Immunomodulation by Endotoxin Tolerance in Murine Models of Inflammation and Bacterial Infection

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Dedicated to Cristina and my family

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Abbreviations

ALT	alanine aminotransferase
AP-1	activation protein-1
CFU	colony forming units
Cl_2MBP	dichloromethylene bisphosphonate
CLP	cecal ligation and puncture
ELISA	enzyme-linked immunosorbent assay
GalN	D-galactosamine
IFN	interferon
I-kB	inhibitor of NF−κB
IL	interleukin
i.p.	intraperitoneal
IL-1R	interleukin-1 receptor
IRAK	interleukin-1 receptor-associated kinase
i.v.	intravenous
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAP	mitogen-activated protein
MPL	monophosphoryl lipid A
mu	murine
MyD88	myeloid differentiation protein
NF−κB	nuclear factor kappa B
NIK	NF−κB-inducing kinase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PMN	polymorphonuclear cells
r	recombinant
RES	reticuloendothelial system
S. aureus	*Staphylococcus aureus*
S. typhimurium	*Salmonella typhimurium*
TBS	Tris buffered saline
TLR	Toll like receptor
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor receptor
TRAF6	TNF receptor-activated factor 6
vs.	versus

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1 Introduction

1.1 Endotoxin tolerance

Endotoxin or lipopolysaccharide, a glycolipid of the cell membrane of Gram-negative bacteria, is one of the most potent known stimulators of immune responses. The immune system responds to LPS with a systemic production of proinflammatory cytokines which recruit and activate immune cells to eliminate invading pathogens (1, 2). Although these cytokines are indispensible for the efficient control of growth and dissemination of the pathogen (2-4), an overshooting inflammatory response is potentially autodestructive and may lead to microcirculatory dysfunction causing tissue damage, shock and eventually death (3, 4). Injection of high dose LPS induces pathological symptoms resembling those of the septic patient (5).

The term “endotoxin tolerance” describes the phenomenon that immune responses and metabolic changes such as fever, inflammation or weight loss as well as lethality in response to LPS challenge are mitigated after repeated LPS administration. Prophylactic subtoxic LPS administration confers protection against inflammatory damage in a number of animal models. Intensive studies attempting to unravel the underlying mechanisms have been conducted over several decades to find a more effective prophylaxis and therapy of Gram-negative infection. A detailed review on two different and apparently contrasting aspects of endotoxin tolerance, i.e. attenuation of inflammatory damage on the one hand and the concomitant modulation of anti-microbial host defense on the other hand is given in chapter 2.

1.2 Lipoteichoic acid

Lipoteichoic acids are amphiphilic polymers of the cell wall membrane complex of many Gram-positive bacteria. Their structure consists of a hydrophilic backbone made of a linear 1,3-linked poly(glycerophosphate) chain of 16-40 glycerophosphate residues on average. The chain is phosphodiester-linked to a glycolipid which anchors the molecule in the cytoplasmic membrane. Since glycolipids vary in structure between different bacterial species, the lipid anchors of LTA vary accordingly. The glycerophosphate residues of most lipoteichoic acids are in part substituted with D-alanine ester and in certain bacteria also contain glycosyl substituents (6-8). Biological activity seems to depend on the amount of D-alanine substituents as demonstrated recently by Morath et al. for LTA from *Staphylococcus aureus* (9). In the last decade, increasing evidence has been provided for a major contribution of LTA

to immunostimulation by Gram-positive bacteria. Several groups reported cytokine and NO production by human monocytes stimulated with LTA preparations from various Gram-positive bacteria (10, 11). The spectrum and kinetics of cytokine release were similar in response to LTA and LPS (10). Simultaneous administration of LTA and peptidoglycan from *S. aureus* could substitute for whole bacteria for induction of nitric oxide, cytokines and lethal shock in rats (12, 13). Recent findings indicated that recognition of LTA was mediated by CD14, a receptor expressed on the surface of monocytes/macrophages that is also involved in LPS recognition (14, 15). However, data are controversial concerning the involvement of different Toll-like receptos (TLR) in LTA-induced signaling. Thus, using transfected cell lines, Schwandner et al. demonstrated signaling in response to LTA via TLR2 (16). In contrast, macrophages from TLR2 deficient mice displayed normal cytokine production upon stimulation with LTA from *S. aureus*, whereas responsiveness to peptidoglycan or whole bacteria was greatly impaired (17). This controversy appears to be the result of different degrees of purity and endotoxin contamination of the LTA employed.

1.3 Infection model

Infection of mice with *Salmonella typhimurium* causes a systemic infection resembling human typhoid fever. Although the natural route of infection is via the uptake of contaminated food or water, i.p. or i.v. injection of salmonellae is widely used to study directly the systemic immune defense. Sublethal infection can be divided into four different phases, which differ regarding the participation of different immune cell populations. During phase I, which lasts approximately one hour postinfection, up to 90% of the inoculated salmonellae are taken up and destroyed by resident phagocytes. In the second phase of infection, salmonellae enter the circulation via lymphatic vessels and colonize liver and spleen, where they start to replicate. *S. typhimurium* is considered a facultative intracellular pathogen but direct evidence for a replication of salmonellae within phagocytes is lacking and the issue is still under discussion (18, 19). Invasion of liver and spleen by salmonellae is paralleled by a massive influx of PMN which eliminate extracellular bacteria (20). Additionally, PMN-mediated lysis of infected hepatocytes was suggested to restrict bacterial growth by releasing salmonellae from the “safe haven” (21). In line, neutrophil depletion prior to injection of bacteria dramatically increased the susceptiblity to *S. typhimurium* infection (21, 22). Besides neutrophils, resident macrophages of the RES contribute to eliminate blood-borne bacteria. This was demonstrated by depletion of macrophages with silica which rendered mice extremely susceptible to

Salmonella typhimurium infection (23). The efficiency of the early inactivation depends on the genetic background of the mouse strain. Macrophages from mice carrying the Ity^s (immunity to typhimurium^{sensitive}) allele are much less efficient in killing *S. typhimurium* than cells from Ity^r (immunity to typhimurium^{resistant}) mice (24). The importance of this factor is reflected by the enormous differences in LD_{50} values between Ity^r and Ity^s strains, due to a faster net growth rate of the salmonellae in the RES of Ity^s mice (25). This gene also controls resistance to *Leishmania donovani*, *Mycobacterium tuberculosis BCG* and *Mycobacterium lepraemurium* and has been designated Ity, Lsh, Bcg and Inr (26). Although professional phagocytes are crucial in the control of salmonellosis, they are insufficient by themselves for initial control of infection, as exponential salmonellae growth is found in the RES. In sublethally infected mice, bacterial growth is suppressed several days after infection and a plateau phase is reached where bacterial replication and inactivation are in balance. This early suppression of exponential bacterial growth must occur for the host to survive. The mechanism is not fully understood yet, though it has been demonstrated that T lymphocytes were not required (27). Experiments using irradiated mice reconstituted with normal or T cell-depleted bone marrow showed that phase 3 requires the presence of radiation sensitive, non-T bone marrow cells, whereas transfer of normal spleen cells will not restore the plateau in irradiated mice (28). Establishment of phase 3 requires production of TNF and IFNγ, as demonstrated by administration of neutralizing antibodies. When either of these cytokines was neutralized, bacteria continued to grow uncontrolled (29-34). The cells responsible for cytokine production have not been pinpointed in the salmonella model. However, results from *Listeria monocytogenes* infection of mice suggested that macrophage derived TNF induced IFNγ production by NK cells, and it is feasible that NK cells exert the same function during *S. typhimurium* infection. Recently Mastroeni et al. suggested that IFNγ release in salmonella infection was regulated via IL-18 produced by macrophages (2). The importance of proinflammatory cytokines for activation of macrophages is stressed by the finding that pretreatment with TNF, IL-1, GM-CSF, M-CSF or IFNγ improved survival of *Salmonella typhimurium* infected mice (35-39).

The plateau is normally followed after a variable time by clearance of the organisms from the RES. This phase clearly requires the presence of T cells. Athymic mice show a progressive increase in colony forming units (CFU) count in the RES in the weeks following the initial plateau (27) and in vivo T cell depletion impairs clearance of attenuated salmonellae from the RES (40).

In our model using infection of inherently susceptible BALB/c mice (Ity^s) with a virulent strain of *Salmonella typhimurium*, phase 3 and 4 do not develop and bacterial growth in the RES continues until mice die. However, the time until death depends on the ability of the immune system to slow down bacterial proliferation and even gradual differences in host defense activity associated with LPS pretreatment can be quantified indirectly by determination of the survival time.

1.4 Aims of the study

Lipopolysaccharide is a potent stimulator of inflammatory responses and injection of experimental animals with endotoxin induces many of the pathophysiological alterations observed in patients suffering from systemic inflammation due to sepsis. Repeated administration of endotoxin, however, results in tolerance to LPS toxicity. Since LPS tolerance is associated with suppression of proinflammatory cytokines *in vivo* as well as diminished cytokine release by isolated macrophages restimulated ex vivo, macrophage hyporesponsiveness represents a crucial mechanism underlying LPS tolerance. It is an attractive hypothesis to consider LPS tolerance as a natural regulatory mechanism aimed to control an otherwise autodestructive systemic inflammation in response to sustained endotoxaemia. Although inflammation induced by Gram-positive bacteria is indistinguishible from that caused by Gram-negative germs, it is not known whether similar mechanisms of tolerance and macrophage desensitization are induced by products from Gram-positive bacteria. The first part of this thesis was initiated with the aim to characterize the effect of repeated administration of purified lipoteichoic acid from *Staphylococcus aureus* on macrophage activity. In detail, the major aims of the first part of this thesis were:

i) Identification of the LTA receptor on macrophages responsible for cytokine induction.

ii) Comparison of the effect of repeated exposure to LTA or LPS on cytokine release *in vitro* by murine macrophages and study of putative cross-desensitization.

iii) Confirmation of the *in vitro* findings *in vivo.*

Results from animal models indicate that induction of LPS tolerance could serve as an interesting tool in sepsis prophylaxis. However, an intact cytokine response is essential for host defense against invading pathogens. Thus, in the second part of this thesis, the following issues were addressed:

iv) Determination of the cytokine response of LPS-tolerant mice challenged with LPS or viable bacteria.

v) Characterization of host resistance to bacterial infections after induction of LPS tolerance.

vi) Identification of mechanisms responsible for enhanced antibacterial defense in LPS-tolerant mice.

2 Endotoxin tolerance – mechanisms and beneficial effects in bacterial infection

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2.1 Endotoxin tolerance

Endotoxin or lipopolysaccharide, a glycolipid of the cell membrane of Gram-negative bacteria, is one of the most potent known stimulators of immune responses. The immune system responds to LPS with a systemic production of proinflammatory cytokines which recruit and activate immune cells to eliminate invading pathogens (1, 2). Although these cytokines are indispensible for the efficient control of growth and dissemination of the pathogen (2-4), an overshooting inflammatory response is potentially autodestructive and may lead to microcirculatory dysfunction causing tissue damage, shock and eventually death (3, 4). Injection of high dose LPS induces pathological symptoms resembling those of the septic patient (5).

The term “endotoxin tolerance” describes the phenomenon that immune responses and metabolic changes such as fever, inflammation or weight loss as well as lethality in response to LPS challenge are mitigated after repeated LPS administration. Prophylactic subtoxic LPS administration confers protection against inflammatory damage in a number of animal models. Intensive studies attempting to unravel the underlying mechanisms have been conducted over several decades to find a more effective prophylaxis and therapy of Gram-negative infection. In this review data are summarized on two different and apparently contrasting aspects of endotoxin tolerance, i.e. attenuation of inflammatory damage on the one hand and the concomitant modulation of anti-microbial host defense on the other hand.

2.1.1 *In vivo* studies

The first reports on acquired resistance to endotoxin derive from physicians, who used vaccines containing whole bacteria to induce fever as a therapeutic measure. In that setting,

the development of tolerance to the pyrogenicity of the vaccines was an annoying problem, as it required the infusion of steadily increasing doses to maintain elevated temperatures. Experimentally, Centanni was the first to demonstrate acquired resistance to a purified pyrogenic preparation from bacterial culture filtrate. Repeated injections of rabbits with this heat-stable, non-protein "pyrotoxina bacterica" resulted in a progressive reduction of its fever-inducing activity (41). In 1942, Centanni postulated that the phenomenon was due to a cellular mechanism and not based on a serological immune response (42). Similar results of antibody-independent desensitization to fever induction by repeated administration of fractions from *Salmonella typhosa* to humans were suggested by Favorite and Morgan (43). In a set of experiments on pyrogenic tolerance to daily endotoxin infusions in rabbits, Beeson provided further evidence for non-immunologic mechanisms of tolerance. This conclusion was based on the findings that pyrogenic tolerance was not specific for the polysaccharide side chain and could not be transferred to naive animals. Furthermore, tolerance rapidly waned after discontinuation of the daily endotoxin infusions (44, 45). Intensive studies performed by Greisman et al. led to the distinction of two phases of endotoxin tolerance (46, 47). As reviewed in detail by Johnston et al. (48), these are a nonspecific early phase, which becomes evident hours or days after endotoxin treatment and an antibody-dependent late phase tolerance induced by repeated injections of endotoxin. The early phase which lasts for about 48 h until several days is associated with hyporesponsiveness to endotoxins as a class, i.e. tolerance extends to endotoxins unrelated to the one used for desensitization. It is independent of antibody formation, as early tolerance develops equally in athymic (nude) mice, B-cell deficient (xid) mice, and splenectomized mice (49). In contrast, several days after endotoxin injection, nonspecific tolerance wanes and hyporesponsiveness is restricted to the endotoxin serotype employed during the pretreatment phase. This late phase tolerance was shown to depend on the formation of LPS-specific antibodies and thus can be passively transferred with serum to naive animals (reviewed by Johnston (48)).

Early reports ascribed the diminished LPS responsiveness after endotoxin pretreatment to increased LPS clearance and degradation, e.g. by stimulation of LPS uptake by the reticuloendothelial system (RES) (44, 45). This view was extended by Freedman, who demonstrated that serum transfer of tolerance to the pyrogenic and lethal activities of endotoxin was related to enhanced RES phagocytic activity of recipient rabbits, as assessed by clearance of colloidal carbon (50, 51). Further studies in contrast demonstrated the development of pyrogenic tolerance in the absence of enhanced phagocytic activity of the

RES (52). Moreover, it was shown later on that the administration of thorothrast, used in the early experiments to demonstrate a critical involvement of the RES in mediating LPS tolerance, equally enhanced the fever response in tolerant and naive animals. Hence, a major contribution of enhanced LPS uptake by the RES is considered unlikely. An alternative explanation was suggested by Moreau et al. who demonstrated enhanced activity of serum esterases resulting in increased intravascular inactivation of endotoxin in LPS-pretreated animals (53). Nevertheless, macrophages play a cardinal role in early endotoxin tolerance as demonstrated by Freudenberg et al. in a set of adoptive transfer experiments: in the model of LPS-induced liver injury in galactosamine-sensitized mice Freudenberg et al. revealed that not only LPS toxicity (54), but also induction of tolerance required the presence of functional, LPS-sensitive macrophages (55). Concomitant with the finding that most of the effects of LPS were transmitted by cytokines, several groups reported decreased levels of macrophage derived mediators in endotoxin-tolerant animals (56-58) and humans (59-61) in response to a second LPS challenge (Table 1). Most studies focused on the production of tumor necrosis factor (TNF), which is almost completely downregulated during LPS tolerance, but other cytokines are also affected by endotoxin pretreatment. Erroi et al. established an order of cytokine inhibition *in vivo* within the same model of LPS tolerance in mice: TNF, interleukin-6 (IL-6) >> colony stimulating factor (CSF) > interferon gamma (IFNγ) > IL-1α and β (62). Downregulation of TNF in spleens and peritoneal macrophages of LPS-tolerant mice appeared already at the level of mRNA production, suggesting a suppression of signaling cascades prior to transcription (63). Whereas downregulation of CSF, IFNγ and IL-6 during LPS tolerance is well established, the effect of repeated LPS injections on IL-1 production is controversial. Several studies showed a partial reduction in circulating IL-1 in response to repeated LPS challenge (63, 64), whereas in one study IL-1 was even increased (65).

LPS tolerance develops rapidly within several hours, depending on the model. Thus, protection against liver damage of galactosamine-sensitized mice could be induced by LPS injection one hour prior to GalN/LPS challenge (55). In contrast, suppression of cytokine production took at least five hours after a single dose of LPS (66). Tolerance to the fever inducing activity of endotoxin even required at least 3 daily injections of endotoxin (44). These kinetic differences suggest distinct mechanisms of LPS-induced protection in the different models, which will be discussed later.

2.1.2 *Ex vivo* studies

Further evidence for a contribution of macrophages to LPS tolerance stemmed from *ex vivo* studies showing impaired cytokine production by macrophages isolated from LPS-tolerant animals restimulated *in vitro* (Table 2). In 1968, Dinarello et al. already demonstrated that Kupffer cells isolated from LPS-tolerant rabbits were unable to produce endogenous pyrogen *in vitro* (67). Peritoneal murine and rat macrophages (resident or thioglycolate-elicited) isolated after *in vivo* administration of LPS displayed a decreased production of TNF (68-71) or IL-1 (68) upon LPS restimulation *in vitro*. Similarly, impaired production of IL-12 and consequently of IFNγ by spleen cells from endotoxin-tolerant mice was reported (72). Additionally, these cells displayed decreased responsiveness to substitution with exogenous IL-12, arguing for a suppression of IFNγ production via two distinct mechanisms (72). Bundschuh et al. demonstrated that suppression of TNF production upon *in vitro* restimulation was a common feature of various macrophage populations (bone marrow cells, peritoneal cells, blood monocytes, alveolar cells and spleen cells) isolated from endotoxin-tolerant mice (73). Monocyte hyporesponsiveness was also reported after administration of endotoxin to humans (74, 75). However, Mackensen et al. reported an increased capacity to release cytokines upon restimulation *in vitro* of PBMC from endotoxin pretreated cancer patients, although serum cytokine levels were significantly reduced after repeated LPS injection. In contrast to the other two studies with human volunteers, Mackensen et al. isolated PBMC from cancer patients 24 hours after the last LPS injection, whereas in the other studies blood was withdrawn one hour or 6 hours, respectively, after LPS injection (61).

