects of T-cell immunity at post-acute stages of systemic inflammation and sepsis

The present study primary focused on adaptive, TCR-dependent facets of T-cell function in background of SIRS and sepsis. Antigen-specific T-cell responses are unarguably the most important aspect of T-cell immunity. However, the capacity of $CD4^+$ and $CD8^+$ T-cells to respond to other immune mediators (e.g. cytokines) is from major interest and plays a significant role in T-cell differentiation and effector function. Although no particular experiments have been discussed in the present study, there is no single evidence for defective cytokine responses at post-acute stages of SIRS and sepsis. For example, non-disturbed proximal TCR/co-receptor signalling and T-cell proliferation indicates proper IL-2 production associated with adequate IL-2 autocrine signalling. In line with that, preliminary experiments using the experimental setup depicted in Fig. 13 indicate that effector T-cells are not compromised in their capacity to respond to *ex vivo* IL-7/IL-15 stimulation at post-acute stages of SIRS and sepsis (data not shown). However, more detailed studies are necessary to investigate this important subject. In particular, the investigation of cytokine receptor signalling (e.g. IL-2, IL-4 or IL-7) and expression/activation of downstream mediators and genes is essential to understand whether SIRS and sepsis impact bystander T-cell activation.

In Germany and many other countries, large-scale vaccination programs protect the recipient against numerous potentially hazardous infectious diseases upon generation of pathogen-specific memory T- and B-cells. Memory $CD4^+$ and $CD8^+$ T-cells represent major cellular components of anti-microbial and –viral immunity as they rapidly recognise and eliminate invading pathogens. Although impaired function of $CD8^+$ T-cell memory has been described for murine sepsis [64], detailed functional analyses have not been carried out yet. In particular, the long-time consequences of SIRS and sepsis on memory T-cell function are poorly understood. The present study can not provide insights into memory T-

cell immunity since all studies were performed with naïve or effector $CD4^+$ and $CD8^+$ T-cells. But one can easily adapt the experimental approaches from this work to study functional aspects of memory T-cell immunity. Immunisation of mice with *Lymphocytic choriomeningitis virus* or *Listeria monocytogenes* generates a profound pool of long-living pathogen-specific memory $CD4^+$ and $CD8^+$ T-cells after the primary effector response has been terminated. Memory T-cells can be identified, and thus purified, by the expression of specific surface marker molecules, e.g. high expression of CD11a for memory $CD8^+$ T-cells. Upon *ex vivo* polyclonal TCR stimulation their functional capacity could be investigated analogous to the experiments depicted in Fig. 10 – 13. *In vivo* T-cell assays (Fig. 16) could also be extended to memory T-cells, just by adoptive transfer of memory P14 T-cells. Evaluation of functional patterns of memory T-cells would provide highly interesting insights in the field of protracted immunosuppression after SIRS and sepsis.

Furthermore, all data from literature point towards impaired cellular T-cell function in acute stages of sepsis, while the data discussed here strongly argue against functional disturbances at later stages. This notion suggests that T-cells can sufficiently recover from acute hyporesponsiveness. This interpretation is from great medical interest, since the critical factors that mediate recuperation of cellular T-cell function might be promising targets for immune-stimulatory therapies in acute episodes of sepsis. Modern techniques of micro-array-based transcriptome analyses would allow screening for differential expression of genes that could mediate T-cell recuperation in tissue samples obtained from the same mouse or patient over the course of several days / weeks. In this manner conceivable molecules could be inhibitory T-cell receptors, such as PD-1, CTLA-4 or BTLA. Currently, only one rodent study is available that describes the gradual reversion of immunosuppression after CLP-induced sepsis, possibly linked with regaining T-cell functionality [86].

Peripheral homeostatic T-cell proliferation is another process that could play a decisive role in the recovery of the T-cell immunity. Under lymphopenic conditions the remaining naïve T-cells replenish T-cell numbers by profound cell division regulated by self-ligand/MHC interaction and various γ_c cytokines, such as IL-7 and IL-15 [133]. However, the impact of SIRS and sepsis on peripheral homeostatic T-cell proliferation is not understood in detail yet and but is from great clinical interest since thymic T-cell output does not play a significant role in adult patients and T-cell homeostasis largely depends on peripheral T-cell proliferation.

5.4.4 Concluding remarks

The results from the present work are an important contribution in the field of clinical sepsis research as they conclusively show that systemic inflammation and sepsis do not lead do enduring cellular defects in T-cell function. This rather unexpected finding indicates a non-decisive role of adaptive T-cell function to the protracted state of immunosuppression after SIRS and sepsis. Under this light, future research studies will focus on other aspects of adaptive immunity, with some of them discussed in the present thesis. Understanding of precise mechanisms of sepsis-induced immunosuppression will finally lead to better therapeutic treatment of immune suppressive patients and hopefully translate into improved long-time outcome with increased health-related quality of life.

VII References

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VIII Danksagung

An dieser Stelle möchte ich mich bei allen Menschen bedanken, die mich bei der Anfertigung dieser Dissertation unterstützt haben.