2.1.3 I*n vitro* studies

Most studies on the mechanism of macrophage desensitisation derive from experiments using primary cells or immortalized cell lines exposed to repeated LPS stimuli *in vitro* (76, 77). As shown for macrophages isolated from endotoxin-tolerant hosts, release of various macrophage mediators in response to LPS stimulation is mitigated after repeated exposure to endotoxin *in vitro*. In this review we will refer to this status of macrophage hyporesponsiveness induced by repeated LPS stimulation *in vitro* as macrophage desensitization or refractoriness, to differentiate it from *in vivo* LPS tolerance, which might involve other mechanisms additional to downregulation of cytokine production. Suppression of cytokine release after LPS exposure was demonstrated for primary cells, such as peritoneal macrophages from mouse or rabbit and human monocytes as well as a variety of murine and human cell lines (Table 3). The spectrum

of cytokines downregulated in desensitized macrophages *in vitro* involves the same mediators shown to be suppressed *in vivo*, although controversial data were provided for most cytokines except TNF. Thus, depending on the experimental setting, downregulation of TNF after exposure to endotoxin was associated with unchanged production as well as suppression or increase of IL-1, IL-6, IL-8, IL-10, and PGE_2 release in response to a subsequent LPS stimulus (78-83). Most controversial data were obtained on the regulation of IL-1. Whereas studies performed with the human cell line THP-1 revealed a downregulation of IL-1 mRNA and protein after repeated LPS stimulation (84-86), data derived from experiments using human or mouse primary cells demonstrated unchanged or even increased IL-1 production in response to a second LPS stimulus (81, 83, 87-91). However, it is possible that despite normal or increased IL-1 levels in desensitized macrophages, the biological activity of IL-1 in the supernatant is suppressed due to sustained or even increased expression of the natural antagonist IL-1 receptor antagonist (IL-1ra) (79, 92).

Suppression of TNF release was associated with decreased mRNA levels, suggesting transcriptional control of cytokine production in cell lines (82, 93-96), human (97), mouse (83, 87, 98-100), and rabbit primary cells (101). This view was challenged by Zuckerman et al. demonstrating inhibition of TNF release despite increased mRNA levels in LPS-pretreated cells (70).

Besides cytokine production, the regulation of nitric oxide (NO) synthesis during LPS tolerance has been studied in detail, but the results are as controversial as for regulation of IL-1. Expression of inducible NO synthase (iNOS) and NO production in response to a second LPS stimulus were suppressed (102-104) or increased (105, 106), depending on the experimental settings. In line with these data, Zhang et al. demonstrated that depending on the concentration of the primary LPS stimulus, either suppression or priming of NO production can be found (107).

To sum up, *in vitro* exposure of cells to LPS results in suppression of TNF release and reprogrammed production of various other macrophage mediators in response to subsequent stimulation. Cells desensitized *in vitro* display many features of macrophages isolated from endotoxin-tolerant hosts. Despite the apparent limitations of the *in vitro* setting such as neglection of neuroendocrine regulation, glucocorticoids and the interaction of different cell types *in vivo*, much of our current knowledge concerning the mechanisms of macrophage desensitization is derived from *in vitro* experiments.

2.1.4 Mechanisms of macrophage desensitization

In the last years, our understanding of the molecular mechanisms underlying desensitization of macrophages by exposure to LPS has increased considerably. Although Larsen et al. suggested that LPS preexposure decreased the number of LPS binding sites on monocytes (108), the expression of the LPS receptor CD14 is unaffected or even increased following LPS-stimulation (95, 109, 110). Thus, it is highly unlikely that tolerance is mediated via expression of this LPS receptor. However, recent results by Nomura demonstrated downregulated surface expression of the LPS signaling receptor toll like receptor 4 (TLR4) on LPS-desensitized macrophages (111). Further downstream, refractoriness in response to LPS preexposure has been shown to be associated with altered G-protein content (112, 113), phospholipase D and phosphatidylinositol-3 kinase expression (114). West et al. reported compromised protein kinase C (PKC) activation in LPS desensitized cells (115) and receptor independent stimulation of the PKC by phorbol myristate acetate could overcome the suppression of cytokine production associated with refractoriness. Others described suppressed signal transduction via both the mitogen-activated-protein (MAP) kinase cascade (98, 116-118) and inhibitor of NF-κB (I-κB) kinases, resulting in impaired transcription of nuclear-factor-kappa B (NF-κB)- and activation protein-1 (Ap-1)-regulated genes (85, 116). An alternative mechanism for suppression of NF-κB-dependent gene expression was suggested by Ziegler-Heitbrock et al.. They used a human monocyte cell line (Mono Mac-6) to demonstrate an upregulation of the p50 subunit of NF-κB in LPS refractory cells, leading to a predominance of transactivation-inactive p50/p50 homodimers. These homodimers bind to NF-κB motifs in several promotors and thereby inhibit the transcription of genes such as TNF (94, 110). Support for this hypothesis originates from experiments with p50 deficient mice that are resistant to tolerance induction by LPS (100). Inhibition of gene transcription in response to a second LPS stimulus via the formation of a specific nuclear suppressor of LPS-induced gene transcription was also suggested by others (84, 86). LaRue et al. provided evidence that decreased LPS-induced transcription of IL-1β in LPS-desensitized THP-1 cells was regulated by a labile repressor which required constant protein synthesis and suggested IκB-α as a potential candidate, although then a contribution of p50 had not yet been studied (84).

In contrast, recent data showing decreased surface expression of TLR4 on LPS-tolerized cells (111) and suppression of IL-1 receptor-associated kinase (IRAK) activation and association with myeloid differentiation protein (MyD88) (119), support the notion that already very early

steps in LPS-signaling upstream of NF-κB are altered after LPS exposure. Further evidence for this was provided by Medvedev et al. (116) who re-evaluated *in vitro* desensitization by IL-1 and TNF, showing induction of cross-tolerance to LPS via the IL-1 receptor but not the TNF-receptor. Intriguingly, signal transduction of the IL-1R, the LPS-receptor TLR4, and TLR2 employ similar signaling molecules (120-122). Recent studies from our laboratory demonstrated that preexposure to lipoteichoic acid that induced signaling via TLR2 resulted in hyporesponsiveness to TLR4-mediated LPS signaling and vice versa. This finding adds further indirect evidence for a suppression of common signaling molecules shared by TLR2/4 and IL-1R, i.e. MyD88, IRAK, TNF receptor-activated factor 6 (TRAF6) or NF-κB-inducing kinase (NIK) in desensitized macrophages. The view that inhibition of common signaling pathways of the IL-1R/TLR family and not diminished TLR4 surface expression is mainly responsible for macrophage hyporesponsiveness is corroborated by the finding that preexposure of macrophages to the TLR2-dependent stimulus mycoplasmal lipopeptide (MALP-2) suppressed LPS-induced TNF release without reducing the surface expression of TLR4 (123).

Despite the large number of studies dealing with macrophage hyporesponsiveness in response to LPS pretreatment, the exact mechanism of suppression of cytokine production has not been identified yet. Since there is sound evidence for a contribution of various of the aforementioned factors, it is feasible that i) macrophage desensitization is the result of the orchestrated action of multiple factors activated by the primary LPS stimulus or ii) depending on the model employed to study tolerance (species, cell type, experimental settings) varying distinct mechanisms account for refractoriness in response to inflammatory bacterial components.

2.1.5 Mediators of tolerance

LPS exerts most of its effects via the activity of macrophage mediators released in response to LPS stimulation. The inflammatory response is regulated by a complex network of mediators that directly interact with each other's expression or biological activity. In this context, a number of macrophage mediators such as IL-10, TGFβ or PGE_2 have potent anti-inflammatory activity by suppressing the formation of proinflammatory cytokines (124-129). Thus, it has been presumed that autocrine mechanisms are also involved in suppression of cytokine production during LPS tolerance.

Mediators of *in vitro* desensitization

As outlined before, LPS-pretreatment of cultured macrophages results in hyporesponsiveness to cytokine release in response to a subsequent LPS stimulus. It has been shown that several cytokines could substitute for LPS as the desensitizing stimulus. Cavaillon et al. demonstrated that incubation of human PBMC with recombinant cytokines prior to restimulation with LPS partially suppressed production of TNF to a different extent. Whereas preexposure to TGFβ or IL-10 reduced TNF release by nearly 60% as compared to saline pretreated cells, IL-4 and IL-1 were less effective (35% and 30% inhibition, respectively) and no inhibition at all was found after administration of TNF, IL-6, IL-8 or leukaemia inhibitory factor (76). The lack of macrophage desensitization by exposure to TNF has been reported also by Li et al. (90). The differential role of TNF and IL-1β in desensitization of macrophages *in vitro* was confirmed by recent studies from Medvedev et al. who showed that exposure of murine macrophages to LPS or IL-1, but not to TNF, resulted in inhibition of transcription factor activation and suppressed transcription of GM-CSF and several chemokines in response to a second LPS stimulus (116). Unfortunately, no information on the regulation of TNF mRNA and protein was given in this study. Convincing data on the contribution of soluble mediators in desensitization of macrophages were derived from experiments with human PBMC. Randow et al. demonstrated that a combination of recombinant human IL-10 and TGFβ was as effective as low-dose LPS pretreatment in terms of reduction of TNF release upon subsequent high-dose LPS stimulation, whereas preexposure to either cytokine alone only partially suppressed the release of TNF (79). In the same setting, addition of neutralizing antibodies to IL-10 and TGFβ inhibited desensitization in response to the first LPS stimulus, providing direct proof for a contribution of these two anti-inflammatory cytokines in LPS-induced monocyte/macrophage refractoriness *in vitro* (79). The critical role of IL-10 and TGFβ in downregulation of TNF production was confirmed by Karp et al., whereas inhibition of IL-12 production in LPS-pretreated human monocytes was independent of these cytokines (130). In line, antibodies against IL-4 or IL-10 as well as addition of indomethacin or a iNOS inhibitor did not abrogate suppression of IL-12 p40 mRNA and protein expression in LPS-desensitized macrophages (131).

The production of a yet unidentified suppressor of TNF formation not identical with IL-1, IL-10 or TGFβ during endotoxin tolerance was reported by Schade et al (132, 133). They showed that addition of culture supernatants of LPS-stimulated peritoneal murine macrophages from endotoxin pretreated mice suppressed TNF release by naive macrophages. Similar results on a

selective inhibitor of TNF, but not of IL-1 or IL-6 synthesis in supernatants of LPS-desensitized macrophages were provided by Fahmi et al. (134). The idea of a negatively acting autocrine mediator in macrophage desensitization was extended by more recent results from Baer et al. who demonstrated the production of a yet unidentified "TNF-inhibiting factor" (TIF) in supernatants of a LPS-stimulated macrophage cell line. Inhibition of TNF-alpha expression by macrophage conditioned medium was associated with selective induction of the NF-κB p50 subunit which selectively inhibited a TNF-promoter reporter construct (135). Since a contribution of IL-4, IL-10 and TGFβ was excluded, these findings provide evidence for LPS induction of a novel cytokine with selective TNF-inhibitory potential participating in endotoxin desensitization (135).

Besides cytokines, arachidonic metabolites were shown to influence the responsiveness of macrophages. It is well established that prostaglandin E_2 (PGE_2) downregulates TNF production by macrophages, probably via the elevation of cAMP (127, 128, 136). Thus it is feasible that PGE_2 produced in response to the primary desensitizing dose of LPS contributes to macrophage hyporesponsiveness. This view was supported by the finding that PGE_2 production was increased in LPS-desensitized macrophages (77, 80, 90, 137). However, direct addition of PGE_2 during primary culture failed to suppress TNF production upon subsequent LPS stimulation of cultured human monocytes (77). In addition, in three different studies the addition of the cyclooxygenase inhibitor indomethacin neither prevented the development of hyporesponsiveness nor restored TNF production upon LPS restimulation (83, 101, 138). In contrast, by using higher concentrations of indomethacin (10-100 µM), Haas et al. could inhibit the suppression of TNF production by LPS-pretreatment (139). Thus, the contribution of arachidonic acid derivatives in desensitization of macrophages still remains to be clarified. Our recent results derived from co-culture experiments argue against a major role of soluble mediators in acquired hyporesponsiveness. Cross-desensitization induced by pre-exposure to LPS or LTA in wild-type macrophages was not transferred to co-cultured macrophages from mice lacking functional TLR2 or TLR4 as evidenced by sustained TNF release upon re-challenge with the other stimulus. However, as we did not perform any neutralization experiments, we cannot rule out that, besides ligand-TLR interaction additional signals provided by soluble mediators were required for desensitization.

Mediators of *in vivo* tolerance

As pointed out for macrophage desensitization *in vitro*, the involvement of soluble mediators in establishing LPS tolerance *in vivo* has also been discussed controversially. Attempts to induce tolerance to the pyrogenicity of subsequent endotoxin injection by repeated administration of endogenous pyrogen (EP) were not successful (140). In contrast, pretreatment of rabbits with IL-1 partially abolished hypotension and TNF release in response to subsequent endotoxin challenge (141). When mice were treated with recombinant TNF or IL-1α, neither cytokine alone was able to mimic LPS induction of tolerance. However, the two cytokines synergized to induce features of early endotoxin tolerance, such as alterations of the monocyte/macrophage bone marrow pool and suppression of CSF release upon subsequent LPS challenge (142). In addition, suppression of CSF release associated with LPS-tolerance was partially reversed by administration of recombinant IL-1 receptor antagonist (IL-1ra) during LPS pretreatment (143). Administration of IL-1α or TNF but not of IL-6 to mice for four days partially inhibited the production of IL-6 and TNF in response to a subsequent LPS challenge, although to a lesser extent than LPS (62). In line with this finding, TNF infusion in rats resulted in a reduced capacity of isolated bone marrow cells to produce TNF, IL-6 or PGE_2 upon LPS stimulation *in vitro* (144). In contrast, Mathison et al. failed to suppress the production of TNF in response to LPS by pretreating rabbits with TNF infusions (145). Pretreatment with IL-1 conferred protection to subsequent high dose LPS challenge (146, 147) and sepsis induced by cecal ligation and puncture (CLP) (148) as well as *E. coli* induced peritonitis (149). Similar results were obtained for TNF, which induced tolerance to the lethality of subsequent LPS challenge (146, 150). In the model of inflammatory liver damage in galactosamine (GalN)-sensitized mice pretreatment with TNF or IL-1 was equally protective as LPS in reducing the extent of liver damage and lethality (151-153). Moreover, administration of IL-1, TNF or LPS induced tolerance to the toxicity of TNF injection itself, as shown for the metabolic changes, weight loss, temperature increase and lethality in response to high-dose TNF injection (only TNF pretreatment) (150, 154), as well as for low-dose TNF-induced hepatocyte apoptosis in GalN-sensitized mice (TNF or IL-1 pretreatment) (151-153). Since enhanced clearance or neutralization of TNF in LPS- or cytokine-pretreated animals was excluded (150, 154) hyporesponsiveness of target cells to TNF activity itself, e.g. by downregulation of TNF receptors and by the production of acute phase proteins or anti-apoptotic factors was suggested as an additional mechanism contributing to LPS tolerance (151-153, 155). This view was corroborated by the finding that addition of acute phase

proteins attenuated the GalN/TNF induced liver damage (156-158). Thus, the protection afforded by LPS pretreatment in the GalN/LPS model is likely to be mediated by two independent mechanisms differing in their requirement of endogenously produced cytokines. On the one hand, the reduction of TNF levels in mice pretreated with LPS suggests macrophage hyporesponsiveness similar to *in vitro* desensitization. As discussed before, although a role of soluble mediators in macrophage desensitization *in vitro* has not been fully identified, yet, evidence has been provided that soluble mediators do not suffice for downregulation of macrophage responsiveness. This view is substantiated by our unpublished results showing suppression of TNF release in TNFR1 deficient mice in response to repeated LPS injections. On the other hand, it is likely that TNF and IL-1 produced upon LPS pretreatment induce hyporesponsiveness of hepatocytes to TNF activity itself as an additional mechanism of protection.

As outlined before, several *in vitro* studies suggested that LPS-induced desensitization of macrophages was mediated via formation of IL-10. In line with this, administration of IL-10 protected mice against a lethal endotoxin challenge (125). However, a major role of the antiinflammatory cytokine IL-10 in mediating LPS tolerance *in vivo* was excluded by Berg et al. using IL-10 deficient mice. Although these mice were LPS-hyperresponsive in terms of TNF production and lethality, tolerance after an initial sublethal LPS dose developed normally as determined by decreased lethality and diminished levels of TNF and IL-6 after subsequent high dose LPS challenge. In addition, infusion of recombinant IL-10 could not substitute for the initial desensitizing dose of LPS (126). In conclusion, although evidence has been provided that cytokines such as TNF or IL-1 have the potential to mimic some of the beneficial effects of LPS pretreatment *in vivo* the actual role of these cytokines in LPS-induced macrophage desensitization still has to be characterized. One important point is that most investigators used recombinant cytokines produced in *E. coli*. Since a possible endotoxin contamination of these recombinant cytokines had not always been excluded, it is difficult to ascribe the observed effects of recombinant proteins to cytokine activity.

Besides cytokines, glucocorticoids possess a strong anti-inflammatory potential. Administration of cortisone prevented lethality after high dose LPS challenge (159) and suppressed the release of TNF, IL-1 and IL-6 (160-165). In line, adrenalectomy sensitized mice to the toxicity of subsequent LPS injection (161, 166, 167). Moreover, since glucocorticoids are released in response to LPS injection, it was feasible to ascribe endotoxin tolerance to the anti-inflammatory activity of endogenous glucocorticoids (167). Studies by

Evans demonstrated that LPS tolerance could not be induced in adrenalectomized mice (167). However, this view was challenged by the finding that endotoxin tolerance in terms of suppressed TNF release developed normally in adrenalectomized rats (168). This finding confirmed earlier results from Chedid's group. In their experiments, endotoxin tolerance developed equally in the absence of glucocorticoids, as shown by adrenalectomy prior to or directly after the initial desensitizing injection of LPS, albeit on the background of overall increased susceptibility (169). A similar status of LPS-hyperresponsiveness can be induced by repeated injections of cortisone. Also under this condition of decreased glucocorticoid responsiveness, mice were rendered endotoxin-tolerant by a single LPS injection (169). Studies in the GalN/TNF model demonstrated that addition of dexamethasone did not prevent liver injury (153), indicating that at least one aspect of LPS tolerance, i.e. diminished sensitivity of hepatocytes to cytokine activity was not mediated by glucocorticoids.
These results, together with the finding that suppression of cytokine release can also be induced *in vitro* (i.e. in the absence of glucocorticoids), argue against a critical involvement of glucocorticoids in endotoxin tolerance.

2.1.6 Specificity of tolerance

The question, whether early phase nonspecific tolerance is restricted to endotoxins as a class or whether it reflects a general state of altered macrophage activity resulting in diminished cytokine expression in response to non-endotoxin inflammatory stimuli as well, has not been settled. The view that tolerance is restricted to endotoxins as a class originates from experiments performed by Greisman et al. who demonstrated that rabbits rendered LPS-tolerant by infusion of endotoxin for several hours displayed a normal fever reaction in response to pyrogenic non-endotoxin challenges such as influenza virus, old tuberculin and staphylococcal enterotoxin (47). Similarly, Roth et al. showed a lack of cross-reactivity between LPS and muramyl-dipeptides in terms of fever induction and production of TNF and IL-6 in guinea pigs (170). However, the experimental setting used consisting of repeated injections of endotoxin over a period of 15 days with administration of muramyl-dipeptide 3 days after the last LPS injection may have been unsuitable to study the specificity of the early phase tolerance which is most prominent within the first 48 h and then starts to wane. Lack of cross-tolerance was reported also by Mathison et al. who failed to suppress TNF-release in response to *Staphylococcus aureus* by preexposure of rabbit macrophages to LPS (101). Similarly, LPS-tolerant Kupffer cells still produced TNF upon viral infection (171). However,

differential suppression of TNF and IL-1 was reported by Wakabayashi et al. who showed that PBMC isolated from LPS-tolerant rabbits still produced TNF, but no IL-1, in response to *Staphylococcus epidermidis* (65), proposing differential regulation of these cytokines during hyporesponsiveness.

Further evidence that downregulation of monocyte/macrophage function after LPS-pretreatment is not restricted to restimulation with endotoxins was provided by Granowitz et al. (75). They demonstrated a reduction of cytokine release by human PBMC derived from endotoxin pretreated volunteers restimulated *ex vivo* with LPS, IL-1 or TSST-1. Cavaillon et al. reported suppression of TNF-release in response to zymosan, staphylococci and streptococci after exposure of human monocytes to LPS *in vitro* (76, 172). Similar results were obtained more recently by Karp et al. for downregulation of IL-12 production (130). Further support for a general macrophage desensitization not restricted to LPS stemmed from Kreutz et al., who reported TNF suppression upon repeated exposure to whole *S. aureus* or synthetic lipopeptides (173). Recently, we could demonstrate macrophage cross-desensitization in terms of TNF production by LPS and lipoteichoic acids (LTA) from *S. aureus* via different TLR. The same held true for *in vivo* tolerance to liver damage by administration of galactosamine plus LPS or LTA. This extends recent findings by Sato et al., who reported cross-desensitization of macrophages by mycoplasmal lipopeptides and LPS via TLR2 and TLR4 (123). These results suggest that tolerance and macrophage desensitization could represent a general antiinflammatory mechanism induced by selected bacterial stimuli to prevent potentially harmful overshooting inflammation during sustained infection.

2.1.7 Restoration of cytokine response

To study the mechanism of cytokine suppression in LPS-desensitized macrophages, a variety of substances was tested for their ability to overcome suppression of cytokine release in response to a second LPS challenge. Several reports indicated that direct stimulation of protein kinase C by addition of PMA to desensitized macrophages had some potential to restore normal immune functions: In human monocytes pretreated with LPS, TNF release in response to PMA was even increased compared to cells preexposed only to medium (77). Others demonstrated reversal of TNF suppression in desensitized murine macrophages by addition of PMA one hour prior to second LPS activation (115). The restoration of LPS responsiveness by preincubation with PMA was associated with reversed inhibition of MAPK and p38 kinase activation (118). In endotoxin-tolerant mice, injection of PMA 10 min before

secondary LPS challenge counteracted suppression of IL-6 and partially of CSF production, but had no effect on TNF release while IL-1β production was even downregulated (58, 62). More physiological tools to restore cytokine release include the proinflammatory cytokines interferon gamma (IFNγ), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 12 (IL-12): It is well established that IFNγ, produced by T lymphocytes or NK cells upon various inflammatory stimuli, is a potent activator of macrophage functions. These include an upregulation of MHC II expression, enhanced microbicidal activity against intracellular pathogens and release of proinflammatory cytokines (174, 175), reviewed in (176). IFNγ receptor-deficient mice display decreased sensitivity to LPS toxicity, associated with depressed TNF synthesis, diminished expression of CD14, and low plasma LPS-binding capacity (177). This suggests that IFNγ is an important co-stimulus for macrophage gene expression that might overcome hyporesponsiveness. Indeed, addition of IFNγ to LPS-desensitized macrophages prior to or concomitant with LPS restimulation partially reversed suppression of TNF production. This was demonstrated for the Mono Mac-6 cell line (139), human monocyte-derived macrophages (178), human PBMC (179) and mouse macrophages isolated after induction of endotoxin tolerance *in vivo* (73). Also several features of *in vivo* endotoxin tolerance, such as suppression of TNF (58, 73) or IL-6 release (58) and increased resistance to endotoxic shock (73), could be partially abolished when IFNγ was injected additionally to LPS re-challenge. Administration of IFNγ also reversed the suppression of TNF, IL-6 and G-CSF release in LPS-tolerant cancer patients (180).

Like IFNγ, GM-CSF is involved in regulation of LPS-induced cytokine production and lethality (181, 182). Further, addition of GM-CSF partially counteracted macrophage desensitization (179, 183), but the priming efficacy differed compared to IFNγ, depending on the cell type used. Thus, GM-CSF was more effective than IFNγ in restoring TNF production by murine monocytes and bone-marrow cells, but less effective when more differentiated macrophages such as peritoneal cells or alveolar macrophages were used (184). In contrast, suppression of TNF production in LPS-pretreated human PBMC and bone marrow cells was counteracted more efficiently by IFNγ (184, 185). Reversal of TNF suppression in LPS-desensitized human PBMC by addition of IL-12 was also demonstrated recently. This cytokine is normally produced by monocytes/macrophages upon inflammatory stimuli and induces IFNγ release by T lymphocytes and NK cells. Since IL-12 release was shown to be downregulated in desensitized macrophages, substitution with exogenous IL-12 should restore TNF release via production of IFNγ. Direct proof for this hypothesis was provided by

Randow et al. who showed that the effect of IL-12 was dependent on both the presence of nonmonocytic cells and production of IFNγ (179). However, suppression of IFNγ production by spleen cells from LPS-tolerant mice could only be partially reversed by addition of IL-12, suggesting diminished responsiveness to IL-12 as an additional mechanism of tolerance (72).
The mechanism underlying restoration of TNF release in LPS-desensitized cells by pretreatment or coadministration of these proinflammatory cytokines is not fully clear yet. Enhancement of TNF production in response to IFNγ, GM-CSF or IL-12 is not restricted to LPS-desensitized cells, but is also found in naive monocytes/macrophages (179, 183). Thus it is feasible that instead of specifically restoring signaling pathways suppressed during hyporesponsiveness, these cytokines rather act by amplifying the minimal responses that still occur in desensitized cells, using the same pathways involved in the enhancement of primary LPS responses.

2.2 Endotoxin tolerance and infection

2.2.1 Introduction

Dysbalanced production of leukocyte-derived inflammatory mediators such as cytokines, arachidonic acid metabolites, lysosomal enzymes, reactive oxygen or nitrogen intermediates is considered a major mechanism responsible for pathophysiological alterations of the microcirculation, leading to shock, multiple organ failure and eventually death in response to systemic infection or endotoxaemia (186-190). Experimentally induced endotoxin tolerance provides protection against lethality and morbidity in animal models of endotoxic shock and fulminant infection used to simulate the systemic inflammatory response syndrome of the septic patient. As pointed out before, LPS tolerance is associated with suppression of several cytokines, attenuation of leukocyte infiltration and consequently a reduction of organ damage. These findings suggested induction of LPS tolerance to be an interesting tool in sepsis prophylaxis (191-193). However, concern was raised whether suppression of inflammatory responses during LPS tolerance would interfere with normal host defense and thus predispose patients to nosocomial infection (191). Indeed, host defense against infection with small numbers of replicating pathogens requires an intact cytokine response to halt proliferation and dissemination of the pathogen (1, 2). In contrast to models of acute hyperinflammation such as endotoxic shock, neutralization of proinflammatory cytokines worsens the outcome of infection with low numbers of virulent bacteria (29, 194) and many cytokine-deficient mice that are resistant to inflammatory damage rapidly succumb to otherwise sublethal infections

(2, 194-198). Also, mice inherently hyporesponsive to LPS because of a nonfunctional mutation in the tlr4 gene (199-202) display increased susceptibility to Gram-negative pathogens (203, 204). Furthermore, experimentally induced endotoxin tolerance displays features of immunoparalysis observed frequently in post-septic or post–traumatic patients several days or weeks after systemic inflammation: Monocytes from immunoparalysed patients were impaired in their ability to produce TNF upon restimulation with LPS *in vitro* (205-212) and displayed diminished surface expression of MHC II (209-212). These cellular defects were associated with an increased incidence of infectious complications and lethal outcome of disease (211, 213). Since similar alterations of monocyte/macrophage activity were found during experimentally induced LPS tolerance, it was feasible that induction of LPS tolerance equally interfered with host defense.

In contrast, it has been known for a long time that endotoxin is a potent activator of host defense and LPS treatment is associated with protection against the lethality of irradiation, restriction of tumor growth as well as enhanced resistance to subsequent infection with various microbial pathogens (214). The first reports on the curative effect of application of bacterial products on infection stemmed from treatment of patients suffering from abdominal typhus with crude extracts of bacteria at the end of the 19th century (215). By this time, the widespread use of fever therapy, i.e. the injection of pyrogenic bacterial preparations, for the treatment of various diseases was initiated. An excellent review on fever therapy was written by Nowotny (214).

The use of animal models to study the mechanisms underlying enhancement of host defenses by bacterial products was initiated in 1892 by Kanthack, who reported pyrogen-induced changes on leukocytes after injection of *Vibrio metchnikorii* filtrates into rabbits (216). In 1955, Rowley was the first to describe increased resistance of mice to bacterial infection after administration of *E. coli* cell wall extracts 48h prior to challenge (217). The same protection was afforded when isolated endotoxin was injected instead of cell walls (218). Subsequently, this phenomenon of reduced susceptibility after endotoxin application was extended to infections with other bacterial species and even some viral pathogens (219). Pretreatment with endotoxin or cellular components of Gram-negative bacteria induced nonspecific protection against infection with a number of different extra- and intracellular bacteria including both Gram-negative and Gram-positive species (220-226), reviewed in detail by Shilo (219) (table 4). More recently, increased resistance of LPS-pretreated animals to lethality and organ damage associated with multi-germ sepsis, induced e.g. by CLP was reported (192, 227-229).

Experiments performed by Rayhane et al. corroborated the notion that increased resistance is nonspecific by demonstrating improved survival and decreased fungal burden of LPS pretreated mice with disseminated *Cryptococcus neoformans* infection (230).

2.2.2 Mechanisms of enhanced host defense

Humoral factors

It has been demonstrated that enhanced resistance after LPS injection was associated with increased bactericidal activity of serum towards certain Gram-negative bacteria (218). Since evidence was provided that LPS administration enhanced serum bactericidal activity mainly towards properdin-sensitive organisms, increased serum properdin levels were suggested to be a major mechanism of LPS-induced resistance (231). This view was questioned later by findings that LPS pretreatment afforded protection also to properdin-insensitive organisms such as Gram-positive bacteria. Moreover, alterations in host resistance against bacterial infection were not always paralleled by serum properdin levels (232). We recently provided further evidence against a major role of the complement system in LPS-induced increased resistance by demonstrating LPS-induced nonspecific resistance to *S. typhimurium* and *L. monocytogenes* in the absence of any changes in complement activity as determined in a sheep erythrocyte lysate assay (233). Furthermore, depletion of the central C3 protein of the complement cascade by administration of cobra venom factor did not abolish the protective effect of LPS pretreatment on *S. typhimurium* infection (233). However, increased serum opsonization activity after LPS administration was reported by several authors (223, 234). In sum, enhanced resistance to infection is associated with increased serum bactericidal or opsonization activity in some models, although direct proof for a critical contribution of the complement system is still lacking.

Macrophages

On the cellular level of host defense, LPS injection is associated with a transient depression of RES activity, followed by a longer lasting period of enhanced clearance of carbon particles, radioactive LPS, labelled chromium phosphate and viable or heat-killed bacteria by the RES (233, 235, 236). Detailed studies by Chedid's group demonstrated that irradiation- and cyclophosphamide-resistant cells mediated improved survival, enhanced RES phagocytic activity and reduced bacterial burden associated with LPS pretreatment of mice subsequently

submitted to an otherwise lethal *Klebsiella pneumoniae* infection (226, 236, 237). Although definite protection of irradiated mice by LPS injection additionally depended on a further, bone-marrow derived cell type not identical with T lymphocytes, their experiments strongly supported the notion that activation of RES macrophages was a major mechanism of LPS-induced host defense against *Klebsiella pneumoniae* (236, 237).

We have shown recently that enhanced hepatic phagocytosis of bacteria in LPS-pretreated mice was associated with increased numbers of Kupffer cells, the resident macrophage population of the liver (233). Increased Kupffer cell numbers were also reported for LPS-tolerant rats (238). Direct evidence for a contribution of Kupffer cells in LPS-stimulated clearance of bacteria was derived from experiments using chlodronate-liposomes to deplete liver macrophages prior to injection of bacteria (233). Ruggiero et al. used isolated perfused rat livers to demonstrate increased hepatic uptake of *Escherichia coli* after *in vivo* LPS pretreatment due to enhanced phagocytic activity of the liver and improved opsonization by the serum (234). Besides an increase in Kupffer cell numbers, enhanced phagocytic activity of individual liver macrophages could account for improved hepatic clearance after LPS-treatment, as demonstrated by Hafenrichter et al. for isolated Kupffer cells from LPS-pretreated rats (239). Accordingly, peritoneal macrophages exposed to LPS *in vivo* or *in vitro* showed accelerated phagocytosis of *Salmonella typhimurium in vitro* (223). In contrast to studies using murine peritoneal macrophages where an enhancement of oxidative burst activity was reported (147, 240), Kupffer cells from LPS-pretreated rats displayed decreased generation of superoxide anions (238). However, our unpublished data indicate improved antibacterial activity of Kupffer cells from endotoxin-tolerant mice.

Neutrophilic granulocytes

LPS induces a plethora of chemokines leading to accumulation of leukocytes, consisting mainly of neutrophilic granulocytes, at the site of LPS administration. This is of importance when bacteria are injected at the site of previous LPS administration, since the microorganisms are confronted immediately with a large number of phagocytes absent in the naive host. We recently demonstrated that intraperitoneal accumulation of leukocytes and enhanced inactivation of intraperitoneally injected *Salmonella typhimurium* during the first hours postinfection was strictly dependent upon the route of LPS pretreatment. Similar results were obtained by Astiz et al. who studied the therapeutic value of administration of monophosphoryl lipid A (MPL), a detoxified LPS derivative, to mice prior to induction of

peritonitis by CLP. In their setting, intraperitoneal (*i.p.*) injections of MPL were more effective in reducing mortality than intravenous (*i.v.*) MPL administration (241). However, activation of resident peritoneal macrophages by *i.p.* LPS injection could also account for the improved antibacterial activity.

It has long been known that endotoxin is a potent stimulator of hematopoesis. Post-endotoxin serum was shown to have potent colony-stimulating factor (CSF) activity *in vitro* as well as *in vivo*, when transferred to naive animals (242-244). Intensive studies on radioprotection by previous administration of endotoxin suggested an important role of accelerated hematopoiesis, as reviewed by Nowotny et al. (214). Administration of LPS resulted in increased white blood cell numbers (233, 245), neutrophilia (60, 233) and augmented numbers of monocyte/macrophage precursors in the bone marrow (57, 246). We demonstrated that endotoxin-pretreated mice displayed elevated numbers of circulating neutrophils throughout the course of *Salmonella* infection, indicating improved recruitment from the bone marrow and/or decreased rate of apoptosis of these cells after LPS treatment (233). A critical role of diminished neutrophil apoptosis for the survival benefit associated with endotoxin pretreatment prior to induction of multi-germ peritonitis was suggested recently (247). This is in line with previous findings by Yamamoto, showing a delay of neutrophil apoptosis by LPS and LPS-induced cytokines *in vivo* and *in vitro* (248).

Besides an increase in overall PMN numbers, enhanced anti-microbial activity of the individual PMN could contribute to enhanced immune defense of the LPS-pretreated host. Our unpublished data indicate an increased oxidative burst response of blood PMN from LPS-tolerant mice upon stimulation *ex vivo*. The view that neutrophils play a decisive role in LPS-induced resistance to infection is substantiated by our findings that PMN depletion partially abrogated the survival benefit of LPS-pretreated mice infected with *Salmonella typhimurium* (233).

Lymphocytes

Activation of lymphocytes by LPS or LPS-induced mediators is well documented, and Galelli et al. demonstrated that definite protection by LPS treatment of irradiated mice required bone-marrow derived radiosensitive cells (236). However, the adaptive immune system seems to be of minor importance for the establishment of the early phase of LPS-induced nonspecific resistance as suggested by experiments performed with athymic or SCID-mice which showed protection in spite of lacking functional T-and B-lymphocytes (236), our own results).

2.2.3 Mediators of nonspecific resistance

Many of the effects of endotoxin are mediated by endogenous mediators such as cytokines, arachidonic acid metabolites, reactive oxygen or nitrogen radicals. The role of autocrine mediators in the process of inducing or maintaining macrophage refractoriness is still under debate. Similarly, there is evidence that LPS-enhanced nonspecific resistance is the result of the biological activity of several cytokines produced in response to LPS injection.