Insbesondere möchte ich mich bei PD Dr. Ignacio Rubio bedanken, der mir die Möglichkeit gegeben hat in seiner Forschungsgruppe das vorgestellte Promotionsprojekt durchzuführen und immer für anregende Diskussionen offen war. Weiterhin möchte ich mich bei allen aktuellen sowie ehemaligen Mitgliedern der Arbeitsgruppe Anergy für Ihre Unterstützung danken.

Bedanken möchte ich mich bei Prof. Dr. Berit Jungnickel und PD Dr. Lucas Simeoni, die sich als Gutachter für diese Dissertation zur Verfügung gestellt haben.
Des Weiteren bedanke ich mich bei Dr. Katharina Ferrari-Kühne und Dr. Martin Förster als Mitglieder meines jährlichen Thesis-Komitees.

Ein ganz besonderer Dank gilt Assistant Professor Vladimir Badovinac von der University of Iowa, USA, in dessen Gruppe ich einen sehr lehrreichen und fruchtbaren Forschungsaufenthalt hatte. Besonders bedanken möchte ich auch bei den Mitgliedern seiner Arbeitsgruppe, namentlich Stephanie A. Condotta, Shanyia Khan, Deepa Rai, und Matthew D. Martin, die mich mit offenen Armen Empfangen haben und jederzeit unterstützt haben. Die Arbeit und Gespräche mit Assistant Professor Vladimir Badovinac und seinem Team haben mich sehr geprägt.

Für viele wissenschaftliche und nicht-wissenschaftliche Gespräche gilt auch ein Dank für die Kollegen am Universitätsklinikum Jena. Hier möchte ich besonders bei Kerstin Gallert, Ulrich Schröder, Marcus Böhme und Govind Pai nennen.

Abschließend bedanke ich mich sehr bei meiner Familie und Freunden. Ein besonderer Dank gilt meiner Freundin Cora und meinem Bruder David.

IX Curriculum Vitae

Personal Information

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Study

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Course	Biochemistry / Molecular Biology
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PhD project

since 10/2011	*Center for Sepsis Control and Care (CSCC), Jena University Hospital,* Jena, Germany
Research Group	ANERGY (project leader PD Dr. Ignacio Rubio)
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Scientific and academic work

01/2009 – 10/2009	*Max-Planck Institute for Chemical Ecology Jena* student research assistant – molecular laboratory work
04/2010 – 06/2010	*Jena University Hospital* student tutor in practical course "Biochemistry II" – experiment preparation and supervision of students
06/2010 – 07/2010	*Jena University Hospital, Institute for Biochemistry II* student research assistant – biochemical work
02/2011 – 03/2011	*Biological Pharmaceutical Faculty University Jena* student tutor in practical course “Methods in Molecular Biology" - experiment preparation and supervision of students
05/2011 – 06/2011	*Jena University Hospital* student tutor in practical course "Biochemistry II"
since 10/2011	*Center for Sepsis Control and Care (CSCC)* *University Hospital Jena* research fellow, PhD project

International research visits

01/2014 – 04/2014	Research stay at the *University of Iowa, Carver College of Medicine, Department of Pathology, Vladimir Badovinac Laboratory,* Iowa City, United States of America
09/2014	Summer School “Cellular Stress Response” *Sino-German Center*, Beijing, China

Active participations on scientific conferences

07/2013	*16th Meeting on T-cell Subsets and Function*, Marburg - oral presentation
09/2013	*Weimar Sepsis Update Consensus & Controversies*, Weimar - poster presentation
11/2013	*17th Joint Meeting Signal Transduction, Receptors, Mediators and Genes*, Weimar - oral presentation

Robby Markwart
Jena, 12.03.2015

X List of Publications

Markwart R., S.A. Condotta, R.P. Requardt, F. Borken, K. Schubert, C. Weigel, M. Bauer, T. S. Griffith, M. Förster, F.M. Brunkhorst, V.P. Badovinac and I. Rubio 2014 *PLoS One. 2014 Dec 26;9(12):e115094*
Immunosuprression after sepsis: systemic inflammation and sepsis induce a loss of naïve T-cells but no enduring cell-autonomous defects in T-cell function

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Ras palmitoylation is necessary for N-Ras activation and signal propagation in growth factor signaling

Geißler, K.J., M.J. Jung, L.B. Riecken, T. Sperka, Y. Cuia, S. Schacke, U. Merkel, **R. Markwart**, I. Rubio, M.E. Than, C. Than-Breithaupt, S. Peuker, R. Seifert, U.B. Kaupp, P. Herrlich and H. Morrison. 2013 *Proc Natl Acad Sci U S A. 2013 Dec 17;110(51):*20587-92*.*
Regulation of Son of sevenless by the membrane-actin linker protein ezrin

XI Ehrenwörtliche Erklärungen

Hiermit erkläre ich, ehrenwörtlich, dass

1. mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist.
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3. ich alle Personen, die mich bei der Durchführung, Auswertung und Auswahl der Experimente und Daten sowie bei der Herstellung des Manuskripts unterstützt haben an den entsprechenden Stellen angegeben habe.
4. ich nicht die Hilfe eines Promotionsberaters in Anspruch genommen habe und keine Dritte weder unmittelbar noch mittelbar geldwerte Leistung von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.
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6. ich die vorgelegte Dissertation bzw. eine in wesentlichen Teilen ähnliche Arbeit oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Robby Markwart

Jena, 12.03.2015