Injection of IL-1 improved survival of mice infected subsequently with *Listeria monocytogenes* (249), *Pseudomonas aeruginosa* (249-253), *Klebsiella pneumoniae* (249, 250, 253), *Escherichia coli* (149) and in the sepsis model of CLP (146, 254). Furthermore, the combination of IL-1 and TNF reduced mortality and bacterial load of mice infected with *E. coli* at 20-fold the $LD_{50\ (255)}$. Pretreatment with IL-1, GM-CSF or G-CSF improved survival after aerosol pneumococcal challenge (256-258). This effect could be due to enhanced microbicidal activity of alveolar macrophages and improved clearance of blood-borne pathogens of cytokine-pretreated mice (259). The beneficial effect of G-CSF treatment prior to induction of bacterial peritonitis or *L. monocytogenes* infection was probably mediated via the recruitment or activation of PMN (260-263). Extensive studies on the beneficial effect of cytokine pretreatment on resolution of infection were performed in the model of *Salmonella typhimurium* infection of mice employed also in our studies. It has been shown that administration of TNF resulted in improved survival of otherwise lethal bacterial challenge (30, 35). Protection against salmonella infection was also conferred by pretreatment with IFNγ (30), IL-18 (2) or TGFβ (264), IL-1 or a combination of IL-1 and TNF (37, 265). Since LPS administration induces the formation of all of these mediators, it is feasible that nonspecific resistance is conferred via endogenous formation of these cytokines. However, direct proof for this hypothesis has not been provided yet. Studies on the role of LPS-induced cytokines in enhancing resistance to infection are hampered by the fact that normal host defense initiated by the pathogen itself also depends on an intact cytokine response. Thus, cytokine-deficient mice are unsuitable and the use of cytokine-specific antibodies requires detailed titration experiments in order to selectively neutralize only LPS-induced cytokines during the pretreatment phase but not during infection. Furthermore, because of the plethora of cytokines with similar protective effect, it is unlikely that neutralization of single mediators will abrogate the beneficial effect of LPS-pretreatment.

2.2.4 Outlook

The finding that LPS-pretreated animals were protected against the toxicity of endotoxin in models of septic shock and sepsis and displayed even enhanced resistance to bacterial infection, suggests the therapeutic use of endotoxin tolerance induction as a sepsis prophylaxis. However, the well-known side-effects of endotoxin injection ranging from fever to potentially fatal systemic inflammatory responses hamper the clinical use of endotoxin administration. The use of detoxified derivatives of LPS such as synthetic lipid A could avoid this risk. Several studies have demonstrated that these substances retain the ability to protect against shock and bacterial infection despite strongly decreased toxicity (57, 59, 192, 193, 241, 266-270). Future experiments will evaluate the clinical value of prophylactic induction of LPS tolerance in reducing the risk of postoperative sepsis.

Table 1: Endotoxin tolerance *in vivo*

Effect			**References**
Survival		↑	mouse (1-6); rat (271-273)
fever		↓	human (41-43, 59); guinea pig (154, 170); rabbit (67)
TNF	protein	↓	human (59-61); mouse (58, 62-64, 72, 99, 132, 147, 167, 193, 233, 274, 275); guinea pig (170); rat (271); rabbit (65); pig (66)
	mRNA	↓	mouse (63, 167); rat (276)
IL-1β	protein	↓	human (60); mouse (62); rabbit (65)
		↑	mouse (64)
	mRNA	↓	mouse (63)
IL-6	protein	↓	human (59, 60), mouse (58, 62, 147, 233, 274); guinea pig (170)
		↑	human (61)
IL-8	protein	↓	human (59-61)
IL-10	protein	↓	mouse (274); rat (276)
IL-12	protein	↓	mouse (72)
CSF	protein	↓	human (60, 61); mouse (49, 57, 62, 143, 246, 269, 277, 278)
IFNγ	protein	↓	mouse (72, 193, 233, 279)
chemokines	protein	↓	rat (280)
arachidonic acid metabolites		↓	rat (272); pig (66)
nitric oxide derivatives		↓	rat (281, 282); chicken (104)
		=	mouse (193)
angiotensinogen		↓	rat (283)

Table 2: Mediator dysregulation in endotoxin tolerance *ex vivo*

Effect			References
TNF	protein	↓	human blood (284), human PBMC (75); mouse PMΦ (133, 183); mouse blood (132); other mouse macrophages (183); rat PMΦ (71); rat Kupffer cells (155, 239); rabbit PBMC (65)
		↑	human PBMC (61)
IL-1β	protein	↓	human PBMC (75); mouse PMΦ (68); rabbit PBMC (65)
		↑	human PBMC (61)
IL-6	protein	↓	human PBMC (75); rat Kupffer cells (239), rat PMΦ (285)
		↑	human PBMC (61)
IL-10, TGFβ	protein	↓	mouse PMΦ (133)
IL-12, IFNγ	protein	↓	mouse spleen cells (72)
arachidonic acid metabolites		↓	mouse PMΦ (69); rat PMΦ (71, 113, 239, 273, 286, 287)
nitric oxide derivatives		↑	rat PMΦ (273, 285)
superoxide		↓	rat non parenchymal cells, perfused liver (238)

Table 3: Mediator dysregulation in endotoxin desensitization *in vitro*

Effect			References
TNF	protein	↓	human PMΦ (97); human monocytes (76, 288)(78, 130)(77, 81); human PBMC (79, 88, 185, 289); human dendritic cells (130); mouse RAW 264.7 cell line (96); mouse PMΦ (83, 89, 90, 99, 106, 115, 117, 118, 123); rabbit PMΦ (101)
	mRNA	↓	human Mono Mac 6 cell line (82, 93-95); human PMΦ (97); mouse RAW 264.7 cell line (96); mouse PMΦ (83, 87, 98-100)
IL-1β	protein	↓	human THP-1 cell line (84, 85)
		=	human monocytes (81); mouse PMΦ (83)
		↑	human PBMC (88); human PMΦ (97); mouse PMΦ (89, 90)
	mRNA	↓	human THP-1 cell line (84-86)
		=	mouse PMΦ (83, 87)
IL-6	protein	↓	human PMΦ (97); human PBMC (289); human monocytes (290); mouse PMΦ (98, 111)
		=	human monocytes (81) (131); mouse PMΦ (90)
IL-10	protein	↓	human PBMC (289) (79, 185)
		↑	human Mono Mac 6 cell line (82); human monocytes (290)
IL-12	protein	↓	human monocytes (130), human dendritic cells (130)
IL-1ra	protein	↑	human THP-1 cell line (92)
		=	human PBMC (79)
CSF	protein	↓	human PBMC (289); mouse PMΦ (116)
		↑	human PMΦ (97)
arachidonic acid metabolites		↓	mouse PMΦ (90)
nitric oxide derivatives		↓	mouse PMΦ (102, 103) (107) (291); chicken macrophages (104)
		↑	mouse PMΦ(105-107)
MHC II expression		↓	human PBMC (292)

Table 4: Effect of endotoxin on host defense

	Challenge	Survival	Mechanism	Reference
Gram-negative bacteria	*Escherichia coli* *Proteus vulgaris* *Pseudomonas aeruginosa* *Klebsiella pneumoniae* *Salmonella typhi*	↑ ↑ ↑ ↑ ↑	CFU↓ (*S. typhi*), Properdin levels ↑	(231)
	Escherichia coli	↑	serum bactericidal activity ↑, RES phagocytosis ↑	(218)
	Salmonella typhi	↑		(293)
	Salmonella dublin	↑	not transferred with serum --> antibody independent	(294)
	Salmonella typhimurium *Salmonella enteritidis*	↑ ↑	CFU ↓, bacterial clearance↑	(295)
	Salmonella typhimurium		serum opsonic activity ↑ MΦ phagocytic acitivity ↑	(223)
	Salmonella typhimurium	↑	CFU ↓, phagocyte accumulation ↑ phagocytosis/bactericidal activity of PMΦ ↑	(240)
	Salmonella typhimurium	↑	CFU ↓, PMN recruitment ↑, PMN accumulation ↑, RES phagocytosis ↑	(233)
	Klebsiella pneumoniae	↑	CFU ↓, RES phagocytosis ↑	(226, 236, 237)
	Klebsiella pneumoniae	↑		(296)
	Pasteurella tularensis	↑		(297, 298)
Mycobacteria	*Mycobacterium fortuitum*	↑	CFU ↓, RES phagocytosis↑	(221)
Gram-positive bacteria	*Staphylococcus aureus*	↑	CFU ↓	(222)
	Staphylococcus aureus *Streptococcus pyogenes* *Diplococcus pneumoniae*	no effect	porperdin insensitive bacteria	(231)
	Staphylococcus aureus	↑	cytokine production ↓	(268)
	Streptococcus agalactiae	↑	not transferred with serum--> antibody independent	(294)
	Listeria monocytogenes	↑	CFU ↓,	(233)
Mixed infection	CLP	↑	CFU ↓, granulopoiesis ↑	(227, 228)
	CLP	↑	cytokine production ↓	(241)
Fungi	*Cryptococcus neoformans*	↑	CFU ↓, TNF-mediated effect	(230)
Parasites	*Plasmodium berghei*	↑		(299)

3 Induction of cross-tolerance by LPS and highly purified lipoteichoic acid via different Toll like receptors independent of paracrine mediators

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3.1 Abstract

Exposure of macrophages to lipopolysaccharide induces a state of hyporesponsiveness to subsequent stimulation with LPS termed LPS-desensitization or tolerance. To date it is not known whether similar mechanisms of macrophage refractoriness are induced upon contact with components of Gram-positive bacteria. In the present study we demonstrate that pretreatment with highly purified lipoteichoic acid (LTA) results in suppression of cytokine release upon restimulation with LTA *in vitro* and *in vivo* in both C3H/HeN and C3H/HeJ mice, but not in macrophages from Toll like receptor 2 (TLR2) -deficient mice. Furthermore, desensitization in response to LPS- or LTA-exposure also inhibits responses to the other stimulus ("cross-tolerance"), suggesting that signaling pathways shared by TLR2 and TLR4 are impaired during tolerance. Finally, we show that LPS- or LTA induced cross-tolerance is not transferred to hyporesponsive cells co-cultured with LPS/LTA-responsive macrophages, showing that soluble mediators do not suffice for tolerance induction in neighbouring cells.

3.2 Introduction

Endotoxin or lipopolysaccharide, a glycolipid of the cell membrane of Gram-negative bacteria, is one of the most potent known stimulators of immune responses, inducing cytokine production by monocytes and macrophages (300). Although cytokine production is indispensible for the efficient control of growth and dissemination of invading pathogens, a dysbalanced inflammatory response is potentially harmful to the host and may lead to microcirculatory dysfunction causing tissue damage, septic shock and eventually death (3, 301). The phenomenon of endotoxin tolerance could represent a host mechanism aimed at limiting inflammatory damage upon activation of the immune system by Gram-negative bacteria or their products. The term endotoxin tolerance (also called LPS hyporesponsiveness or refractoriness) describes the observation that pretreatment of experimental animals with an initial low dose of LPS confers protection against the detrimental consequences of a subsequent high dose of LPS (44, 47). This protection is associated with an attenuated inflammatory response to LPS due to a downregulation of macrophage responsiveness (62, 75, 302). A similar status of refractoriness to endotoxin stimulation is induced *in vitro* by exposure of macrophages to LPS several hours or days prior to re-challenge (83, 91, 101)).

In contrast to the endotoxin of Gram-negative bacteria, no single constituent has so far been identified as the major immunostimulatory element in Gram-positive bacteria. In the last years, increasing evidence was provided that lipoteichoic acids and peptidoglycans could substitute for whole Gram-positive bacteria in activation of macrophages and induction of multi-organ dysfunction syndrome (10, 12). Recently, it has been shown that signal transduction in response to several Gram-positive bacteria and peptidoglycan is mediated by a cellular transmembrane receptor termed toll-like receptor (TLR) 2 (16, 17, 303, 304). However, data have been controversial regarding the involvement of TLR2 or TLR4 in LTA-induced signaling (16, 17).

In spite of the efforts aimed at characterizing the interaction of Gram-positive components with the immune system, the question whether similar mechanisms of macrophage desensitization, as reported for endotoxin tolerance, are also induced by Gram-positive components, has not been addressed in detail. For this purpose we studied the effect on cytokine produciton of low dose pretreatment with LTA or LPS followed by a second LTA/LPS challenge *in vivo* and in primary murine macrophages *in vitro.* Using mice lacking

functional TLR4 (C3H/HeJ) or TLR2 (TLR2 knock-out) we demonstrate macrophage desensitization upon pre-exposure to LTA via TLR2 but independent of TLR4.

3.3 Materials and Methods

3.3.1 Mice

Male C3H/HeN and C3H/HeJ and female CD1 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). TLR2 knockout mice were generated by homologous recombination by Deltagen, Menlo Park, USA and kindly provided by Tularik Inc., South San Francisco, USA. Heterozygous mice were interbred with CD1 mice to obtain homozygotes. Female TLR2-deficient mice and CD1 control mice were used for experiments. They were kept at 24 °C, 55% humidity, 12-h day-night rhythm on a diet of Altromin C 1310 (Altromin Co., Lage, Germany) and were used at 8-10 weeks of age. All animals received humane care in accordance with the NIH guidelines and the legal requirements in Germany.

3.3.2 Preparation of LTA

Staphylococcus aureus (DSM 20233) was grown in a 35 l fermentor, washed and stored at -80 °C. A defrosted aliquot of bacteria was mixed with an equal volume of n-butanol (Merck, Darmstadt, Germany) under stirring for 30 min at RT. After centrifugation at 13000 g for 20 min, the aquatic phase was lyophilized, resuspended with chromatography start buffer (15 % n-propanol in 0.1 M ammonium acetate, pH 4.7) and centrifuged at 45000 g for 15 min. The supernatant was subjected to hydrophobic interaction chromatography (HIC) on octyl-sepharose. This LTA preparation contained less than 0.08 endotoxin units per mg LTA (6 pg LPS/mg LTA), as assessed by Limulus amoebocyte lysate assay (Bio Whittaker, Verviers, Belgium).

3.3.3 In vitro experiments

Mice were killed by terminal pentobarbital anesthesia (Narcoren™; Merial, Hallbergmoos, Germany) and 10 ml of ice-cold PBS (Gibco Life Technologies, Karlsruhe, Germany) were injected into the peritoneal cavity. Animals were shaken gently and the lavage liquid was transferred to siliconized glas tubes (Vacutainer®, Becton Dickinson, Heidelberg, Germany) for isolation of peritoneal cells. After centrifugation, cells were resuspended in medium (RPMI 1640 BioWhittaker, Verviers, Belgium) containing 10% FCS (Boehringer-Mannheim,

Mannheim, Germany) and 100 IU/ml penicillin/streptamycin (Biochrom, Berlin, Germany), and transferred to 96-well cell culture plates (5×10^5 cells/ ml). For determination of cytokine induction by LPS or LTA, cells were stimulated immediately with pyrogen-free saline (Braun, Melsungen, Germany), increasing concentrations of LPS (*Salmonella abortus equi;* Metalon, Wustenhofen, Germany), or LTA (*Staphylococcus aureus*), respectively, at 37°C, 5% CO_2 in a humidified atmosphere for 6 h.

To study desensitization, cells from C3H/HeN and HeJ mice were incubated in medium with increasing concentrations of LPS or LTA for 20 h. Then cells were washed twice with PBS and new medium was added. Cells were challenged with either 10 ng/ml LPS or 10 µg/ml LTA for 6 h.

To determine the involvement of soluble factors in tolerance induction equal numbers of peritoneal cells from C3H/HeN and HeJ mice were pooled and pre-incubated with medium, 1 ng/ml LPS or 10 µg/ml LTA for 20 h prior to re-stimulation with 10 µg/ml LTA for 6 h.

In analogy, equal cell numbers from CD-1 wild-type and TLR-$2^{-/-}$ mice were pre-exposed to medium, 10 ng/ml LPS or 10 µg/ml LTA for 20 h and re-stimulated with 100 ng/ml LPS for 6 h. TNF was determined in supernatants by ELISA.

3.3.4 In vivo experiments

Mice were injected intraperitoneally with pyrogen-free saline, 3 µg/kg LPS or 15 mg/kg LTA in pyrogen-free saline 24 h prior to challenge. For induction of liver damage, mice were starved overnight and injected intraperitoneally with 5 µg/kg LPS or 25 mg/kg LTA with 1 g/kg galactosamine (GalN) (Roth Chemicals, Karlsruhe, Germany) diluted in saline. After 90 min blood was obtained from the tail vein for determination of plasma TNF. The extent of liver damage was assessed 8 h after challenge by measuring plasma alanine amino-transferase (ALT) activity with an EPOS 5060 analyzer (Netheler & Hinz, Hamburg, Germany) (305).

3.3.5 Cytokine ELISA

TNFα in supernatants was determined by ELISA, using specific antibodies purchased from PharMingen (Hamburg, Germany). For the detection of TNF in plasma samples the OptEia™ kit from PharMingen was used. The detection limits were 10 pg/ml.

3.3.6 Statistics

Data are presented as means ± standard error (SEM). Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test or Dunnett's Multiple Comparison Test of all groups versus the control group. Data derived from experiments using non-pooled cells from individual mice were analyzed using Dunnett's Multiple Comparison Test after repeated measures ANOVA to account for the interindividual differences. Statistical evaluation of results presented as % was performed with raw data. In case of unequal variances (Bartlett's Test $p < 0.05$), data were first log-transformed. $P < 0.05$ was considered significant. All tests were performed with GraphPad Prism, version 3.0 for Windows (GraphPad Software, San Diego, CA, USA).

3.4 Results

3.4.1 Cytokine production in response to lipoteichoic acid depends on functional TLR2 but not TLR4

Published results are controversial concerning the requirement of TLRs for signal transduction in response to LTA from Gram-positive bacteria (16, 17). To determine the involvement of TLR4 in signaling upon stimulation with LTA, we compared TNFα production by peritoneal macrophages from C3H/HeJ mice lacking functional TLR4 (199, 200, 202) and wild-type C3H/HeN mice. Macrophages from wild-type mice exhibited TNF release upon LPS-stimulation employing concentrations as low as 10 pg/ml, whereas no TNF was released from cells of C3H/HeJ mice when LPS-concentrations up to 100 ng/ml were used, confirming the well-known status of LPS-hyporesponsiveness of this strain (Fig. 1A). In contrast to stimulation with LPS, macrophages from HeJ mice were equally responsive as compared to HeN mice in terms of LTA-induced TNF production (Fig. 1B).

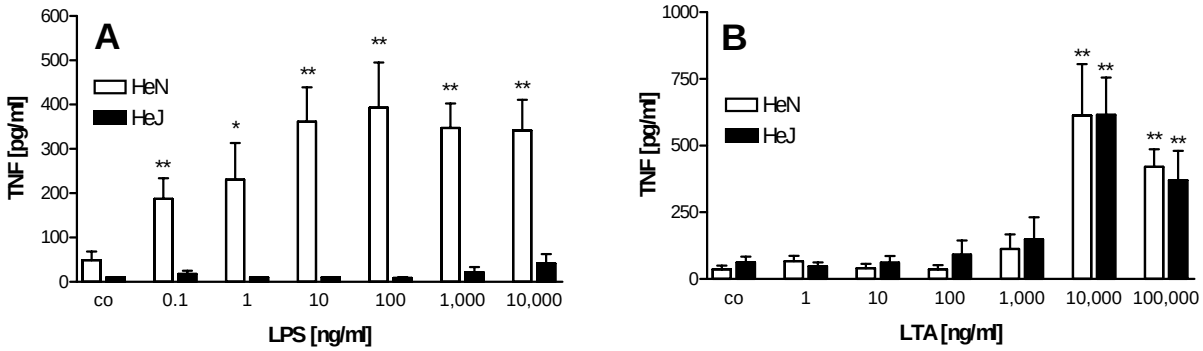

Figure 1. Peritoneal macrophages from LPS-hyporesponsive C3H/HeJ mice exhibit normal TNF-release upon stimulation with LTA.
5×10^5/ml peritoneal cells from C3H/HeN (n = 6) and C3H/HeJ mice (n = 6) were stimulated with increasing concentrations of LPS (A) or LTA (B) for 6 h. Data are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. respective control group (NaCl) based on ANOVA, followed by Dunnett's multiple comparison test.

To test the involvement of TLR2 in LTA signaling, we compared TNF production by peritoneal cells from TLR2 deficient and control mice stimulated with LPS or LTA. In contrast to the previous experiments with cells from C3H/HeJ mice, $TLR2^{-/-}$ cells were normoresponsive to LPS-induced TNF release, whereas no TNF-production in response to LTA could be observed (Fig. 2).

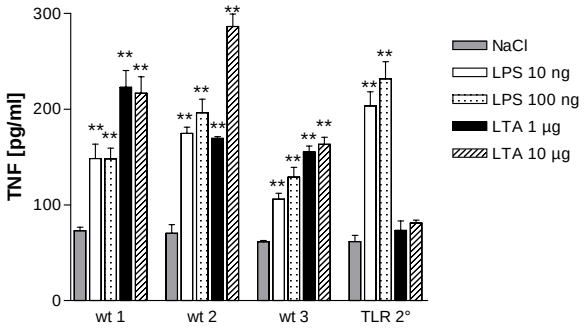

Figure 2. Impaired TNF release in response to LTA by TLR2$^{-/-}$ macrophages.
5×10^5/ml peritoneal cells from 3 wild-type CD-1 mice and one TLR2$^{-/-}$ mouse were stimulated with different concentrations of LPS or LTA for 6 h in triplicates. Data are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. respective control group (NaCl) based on ANOVA, followed by Dunnett's multiple comparison test.

3.4.2 LTA and LPS induce cross-desensitization in vitro via different TLRs

Prior exposure to LPS renders cells hyporesponsive to subsequent stimulation with LPS. We tested whether prior exposure of cells to highly purified LTA induced refractoriness to subsequent stimulation with LTA comparable to LPS-tolerance. Release of TNF in response to 10 µg/ml LTA was decreased in a dose-dependent fashion when primary murine peritoneal cells were pretreated with different concentrations of LTA for 20 h prior to restimulation (Fig. 3A, right panel). Previously it has been shown that interleukin-1 and LPS could substitute for each other in the induction of refractoriness in vitro (76, 116), suggesting that hyporesponsiveness was due to impaired function or expression of signaling intermediates shared by IL-1 and LPS. As many of these factors are involved also in signal transduction via TLR2 (120, 122, 306), we sought to determine whether, in analogy to IL-1, pretreatment with LTA had an inhibitory effect on LPS-induced signaling and vice versa. Indeed, exposure of cells to LTA also induced hyporesponsiveness to subsequent stimulation with LPS (Fig 3B, right panel). Similarly, LPS induced cellular refractoriness to both LTA and LPS (Fig. 3A, left panel, Fig 3B, left panel). The same experiments, as controls, were performed with cells from LPS-hyporesponsive C3H/HeJ mice. As expected, no cytokine release was found upon restimulation with 10 ng/ml LPS (Fig. 3D), whereas LTA-induced release of TNF from saline-preexposed HeJ macrophages was comparable to cells derived from HeN mice (Fig. 3C, left panel). In contrast to wt cells, HeJ macrophages were not rendered LTA-tolerant by preexposure to LPS, whereas pretreatment with LTA inhibited cytokine release (Fig. 3 C). These results clearly demonstrate the requirement of functional TLR4 for induction of LTA-tolerance by LPS but not by LTA itself, which renders cells refractory independently of TLR4.

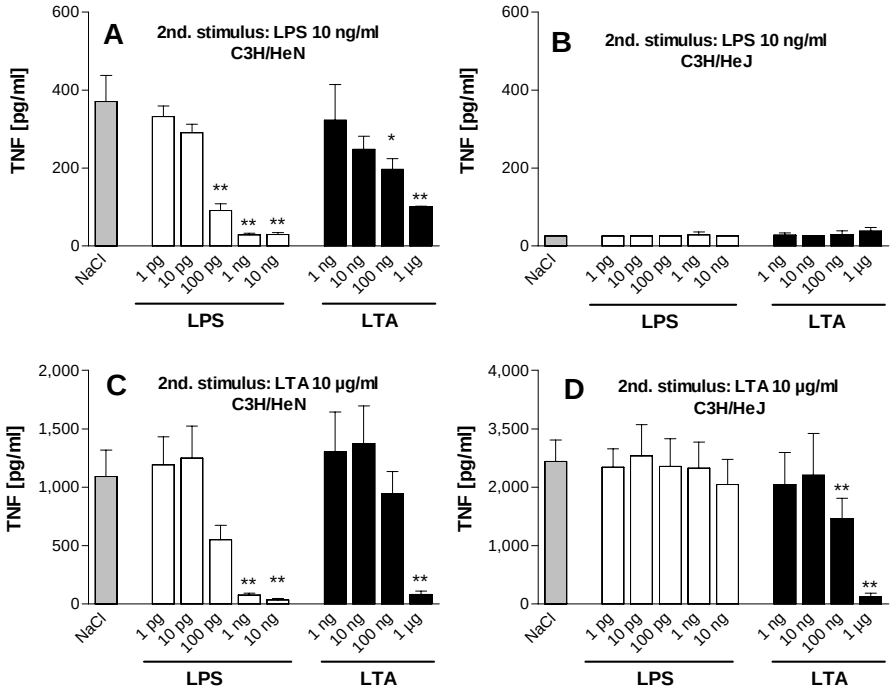

Figure 3. Induction of cross-tolerance by LPS and LTA.
5×10^5/ml peritoneal cells from C3H/HeN (n = 4) and C3H/HeJ mice (n = 4) were pre-exposed to saline (hatched bars) or increasing concentrations of LPS (white bars), or LTA (black bars) for 20 h. After washing, cells were re-stimulated with 10 µg/ml LTA (3A, 3C), or 10 ng/ml LPS (3B, 3D) for 6 h and TNF was determined in supernatants by ELISA. Representative data from one of three independent experiments are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. respective control group (NaCl) based on repeated measurement ANOVA, followed by Dunnett's multiple comparison test.

3.4.3 Cross-tolerance induction with LPS and LTA *in vivo*

Administration of endotoxin in vivo induces a state of tolerance to the toxicity of subsequent endotoxin challenge in models of LPS shock and LPS induced liver injury (55). Although suppression of LPS-induced cytokine production, especially of TNF, in tolerant animals is often put forward to explain the protective effect of endotoxin tolerance and ex vivo data confirm hyporesponsiveness of macrophages isolated from LPS-pretreated animals (183), the relationship between in vitro desensitization of macrophages and in vivo tolerance induction has not been delineated completely (307).

To determine whether the phenomenon of cross-desensitization by LPS and LTA observed *in vitro* can also be reproduced *in vivo*, we performed animal studies, injecting LTA or LPS 24 h prior to induction of liver damage by galactosamine plus LPS/LTA. Saline pretreated mice exhibited elevated TNF-plasma levels in response to GalN + LPS (5 µg/kg) (only C3H/HeN mice Fig. 4A) or GalN + LTA (25 mg/kg) (C3H/HeN (Fig 4B) and C3H/HeJ mice (Fig. 4C)) and developed liver injury as assessed by plasma transaminase activity 8 h after challenge. In

contrast, TNF levels and liver damage were significantly lessened when animals were injected with LPS (3 µg/kg) or LTA (15 mg/kg) 24 h prior to challenge. Similarly, as shown for isolated macrophages *in vitro*, LPS-pretreatment was ineffective in C3H/HeJ mice challenged subsequently with GalN/LTA, confirming the requirement of TLR4-mediated signaling in tolerance induction by LPS *in vivo* (Fig. 4C).

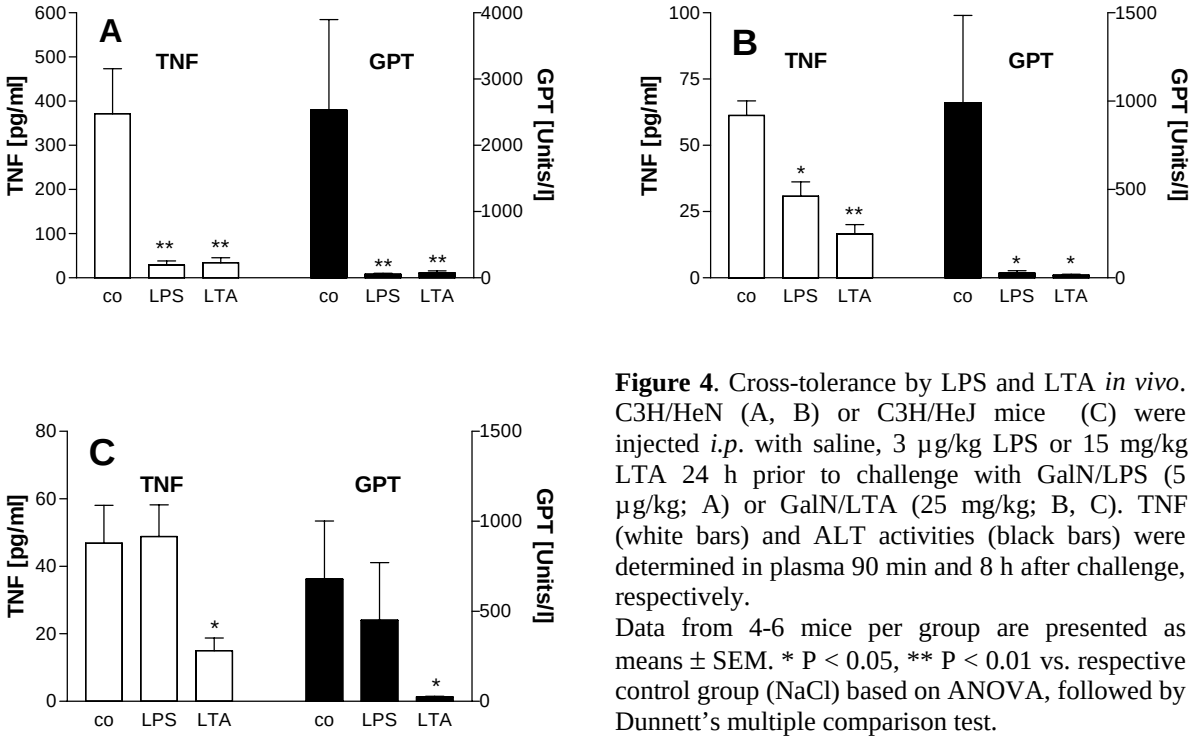

Figure 4. Cross-tolerance by LPS and LTA *in vivo*. C3H/HeN (A, B) or C3H/HeJ mice (C) were injected *i.p.* with saline, 3 µg/kg LPS or 15 mg/kg LTA 24 h prior to challenge with GalN/LPS (5 µg/kg; A) or GalN/LTA (25 mg/kg; B, C). TNF (white bars) and ALT activities (black bars) were determined in plasma 90 min and 8 h after challenge, respectively.
Data from 4-6 mice per group are presented as means ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. respective control group (NaCl) based on ANOVA, followed by Dunnett's multiple comparison test.

3.4.4 LPS-induced in vitro desensitization is not conferred by soluble mediators

Earlier reports suggested that refractoriness after prexposure of cells to endotoxin was mediated by endogenous factors such as IL-10, TGFβ or IL-1 (76, 308). This led us to study the role of soluble mediators in tolerance induction in a co-culture system using mixed macrophages from wild-type and TLR-deficient mice. If desensitization was mediated by soluble factors released upon stimulation with LPS, macrophages from TLR4-mutated C3H/HeJ mice co-cultured together with wild-type macrophages should be rendered LTA-tolerant by LPS pretreatment. In cultures of macrophages from either C3H/HeN or HeJ mice and in co-culture of both cell types, LTA-pretreatment completely inhibited cytokine production in response to LTA challenge (Fig. 5A). In contrast, preexposure to LPS only partially suppressed cytokine release by approximately 50 % in the co-culture system, whereas complete inhibition was found in HeN cells (Fig. 5A). Similar results were obtained

with co-culture of TLR2$^{-/-}$ and wild-type cells restimulated with LPS. In this setting, LPS-pretreatment suppressed TNF production in wild-type, TLR2$^{-/-}$ and mixed cells (Fig. 5B). LTA pretreatment impaired cytokine production by wild-type cells but had no effect on TLR2$^{-/-}$ cells, whereas a 50 % reduction of TNF was found in supernatants of co-cultured cells (Fig. 5B). These results indicate that in the co-culture system only the cells carrying functional TLR were desensitized, whereas the TLR4 or TLR2-deficient cells were unaffected by the LPS/LTA pretreatment. These findings suggest that soluble factors such as IL-10, TGFβ or IL-1β, that are released upon contact with LPS or LTA are not responsible for suppression of TNF formation upon secondary stimulation. Hence, direct signaling via the adequate TLR seems to be necessary for *in vitro* desensitization by LPS or LTA.

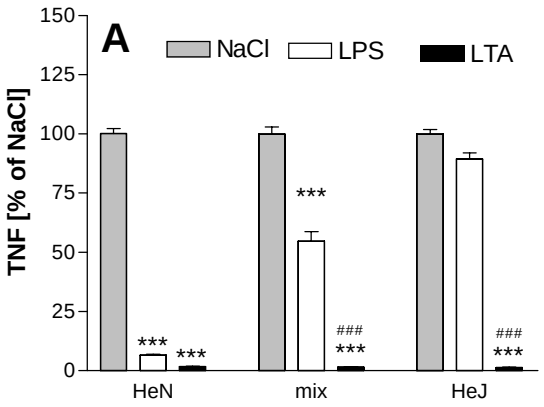

Figure 5A. LPS-induced cross-tolerance is not transferred to co-cultured C3H/HeJ cells.
5×10^5/ml peritoneal cells from C3H/HeN or C3H/HeJ mice or mixed cells from both mouse strains (1×10^6/ml) were pre-exposed to medium, 1 ng/ml LPS or 10 µg/ml LTA for 20 h, washed and re-stimulated with 10 µg/ml LTA for 6 h for determination of TNF. Pooled data from 2 independent experiments performed in quadruplicates are expressed as % of respective saline control (NaCl) and are shown as means ± SEM. 100 % of NaCl group corresponds to 1180 pg/ml (C3H/HeN), 2570 pg/ml (C3H/HeN + C3H/HeJ) and 3180 pg/ml (C3H/HeJ). *** $P < 0.001$ vs control group (NaCl), ### $P < 0.001$ vs. LPS pretreated cells (LPS) based on ANOVA, followed by Tukey's Multiple Comparison Test.

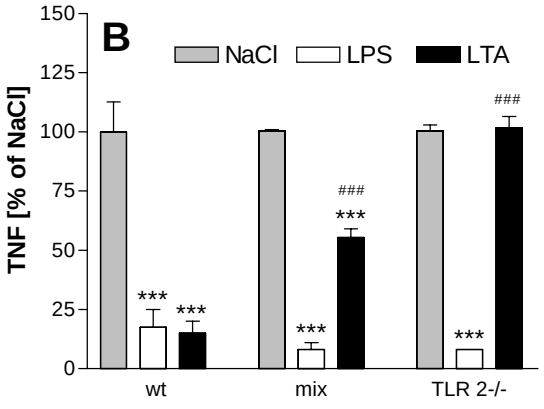

Figure 5B. LTA-induced cross-tolerance is not transferred to co-cultured TLR2$^{-/-}$ cells.
5×10^5/ml peritoneal cells from either CD1 wild-type or TLR2$^{-/-}$ mice, or mixed cells from both mouse strains (1×10^6/ml) were pre-exposed to medium, 10 ng/ml LPS or 10 µg/ml LTA for 20 h, washed and re-stimulated with 100 ng/ml LPS for 6 h for determination of TNF production. Data from one experiment performed in triplicates are expressed as % of respective saline control and are shown as means ± SEM. 100% TNF of NaCl group correspond to 20 pg/ml (CD1), 50 pg/ml (CD1 + TLR2$^{-/-}$) and 25 pg/ml (TLR2$^{-/-}$)). *** $P < 0.001$ vs. control group (NaCl), ### $P < 0.001$ vs. LPS pretreated cells (LPS) based on ANOVA, followed by Tukey's Multiple Comparison Test.

3.5 Discussion

Overactivation of the immune system in the course of systemic infection creates a life-threatening situation characterized by pathophysiological alterations of the microcirculation that finally can lead to multi organ failure and death (309, 310). Inhibition of inflammatory processes after a primary proinflammatory response may represent a mechanism of the host´s immune system to limit autodestruction caused by sustained systemic inflammation. In animal models of endotoxic shock, LPS- and TNFα-mediated liver damage as well as fulminant infection with high numbers of bacteria, suppression of cytokine production and prevention of tissue damage as well as of mortality were observed when a nontoxic dose of LPS was administered several hours or days prior to challenge (192, 307). We demonstrate here that acquired tolerance to the immunostimulatory activity of a bacterial component is not restricted to LPS, but also is found in response to LTA from Gram-positive bacteria. Moreover, our data showing cross-desensitization of LPS and LTA suggest a general mechanism of suppressing proinflammatory responses after repeated contact with components of Gram-positive or Gram-negative bacteria.

Our findings are in line with a previous report showing suppressed cytokine production upon stimulation of LPS-pretreated human monocytes with whole *Staphylococcus aureus* (130). However, contrasting results were obtained by others showing unaltered or even increased cytokine production upon stimulation with muramyl dipeptide or whole *S. aureus* after LPS-tolerance induction (101, 170). These discrepancies may be due to species differences as guinea pig and rabbit macrophages were employed there or due to overall different experimental settings.

In the last years, our understanding of the molecular mechanisms underlying desensitization of macrophages by exposure to LPS has increased considerably. Expression of CD14 is unaffected or even increased following LPS-stimulation, thus it is highly unlikely that tolerance is mediated via expression of this LPS receptor (95, 109). Refractoriness in response to LPS preexposure has been shown to be associated with altered G-protein content (112, 113), phospholipase D and phosphatidylinositol-3 kinase expression (114), and compromised protein kinase C activation (115). Others described suppressed signal transduction via both the MAP kinase cascade (98, 116-118) and I-κB kinases, resulting in impaired transcription of NF-κB- and Ap-1-regulated genes (85, 116). A predominance of transactivation-inactive p50/p50 homodimers of NF-κB has also been found in LPS-tolerance (94, 100). Recent data showing suppression of IL-1 receptor-associated kinase (IRAK) activation and association

with myeloid differentiation protein (MyD88) (119) and decreased surface expression of TLR4 on LPS-tolerized cells (111) support the notion that very early steps in LPS-signaling are altered after LPS-exposure. Further evidence for this was provided by Medvedev et al. (116) who re-evaluated *in vitro* desensitization by IL-1 and TNF, showing induction of cross-tolerance to LPS via the IL-1 receptor but not the TNF-receptor. Intriguingly, signal transduction of the IL-1R, the LPS-receptor TLR4, and TLR2 employ similar signaling molecules (120-122). Our findings that preexposure to LTA inducing signaling via TLR2 results in hyporesponsiveness to TLR4-mediated LPS-signaling and vice versa add further indirect evidence for a suppression of common signaling molecules shared by TLR2/4 and IL-1R, i.e. MyD88, IRAK, TNF receptor-activated factor 6 (TRAF6) or NF-κB-inducing kinase (NIK) in desensitized macrophages. Alternatively, activation of the IL-1R/TLR signaling pathway could result in formation of a specific nuclear suppressor of LPS-induced gene transcription, as suggested previously (84, 86).

Previous studies suggested that hyporesponsiveness after pretreatment with LPS was mediated by the action of endogenous mediators such as IL-10, TGFβ or IL-1β, produced upon primary or secondary LPS stimulation (76, 130, 308). Others postulated the existence of soluble yet unidentified suppressor molecules of TNF-expression during LPS-tolerance (133). Our findings that macrophages from mice deficient in TLR2 or carrying a nonfunctional mutant of TLR4 were not rendered refractory by LTA or LPS-pretreatment, respectively, when cocultured with wild-type macrophages, argue against soluble mediators of desensitization. However, we cannot rule out that the effects of LPS/LTA pretreatment *in vivo*, i.e. protection against LPS-shock and liver injury are mediated in part by endogenously produced cytokines. Thus, macrophage-derived products such as TNF and IL-1β, which are released during the LPS/LTA-pretreatment, might contribute to the desensitization of target cells (e.g. hepatocytes) against the toxicity of subsequent LPS/LTA challenge *in vivo*, e.g. by inducing heat-shock and other acute phase proteins (150, 151, 155).

In conclusion, in the present study we demonstrate cross-desensitization of primary murine macrophages by highly purified LTA and LPS *in vitro* and cross-tolerance to LTA/LPS induced TNF production and liver damage *in vivo*. Our data provide evidence that macrophage desensitization is not a LPS-specific phenomenon but that common signaling pathways shared by TLR4 and TLR2 are impaired during macrophage refractoriness. Finally, our findings suggest that endogenously produced soluble factors do not suffice for macrophage desensitization *in vitro*.

4 Improved innate immunity of endotoxin-tolerant mice increases resistance to *Salmonella typhimurium* infection despite attenuated cytokine response

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4.1 Abstract

During infection with Gram-negative bacteria, exposure of immune cells to lipopolysaccharide from the bacterial cell membrane induces a rapid cytokine response which is essential for the activation of host defenses against the invading pathogens. Administration of LPS to mice induces a state of hyporesponsiveness or tolerance characterized by reduced cytokine production upon subsequent LPS-challenge. In the model of experimental *Salmonella typhimurium* infection of mice we assessed the question whether complete LPS tolerance induced by repetitive doses of LPS interfered with cytokine production and host defense against Gram-negative bacteria. Although production of various cytokines in response to *S. typhimurium* was attenuated by LPS-pretreatment, LPS-tolerant mice showed improved antibacterial activity, evidenced by a prolongation of survival and a continuously lower bacterial load. We attribute this protective effect to three independent mechanisms:
Peritoneal accumulation of leukocytes in the course of LPS-pretreatment accounted for enhanced defense against *S. typhimurium* during the first 6 hours of infection, but not for decreased bacterial load in late-stage infection.

LPS-tolerant mice had an increased capacity to recruit neutrophilic granulocytes during infection.

LPS-tolerant mice showed three-fold increased Kupffer cell numbers, enhanced phagocytic activity of the liver and strongly improved clearance of blood-borne *S. typhimurium*.

These results demonstrate that despite attenuated cytokine response, acquired LPS tolerance is associated with enhanced resistance to Gram-negative infections and that this effect is mainly mediated by improved effector functions of the innate immune system.

4.2 Introduction

Endotoxin or lipopolysaccharide, a glycolipid of the cell membrane of Gram-negative bacteria, is one of the most potent stimulators of immune responses known. The immune system responds to LPS with a systemic production of proinflammatory cytokines which recruit and activate immune cells to eliminate invading pathogens (311). Although these cytokines are indispensible for the efficient control of growth and dissemination of the pathogen (203, 204, 312), an overshooting inflammatory response is potentially autodestructive and may lead to microcirculatory dysfunction causing tissue damage, septic shock and eventually death (3, 4). The phenomenon of endotoxin tolerance is known from animal models of ‘sterile infection’ induced by LPS: after an initial low dose of LPS, animals are protected against the detrimental consequences of a subsequent high dose of LPS. This protection is associated with an attenuated cytokine response to LPS (56) due to a downregulation of macrophage responsiveness (134).

The value of endotoxin tolerance induction as a mean of sepsis prophylaxis was studied in animal models of endotoxic shock or polymicrobial sepsis. In these models, protection by tolerance induction was ascribed to the decreased proinflammatory response resulting in less inflammatory cell infiltration and therefore attenuation of organ damage (192, 193, 266, 269). These models simulate the final phase of sepsis but they do not entirely reflect the situation of infection with small numbers of virulent pathogens where activation of host defenses contributes to halt proliferation and dissemination of the pathogen (1, 2). Only if the immune system fails to control the infection, bacterial replication results in overwhelming and finally lethal pathogen numbers. Therefore, it is not surprising that in contrast to models of acute hyperinflammation, neutralization of proinflammatory cytokines worsens the outcome of infection with low numbers of virulent bacteria (29, 194). Moreover, whereas depletion of various leukocyte populations confers protection against endotoxic shock or inflammatory

liver damage (313, 314), this treatment renders animals more susceptible to bacterial infection (22, 23).

Considering the obvious differences between models of hyperinflammation versus infection with low numbers of virulent bacteria, we were interested whether attenuation of cytokine release by induction of endotoxin tolerance would affect susceptibility of mice to infection with virulent bacteria and which possible consequences could arise from these model experiments for sepsis prophylaxis.

Infection of mice with *Salmonella typhimurium*, the equivalent to human typhoid fever, is one of the best characterized models of systemic and lethal infection (25). This model was chosen for two reasons:

The murine pathogen *S. typhimurium* can replicate and cause systemic infection starting from very few inoculated bacteria (18), i.e. host responses can be studied without inducing septic shock.

Efficient host defense against *S. typhimurium* depends on the production of proinflammatory cytokines like TNFα and IFNγ, and susceptibility is increased by neutralization of these mediators (reviewed in (315)). If endotoxin tolerance had a negative impact on host defense, e.g. by impairing bacteria-induced cytokine release, this should be most obvious during infection with this Gram-negative pathogen.

Our study demonstrates that despite impaired systemic release of proinflammatory cytokines in response to viable bacteria, LPS-tolerant mice show increased resistance to *Salmonella typhimurium* infection due to improved antibacterial defense capabilities of the innate immune system.

4.3 Materials and Methods

4.3.1 Mice

Male BALB/c mice, 7-9 weeks of age, from the breeding facility of the University of Konstanz (Konstanz, Germany) were kept at 24 °C, 55% humidity, 12-h day-night rhythm on a diet of Altromin C 1310 (Altromin Co., Lage, Germany). All animals received humane care in accordance with the NIH guidelines and the legal requirements in Germany.

4.3.2 Bacteria

Salmonella typhimurium LT2 strain ATCC 15277 from the American Type Culture Collection (Rockville, MD, USA) were cultured overnight in tryptic soy broth (Difco, Detroit, MI, USA) at 37 °C and gentle rotation. Aliquots of 5 x 10^8 viable bacteria/ml in 25% glycerin were stored at -80 °C. Just before use, aliquots were thawed and diluted in pyrogen-free saline.

4.3.3 LPS tolerance induction

For induction of LPS tolerance, mice were injected *i.p.* or *i.v.* with a dose of 1 mg LPS *(Salmonella abortus equi;* Metalon, Wustenhofen, Germany) per kg body weight diluted in pyrogen-free 0.9% NaCl solution (Braun, Melsungen, Germany) at 72, 48, and 24 hours prior to challenge with LPS (LPS-shock) or *Salmonella typhimurium* (infection).

4.3.4 LPS-shock

Control mice were injected with the same volume of pyrogen-free saline at the same time points. For induction of endotoxic shock, control and LPS-pretreated mice were injected with 10 mg/kg LPS *i.p.* and survival was monitored for 72 h. Blood for determination of plasma TNFα was obtained from the tail vein 90 min after challenge.

4.3.5 Experimental infection

Salmonella typhimurium infection was initiated by *i.p.* inoculation with 10^7 bacteria per kg body weight and survival was monitored for 10 days. Bacterial load, leukocyte counts, MPO activity and cytokine levels were analyzed at various time points in parallel experiments.
PMN depletion. Anti-Ly-6G (RB6-8C5) IgG or control rat IgG (Biotrend, Cologne, Germany) were administered 16 h (0.6mg/mouse *i.p.*) prior to and 6 h as well as 30 h (0.3mg/mouse *i.p.*) after infection of LPS- tolerant (i.v.) and control mice with *S. typhimurium* (10^6/kg i.p.). 24 h after injection of bacteria, blood was obtained from the tail vein for determination of total and differential leukocyte counts and survival was monitored for 10 days. Anti-Ly6-8C5 rat IgG2b were purified from supernatants of the RB6-8C5 clone (provided by Dr. R. Coffman, DNAX, Palo Alto, CA) grown in 350 ml culture flasks (CL 350, Integra Biosciences, Fernwald, Germany).

4.3.6 Determination of bacterial clearance

Control and LPS-tolerant mice were infected with 10^8 *S. typhimurium*/kg *i.v.*. Recovery of injected bacteria from blood, liver and spleen was determined 5, 20, 40 and 90 min after infection. To study the role of macrophages, liver and spleen macrophages were depleted by treatment with liposomes containing dichloromethylene bisphosphonate (Cl_2MBP) (Roche Diagnostics GmbH, Mannheim, Germany) (316) prior to injection of bacteria: Cl_2MBP liposomes were injected *i.v.* in 0.2 ml at 24 h, 48 h and 71 h after the third injection of saline or LPS. At 72 h, *Salmonella typhimurium* (10^8 bacteria/kg) were injected *i.v.* and recovery of bacteria was determined 10 min after infection in blood and various organs.

4.3.7 Leukocyte counts

Peritoneal lavage cells obtained with 10 ml of ice-cold PBS under terminal pentobarbital anesthesia (Narcoren™; Merial, Hallbergmoos, Germany) were counted in a Neubauer chamber. Differential cell counts were performed microscopically after May-Grünwald/ Giemsa staining (Merck, Darmstadt, Germany) of cytospin preparations. Blood was obtained by cardiac puncture with heparinized syringes. White blood cell counts were determined microscopically in a Neubauer chamber after erythrocyte lysis with Türk´s solution (Merck). Leukocyte differential counts were done on May-Grünwald/Giemsa stained smears.

4.3.8 Determination of CFU

CFU were determined from serial dilutions of organ homogenates, blood or peritoneal lavage fluid plated on Columbia blood agar plates (Heipha, Heidelberg, Germany) and incubated at 37°C for 24 hours before colonies were counted.

4.3.9 Immunohistological staining of macrophages

Liver samples were fixed for 24 h in 10% neutral buffered formalin (Sigma, Deisenhofen, Germany), dehydrated and embedded in paraplast (Sherwood Medical Co., St. Louis, USA). 3 µm slices were cut with a microtome (Microm International, Walldorf, Germany). After rehydration, slices were incubated with 1% w/v of trypsin (Sigma) for 20 min at 37 °C to retrieve antigen followed by inactivation of endogenous peroxidase activity by treatment with 1% H_2O_2 v/v in methanol for 10 min at room temperature. Unspecific binding was blocked by incubation with 0.2 mg/ml of goat IgG (Biotrend) in 5% w/v milk powder in TBS for 1 h at 37 °C. Then, slices were incubated overnight at 4 °C with the primary monoclonal rat anti-

mouse F4/80 IgG2b antibody (Serotec, Oxford, UK) in a 1:50 dilution. The secondary polyclonal goat anti-rat IgG antibody coupled to horseradish peroxidase (Biotrend) was diluted 1:50 in Tris buffered saline (TBS) (final protein concentration 40 µg/ml) and slices were incubated at 37 °C for 30 min. After washing in TBS, the peroxidase substrate 3´-3´diaminobenzidine (DAB) peroxidase substrate (Sigma) was added and the reaction stopped after 20-30 min by washing. Nuclei were counterstained with Mayer´s hemalaun solution (Merck) for 30 sec. Control samples without primary or secondary antibody confirmed the specificity of reaction. F4/80 positive nucleated cells were counted in 20 representative 630x magnification fields per sample.

Cytokine determination. Aliquots of organ homogenates, blood and peritoneal lavage fluid were centrifuged at 14,000g for 7 min and the supernatants were used for the determination of cytokines in a sandwich ELISA: Flat-bottomed high-binding polystyrene microtiter plates (Greiner, Nürtingen, Germany) were coated with a sheep anti-mouse tumor necrosis factor α (TNFα) capture polyclonal antibody (protein solution 20mg/ml, in-house preparation). Recombinant murine TNFα served as standard (gift of Dr. G. Adolf, Bender & Co, Vienna, Austria). The biotinylated anti-TNFα tracer antibody was purchased from Pharmingen (Hamburg, Germany). For determination of interleukin-6 (IL-6) and interferon γ (IFNγ), matched antibody pairs and standards were purchased from Pharmingen. The quantity of tracer antibody bound was determined using streptavidin-peroxidase (Jackson Immuno Research, West Grove, PA, USA) and TMB liquid substrate solution (Sigma). The detection limit was 10 pg (TNFα, IFNγ) and 25 pg (IL-6), respectively.

4.3.10 Determination of myeloperoxidase (MPO) activity

Samples from liver and spleen were excised, weighed, frozen in liquid nitrogen and stored at –70°C. Tissues were homogenized with a polytron homogenizer (PT 1200, Kinematica, Luzern, Switzerland) in 50 mM potassium phosphate buffer (ph 6.0) containing 0.5% (w/v) hexadexylammonium bromide (Sigma). Homogenates were shock frozen in liquid nitrogen, thawed rapidly and centrifuged at 14,000 g for 7 min. Serial dilutions of the supernatants were added to TMB liquid substrate (Sigma) for determination of MPO activity. The reaction was stopped by addition of H_2SO_4 and the absorption determined at 450 nm. MPO from human leukocytes (Sigma) served as standard.

4.3.11 Statistics

Data in the tables are given as means ± SD and in the figures as means ± SEM. Analysis of pretreatment effects were done with the two-sided, unpaired Student´s t-test, the two-sided Welch test or the Mann-Whitney test for two groups. In case of unequal variances, data were first transformed by log (X + 1). For experiments with three groups, one-way ANOVA ($P < 0.05$) was performed, followed by the two-sided, unpaired Student´s t-test according to Shaffer (317) or Dunnett´s test for comparison with the control group. For more than three groups, Bonferroni´s multiple comparison test for selected groups was used. The survival curves were created by the method of Kaplan and Meier. For statistical comparison, survival curves were analyzed using the log-rank test. All tests were done with GraphPad Prism, version 3.0 for Windows (GraphPad Software, San Diego, CA, USA).

4.4 Results

4.4.1 Effect of LPS-pretreatment on cytokine production and sensitivity to endotoxic shock

Mice were injected one to three times with 1 mg LPS/kg body weight at 24 h intervals. Groups of three mice were sacrificed 90 min after the single, double or triple LPS injection regimen and samples from liver, spleen and blood were taken for determination of cytokine levels. High levels of TNFα, IFNγ and IL-6 were detected in plasma and homogenates of liver and spleen after single LPS injection. Cytokine production was attenuated upon the second LPS treatment and strongly reduced or even completely suppressed after the third LPS injection (Table I).

Table I. *Effect of repeated LPS injections on cytokine production in vivo*[a]

Treatment	TNFα		IL-6		IFNγ	
	plasma	liver	plasma	liver	plasma	spleen
Saline	< 20 [b]	610 ± 150	< 50	1,150 ± 90	< 20	2,120 ± 980
1 x LPS	1,980 ± 550	4,150 ± 290	44,700 ± 17,400	6,500 ± 500	8,010 ± 2,670	18,540 ± 12,480
2 x LPS	< 20 [c]	360 ± 130 [c]	17,000 ± 5,800	3,300 ± 550 [d]	< 20 [c]	2,200 ± 1,250 [c]
3 x LPS	< 20 [c]	20 ± 30 [c]	2,800 ± 2,250 [c]	1,330 ± 390 [c]	< 20 [c]	1,500 ± 170 [c]

[a] Mice were treated with *Salmonella abortus equi* LPS (1 mg/kg; *i.p.*) one to three times at 24 h intervals. 90 min (TNFα) or 6 h (IL-6, IFNγ) after the last LPS injection, cytokines were determined in plasma and liver (TNFα, IL-6) or spleen (IFNγ). Saline injected mice served as controls.
[b] Data are expressed as means ± SD with n = 3 and given in [pg/ml] for plasma and [pg/g] for liver and spleen.
[c] Significantly different from 1 x LPS ($P < 0.01$; Dunnett´s test after one-way ANOVA).
[d] Significantly different from 1 x LPS ($P < 0.05$).

Confirming the well-known state of LPS tolerance, LPS-pretreated mice were protected from an otherwise lethal dose of LPS (10 mg/kg *i.p.*: 100% survival of LPS-tolerant *vs.* 0% of control mice within 72 hours, n = 7; peak TNFα in plasma: 0.3 ± 0.2 ng/ml in LPS-tolerant *vs.* 10.1 ± 2.2 ng/ml in controls, $P < 0.001$).

4.4.2 Attenuation of cytokine production in response to *Salmonella typhimurium* infection in LPS-tolerant mice

As immune cells isolated from LPS-tolerant mice displayed impaired cytokine release upon *ex vivo* stimulation with heat killed *Salmonella typhimurium* (data not shown), we were interested whether this would hold true for infection *in vivo.* Control mice inoculated intraperitoneally with 10^7 *S. typhimurium*/kg responded to infection with an early release of

various cytokines such as TNFα, IL-6 and IFNγ with maximal concentrations at 3 hours post infection. This increase in cytokine levels in plasma, liver and spleen in the initial phase of infection was strongly attenuated in LPS-tolerant mice (Fig.1). Since proinflammatory cytokines were shown to be essential for activation of host defenses against *Salmonella typhimurium (315)*, we were interested whether the impaired cytokine response of LPS-tolerant mice to live *S. typhimurium* affected their susceptibility to infection with these Gram-negative bacteria.

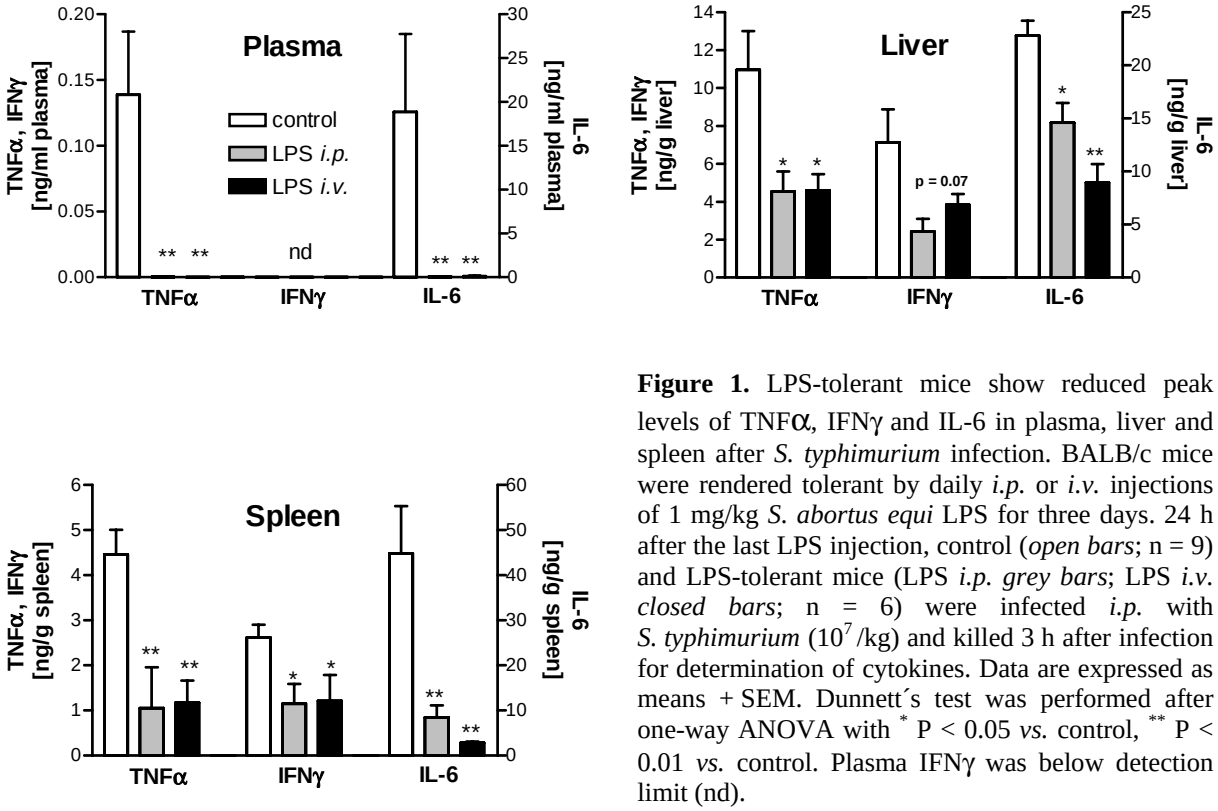

Figure 1. LPS-tolerant mice show reduced peak levels of TNFα, IFNγ and IL-6 in plasma, liver and spleen after *S. typhimurium* infection. BALB/c mice were rendered tolerant by daily *i.p.* or *i.v.* injections of 1 mg/kg *S. abortus equi* LPS for three days. 24 h after the last LPS injection, control (*open bars*; n = 9) and LPS-tolerant mice (LPS *i.p. grey bars*; LPS *i.v. closed bars*; n = 6) were infected *i.p.* with *S. typhimurium* (10^7 /kg) and killed 3 h after infection for determination of cytokines. Data are expressed as means + SEM. Dunnett´s test was performed after one-way ANOVA with * $P < 0.05$ *vs.* control, ** $P < 0.01$ *vs.* control. Plasma IFNγ was below detection limit (nd).

4.4.3 Prolonged survival of LPS-tolerant mice after lethal infection with *Salmonella typhimurium*

Mice were pretreated with saline or LPS as described above and infected with *S. typhimurium* (10^7 /kg *i.p.*) 24 h after the last LPS injection. LPS-tolerant mice survived significantly longer than non-tolerant control mice (154 ± 13 h *vs.* 76 ± 8 h, n = 12; $P < 0.001$). The onset of weight loss observed in control mice already one day after injection of bacteria was delayed by 2 days in LPS-tolerant mice. In addition, LPS-tolerant mice showed no symptoms of disease, i.e. no piloerection and apathy, during the first 3 days.

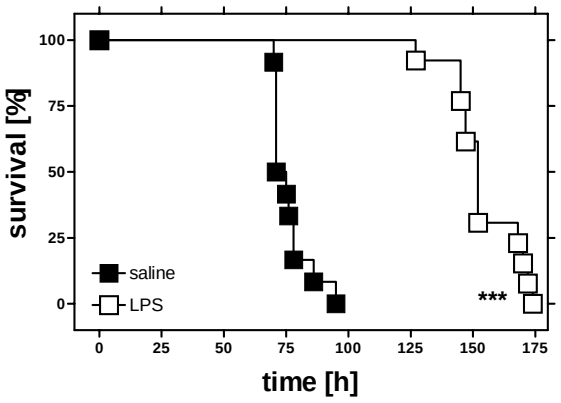

Figure 2. Mice were injected with i.p. with 1 mg/kg LPS (□) or saline (■) at - 72, -48, -24 h prior to *Salmonella typhimurium* infection (10^7/kg, i.p.)

4.4.4 Reduction of bacterial load in LPS-tolerant mice

We next examined whether the prolongation of survival resulted from improved bacterial killing or from tolerance towards higher numbers of *S. typhimurium.* Therefore, we determined the time course of bacterial load in different organs of control and LPS-tolerant mice infected *i.p.* with *S. typhimurium* (10^7 /kg). In control mice, after a negligible early reduction of CFU in the peritoneum, bacterial numbers increased, and after 6 h strongly outnumbered the primary inoculum. In contrast, in LPS-tolerant mice we found a continuous decrease in bacteria numbers in the peritoneal cavities during the first 6 h of infection. At the end of this period, the peritonea of LPS-tolerant animals contained about 10^4 times less CFU compared to those of control mice. In addition, dissemination of bacteria into blood, liver and spleen, which became apparent 30 minutes after induction of infection, was strongly reduced in LPS-tolerant mice (Fig.3).

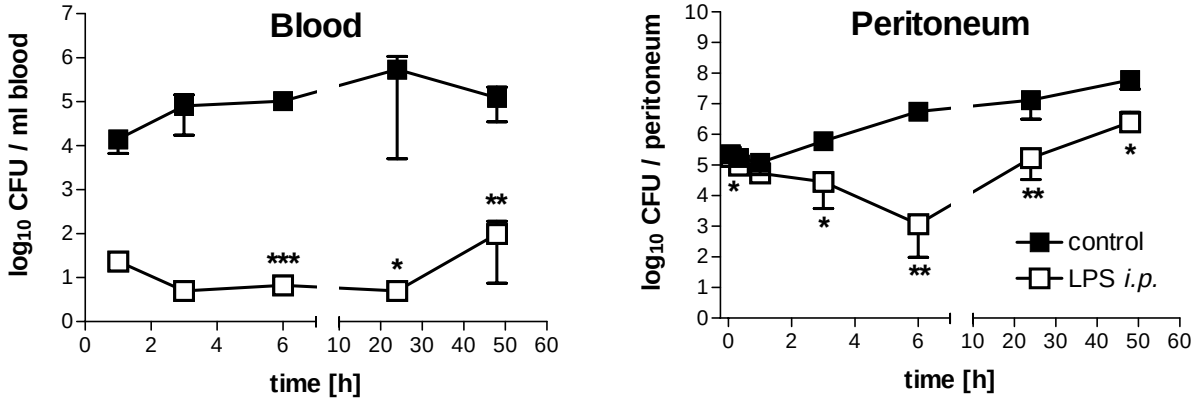

Figure 3. Time course of bacterial load in peritoneal lavage and blood of control (■) and LPS-tolerant mice (LPS *i.p.* □) during *S. typhimurium* infection (10^7 /kg, *i.p.*). Data are shown as means ± SEM, n = 3. For statistical analysis, the unpaired two-sided Student´s t-test was performed for each time point with log transformed data (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ all *vs.* control).

The reduction of bacterial load was associated with up to five times the numbers of leukocytes in the peritoneal cavity of LPS-tolerant mice compared to controls (Fig.4). As those elevated numbers of peritoneal leukocytes were already observed at the onset of infection, we studied the interrelationship between the LPS-pretreatment and these leukocytes in more detail.

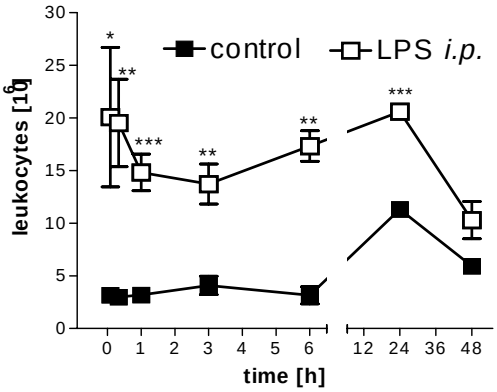

Figure 4. Time course of peritoneal cell numbers in control (■) and LPS-tolerant mice (LPS *i.p.* □) infected with *S. typhimurium* (10^7 /kg *i.p.*). Total peritoneal cell numbers are expressed as means ± SEM, n = 3. For statistical analysis, log transformed data were tested with the unpaired two-sided Student´s t test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ all *vs.* control).

4.4.5 Intraperitoneal LPS-pretreatment induces local accumulation of neutrophils

Analysis of number and composition of peritoneal leukocytes prior to and subsequent to the LPS injections revealed a steady accumulation of leukocytes during the pretreatment phase which was mainly due to an influx of polymorphonuclear leukocytes (PMN). At the time of induction of infection, LPS-pretreated mice had total numbers of peritoneal leukocytes that were about 5-fold higher than control values. In contrast to control mice where less than 2% of total peritoneal leukocytes were PMN, the locally elicited cells in LPS-pretreated animals comprised 80% PMN (Table II). It became conceivable that the intraperitoneal administration of LPS during tolerance induction, the local accumulation of PMN in the peritoneal cavity, and the early inactivation of bacteria represent a causal sequence of events in this model.

Table II. *Effect of repeated LPS injections on peritoneal leukocyte counts* [a]

Treatment	Total leukocytes [10^6]	PMN [10^6]	Mononuclear cells [10^6]
Naive	1.7 ± 0.2 [bb]	< 0.02	1.7 ± 0.2
1 x LPS	4.5 ± 1.0 [c]	3.7 ± 0.9 [d]	0.8 ± 0.1
2 x LPS	7.9 ± 4.3 [d]	6.5 ± 3.7 [d]	1.5 ± 0.6
3 x LPS	23.3 ± 2.4 [d]	17.4 ± 3.2 [d]	5.9 ± 1.0 [d]

[a] Mice were treated with *Salmonella abortus equi* LPS (1 mg/kg; *i.p.*) one to three times at 24 h intervals. 24 h after the last LPS injection, total and differential counts of peritoneal cells were performed. Saline injection had no effect on peritoneal cell numbers as compared to naive mice.
[b] Data are expressed as means ± SD with n = 3.
c Significantly different from control ($P < 0.05$).
d Significantly different from control ($P < 0.01$).

4.4.6 Effect of intravenous LPS administration

In order to test the hypothesis that intraperitoneal leukocyte accumulation represents the protective mechanism of tolerance induction, we changed the route of LPS-administration: instead of *i.p.* administration, LPS was injected via the tail vein, thus circumventing local accumulation of leukocytes prior to *i.p. S. typhimurium* infection. In contrast to *i.p.* LPS-pretreatment, the total number and composition of peritoneal leukocytes was not increased after the intravenous LPS injections. In parallel, the reduction of the bacterial load in the peritoneal cavity, blood, liver and spleen was much less pronounced in the LPS *i.v.* mice compared to LPS *i.p.* pretreated animals. Six hours after injection of bacteria, LPS *i.v.* mice contained 10^2-10^3 times more CFU than LPS *i.p.* animals. However, at 24 h and 48 h after infection, comparable numbers of *S. typhimurium* were recovered from blood and peritoneal lavage of LPS *i.v.* and LPS *i.p.* pretreated mice. (Fig. 5). Taken together, accumulation of leukocytes due to intraperitoneal administration of LPS seems to be a prerequisite for improved inactivation of *S. typhimurium* in the peritoneum during the early course of infection, but does not explain the systemic reduction of bacteria in LPS i.v. mice at late stages of infection.

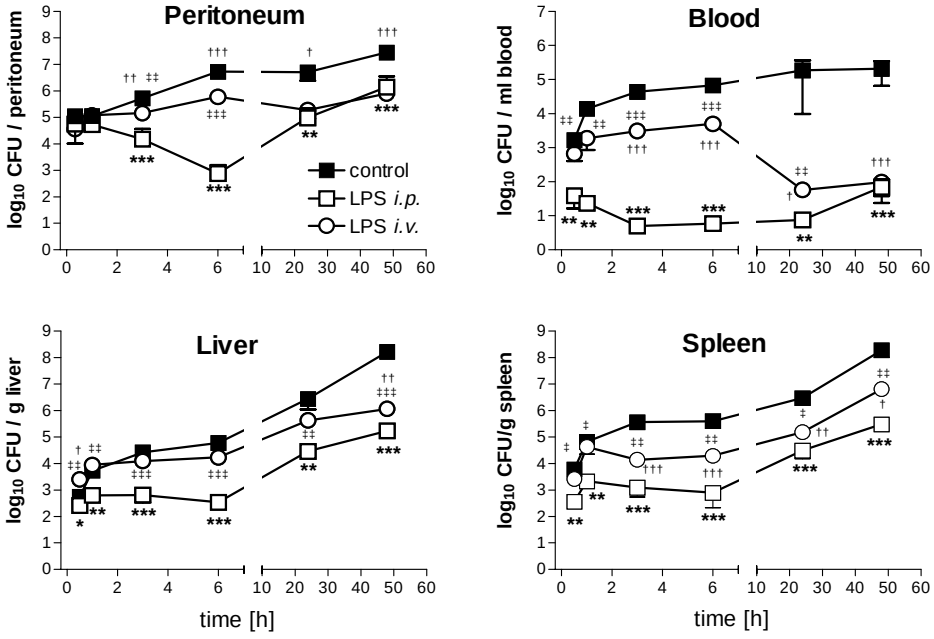

Figure 5. Effect of different LPS-administration routes for tolerance induction on time course of bacterial load. Control (■) and LPS-tolerant mice (LPS-pretreatment *i.p.* (□) or *i.v.* (○)) were infected *i.p.* with *S. typhimurium* (10^7 /kg). Data are means ± SEM with n = 9 for controls and n = 6 for either of the LPS groups. For statistical analysis, an unpaired two-tailed Student´s t-test was done after one-way ANOVA of log transformed data to compare the three groups at each time point individually (* P < 0.05 LPS *i.p. vs.* control; ** P < 0.01 LPS *i.p. vs.* control; *** P < 0.001 LPS *i.p. vs.* control; † P < 0.05 LPS *i.v. vs.* control; †† P < 0.01 LPS *i.v. vs.* control; ††† P < 0.001 LPS *i.v. vs.* control; ‡ P < 0.05 LPS *i.v. vs.* LPS *i.p.*; ‡‡ P < 0.01 LPS *i.v. vs.* LPS *i.p.*; ‡‡‡ P < 0.001 LPS *i.v. vs.* LPS *i.p.*).

4.4.7 Increased emergency recruitment of leukocytes in LPS-tolerant mice

We determined the numbers of circulating leukocytes in either tolerant or control mice during lethal *Salmonella* infection (10^7 /kg *i.p.*). We found sustained leukocytosis with an increased proportion of neutrophilic granulocytes in LPS-pretreated mice throughout the course of infection. The difference between tolerant and control mice in white blood cell counts was most prominent at late phases of infection: 48 hours post infection, blood leukocytes were up to 4-fold higher in LPS-tolerant mice ($P < 0.01$). The percentage of PMN steadily increased in all groups during the course of infection, but was consistently higher in LPS-pretreated animals (Table III).

Table III: Effect of LPS pretreatment on blood leukocytes during S. typhimurium infection [a]

Time point [h]	Pretreatment	Total leukocytes [10^6 /ml]	PMN [%]	Lymphocytes [%]	Monocytes [%]
1h	control	2.3 ± 1.6 [b]	18 ± 8	70 ± 11	9 ± 5
	LPS *i.p.*	5.2 ± 1.9 [c]	76 ± 6 [c]	14 ± 6 [d]	10 ± 2
	LPS *i.v.*	2.9 ± 1.0	51 ± 8 [c]	35 ± 6 [d]	14 ± 4
48h	control	1.2 ± 1.0	51 ± 14	34 ± 15	12 ± 8
	LPS *i.p.*	4.9 ± 2.4 [d]	61 ± 11	27 ± 7	10 ± 7
	LPS *i.v.*	3.3 ± 1.3 [d]	68 ± 3	20 ± 4	12 ± 2

[a] Mice were treated *i.p.* or *i.v.* with *Salmonella abortus equi* LPS (1 mg/kg) three times at 24 h intervals. 24 h after the last LPS injection, LPS-tolerant mice and saline pretreated controls were infected with *Salmonella typhimurium* (10^7 /kg; *i.p.*). Total and differential counts of blood leukocytes were performed 1 h and 48 h after infection.
[b] Data are expressed as means ± SD with n = 9 for saline controls and n = 6 for either of the LPS groups.
[c] Significantly different from saline control ($P < 0.05$).
[d] Significantly different from control ($P < 0.01$).

Additionally, determination of MPO activity suggested significantly increased tissue PMN numbers in livers (six-fold increase by 3 h, $P < 0.01$) and spleens (two-fold increase by 24 h, $P < 0.001$, three-fold increase by 48 h, $P < 0.05$) of LPS-pretreated mice during infection. It thus seemed probably that the enhanced capacity to recruit phagocytes to the major sites of bacterial proliferation contributed to reduction of bacterial load in LPS-tolerant mice.

4.4.8 Partial reversal of survival benefit by depletion of PMN

To assess the contribution of PMN to the prolonged survival of LPS-tolerant mice we depleted neutrophils by administration of anti-PMN antibodies. Injection of anti-Ly-6G rat IgG2b (clone RB6-8C5) (22) at –16 h, +6 h and +30 h efficiently depleted circulating PMN numbers by > 90% ($P < 0.001$ for saline and LPS pretreatment + RB6-8C5 vs. respective control IgG) and partially reversed the beneficial effect of LPS pretreatment on survival time (Table IV). Although the survival benefit of LPS-tolerant mice was decreased by approximately 60 % (26 h survival prolongal vs. 68 h) by depletion of neutrophils, tolerant animals still survived significantly longer than PMN-depleted controls ($P < 0.001$). It is concluded that increased PMN numbers only partially account for prolonged survival of LPS-tolerant mice.

Table IV: Effect of PMN-depletion on survival time [a]

Treatment	Survival time [h] mean ± SD	Survival time [h] median
Saline + control IgG	123 ± 22	115
Saline + anti-Ly-6G IgG	47 ± 3	47
LPS + control IgG	191 ± 60	168[b]
LPS + anti-Ly-6G IgG	73 ± 3	74[c]

[a] Mice were pretreated *i.v.* with saline or *Salmonella abortus equi* LPS (1 mg/kg) three times at 24 h intervals. 24 h after the last LPS injection, LPS-tolerant mice and saline pretreated controls were infected with *Salmonella typhimurium* (10^6 /kg; *i.p.*) and survival time was determined. To study the role of PMN, mice received anti-Ly-6G IgG (RB6-8C5) or normal rat IgG *i.p.* 16 h prior to (0.6 mg) and 8 h and 32 h after initiation of infection (0.3 mg), respectively. Data are from one of two experiments with n = 6.
[b] Significantly different from saline + control IgG ($P < 0.05$).
[c] Significantly different from saline + anti-Ly-6G ($P < 0.001$).

4.4.9 Increased hepatic uptake of *Salmonella typhimurium* in LPS-tolerant mice

Besides PMN, the macrophages of the reticuloendothelial system (RES) are involved in elimination of bacteria. To test whether the activity of the RES was altered by LPS-pretreatment, we determined the clearance of systemically injected *S. typhimurium* (10^8 /kg) in LPS-tolerant and control mice. *S. typhimurium* were cleared much more rapidly in LPS-tolerant mice (0.1 ± 0.1% of inoculum recovered from blood of LPS-tolerant mice 20 min after intravenous administration *vs.* 28.6 ± 13.5% in controls; n = 6; $P < 0.001$; Fig. 6). Simultaneously, livers of LPS-pretreated mice contained approximately 2-3 times more bacteria than livers of control mice after the first 20 min (Fig. 6). At later time points, similar

numbers of bacteria were found in livers of both treatment groups. In contrast, although splenic uptake of bacteria was comparable during the first 10 min, numbers of bacteria continuously increased in spleens of controls but not of LPS-pretreated mice (Fig. 6). We next assessed whether the increased early hepatic uptake of bacteria reflected numerical changes in Kupffer cells, i.e. liver macrophages, resulting from LPS pretreatment. Immunohistological examination demonstrated an approximately three-fold increase of cells positive for the macrophage antigen F4/80 in livers of LPS-tolerant mice compared to controls (15.1 ± 6.3 /630x field in LPS-tolerant mice *vs.* 4.5 ± 0.7 in controls; n = 4; $P < 0.05$).

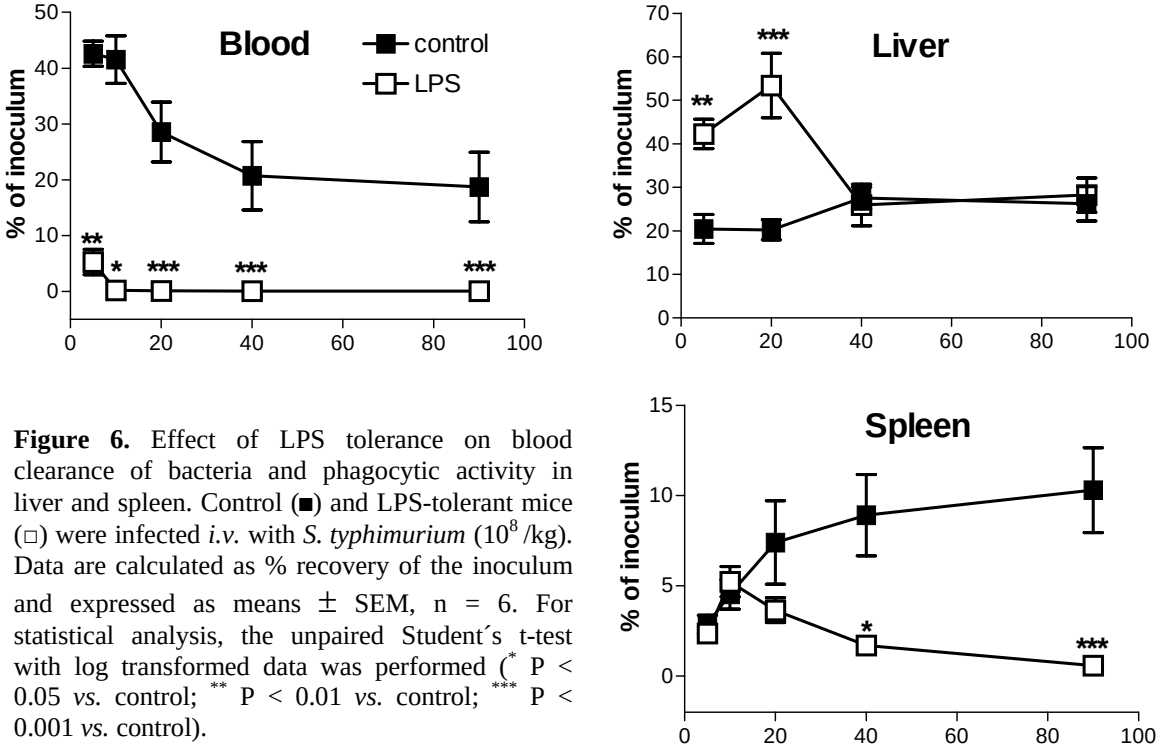

Figure 6. Effect of LPS tolerance on blood clearance of bacteria and phagocytic activity in liver and spleen. Control (■) and LPS-tolerant mice (□) were infected *i.v.* with *S. typhimurium* (10^8 /kg). Data are calculated as % recovery of the inoculum and expressed as means ± SEM, n = 6. For statistical analysis, the unpaired Student´s t-test with log transformed data was performed (* $P < 0.05$ *vs.* control; ** $P < 0.01$ *vs.* control; *** $P < 0.001$ *vs.* control).

In order to examine the possible causal relation of increased numbers of Kupffer cells and improved clearance of blood-born *S. typhimurium*, we depleted macrophages by injection of Cl_2MBP-containing liposomes prior to injection of bacteria. In line with the efficient elimination of F4/80 positive liver macrophages which was controlled by immunohistology, administration of liposomes strongly decreased hepatic uptake of bacteria in control and tolerant mice, resulting in complete ablation of the improved clearance of *S. typhimurium* observed in non-depleted LPS-tolerant mice (Fig. 7). These results suggest that besides leukocyte accumulation in the peritoneum and accelerated neutrophil recruitment, improved activity of the RES due to increased numbers of Kupffer cells, contributes to the systemic reduction of *S. typhimurium* numbers in LPS-tolerant mice.

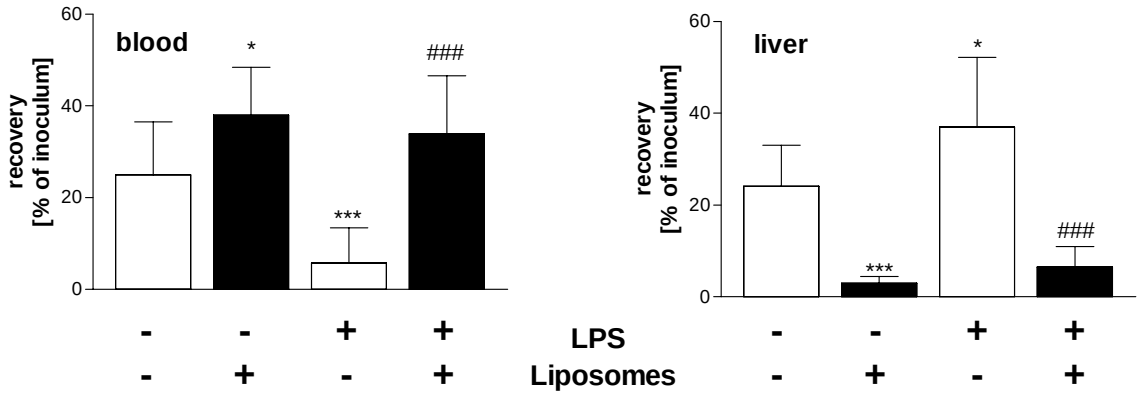

Figure 7. Clearance of *S. typhimurium* in control (co) and LPS-tolerant (LPS) mice after macrophage-depletion with Cl_2MBP-liposomes. LPS tolerance was induced by daily *i.p.* administration of 1 mg/kg *S. abortus equi* LPS for three days. 24 h, 48 h and 71 h after the last LPS injection, liposomes or pyrogen-free saline were injected *i.v.*. One hour after the last injection of liposomes, mice were infected *i.v.* with *S. typhimurium* (10^8 /kg). 10 min after injection of *S. typhimurium*, viable bacteria were determined in blood and liver homogenates and calculated as % recovery of the inoculum. Pooled data from 3 experiments are expressed as means ± SEM with 7-14 mice per group. For statistical analyis, the Bonferroni-test for selected groups was done after one-way ANOVA (* $P < 0.05$, *** $P < 0.001$ *vs.* saline control (co/-), ††† $P < 0.001$ *vs.* LPS control (LPS/-)).

4.5 Discussion

Endotoxin-tolerance is known to protect prophylactically against mortality and morbidity in endotoxic shock, LPS- and TNFα-mediated liver damage and various models of fulminant infection with high numbers of bacteria. In these models, the crucial role of the proinflammatory cytokines TNFα, IL-1 and IFNγ as distal mediators of LPS toxicity leading to shock and death is well documented (177, 318, 319). It was therefore logical to ascribe protection due to tolerance induction to an attenuated response of effector cells, diminished sensitivity of target cells, and to a general limitation of tissue damage by infiltrating leukocytes (192, 241). On the other hand, the pivotal role of an intact cytokine response, in particular regarding the release of TNFα, IL-1, IFNγ and IL-6, for host defense against bacterial infections has been unequivocally shown in different infection models (29, 194, 196, 320, 321). These studies clearly demonstrate that in contrast to the models of hyperinflammatory damage, a successful immune defense against infectious diseases, which normally start with low numbers of virulent bacteria, requires a vigorous inflammatory response.

These experimental differences prompted us to carry out an LPS tolerance/infection study where we created a more drastic situation of hyporesponsiveness to endotoxin by giving repeated injections of a nearly lethal LPS dose (0.3 x LD_{50}). For the infection, we chose a

lethal dose of virulent *Salmonella typhimurium*, a Gram-negative bacterium that causes systemic reactions and symptoms resembling human typhoid fever (18). In contrast to the commonly used single low dose injection of LPS 24 h prior to high dose LPS-challenge, our LPS tolerance induction regimen not only blunted the release of TNFα, but also inhibited or reduced the production of other cytokines, i.e. IFNγ and IL-6 in response to subsequent LPS-challenge (Table I) or intraperitoneal *S. typhimurium* infection (Figure 1). In contrast to mere LPS challenge, cytokine release was not abrogated completely after infection with viable *S. typhimurium*, suggesting that immune stimuli other than LPS are also transmitted by these Gram-negative bacteria.Experimental induction of LPS hyporesponsiveness did not cause increased susceptibility of mice to *S. typhimurium* infection as observed in innately LPS-unresponsive (lps^d) mice (322), but instead improved survival. Since this was associated with a decrease in the bacterial load in the peritoneal lavage, blood, liver and spleen, the prolongation of survival is unlikely to stem from the known dampening of the proinflammatory immune response during LPS tolerance. This view is supported by our observation (unpublished) that immunosuppression by dexamethasone, which protects against LPS shock by blocking the proinflammatory response, failed to increase survival time of *S. typhimurium* infected mice. Moreover, although LPS-tolerant mice survived significantly longer than control animals, the bacterial load of various organs at the time of death did not differ substantially between the different treatment groups. This indicates that prolonged survival was not the result of an improvement in the immune system´s capacity to deal with high numbers of bacteria.

This led us to the assumption that improved early inactivation of *S. typhimurium* might be responsible for the increase in mean survival time, raising the question as to possible mechanisms contributing to enhanced host defense. A comparison of the time course of bacterial proliferation in control and LPS-tolerant mice showed that enhanced inactivation of bacteria in tolerant mice was already observed one hour after inoculation. Consequently, six hours post infection LPS-tolerant mice carried approximately four orders of magnitude fewer CFU in the peritoneal cavity than control animals. Dissemination of bacteria to blood and subsequently to liver and spleen was also diminished. Therefore, we related the accumulation of professional phagocytes in the peritoneal cavity, the later site of injection of bacteria, to the enhanced inactivation of *S. typhimurium* observed in LPS-tolerant mice immediately after infection. The experiments with LPS injections *i.v.* instead of *i.p.* support this interpretation (Figure 4). Others have also previously pointed out the importance of localized therapy in the

prevention of lethal sepsis by tolerance induction: In their experimental setting, intraperitoneal injection of monophosphoryl lipid A was much more efficient in decreasing mortality after lethal cecal ligation and puncture than intravenous administration (241). Surprisingly, mice made tolerant by intravenous LPS injection also showed an extended survival time compared to control mice. Moreover, a similar decrease in bacterial load in blood and peritoneal lavage 48h after *i.p. Salmonella* infection was found in LPS *i.p*. and LPS *i.v.* pretreated animals. This suggests an additional mechanism in fighting the bacteria at later stages of infection.

LPS is a potent stimulator of hematopoiesis and administration of LPS or derivatives is associated with the production of various colony-stimulating factors (CSF) (277, 323), increased total numbers of circulating leukocytes (245), neutrophilia (60) and augmented numbers of monocyte/macrophage precursors in the bone marrow (246). Early reports ascribed increased resistance against infection and lethal irradiation after pretreatment with endotoxin to the leukopoietic properties of endotoxin (227). During infection with *S. typhimurium*, PMN are able to limit bacterial growth within host cells by lysis of infected hepatocytes and subsequent phagocytosis of extracellular bacteria, e.g. in the sinusoids of the liver (21). Since we actually found higher numbers of circulating neutrophils in blood as well as increased tissue infiltration of PMN indicated by enhanced MPO activity in LPS-tolerant mice during the course of infection, it is conceivable that this mechanism contributes to bacteriostasis in liver and spleen, which are the major sites of replication of *S. typhimurium*. Indeed, PMN depletion reduced the increase in survival time associated with LPS tolerance (i.v.) by approximately 60%.

The enhanced clearance of bacteria from blood of LPS-tolerant mice, on the other hand, is due to a more efficient phagocytic activity of the RES as shown by our intravenous inoculation experiments. This interpretation is in line with previous findings that show enhanced phagocytosis of bacteria or latex particles by Kupffer cells of LPS-tolerant animals *in vivo*, or in the perfused liver *ex vivo* (234, 239). By immunohistological examination we demonstrated approximately three-fold augmented numbers of F4/80-positive cells in livers of LPS-tolerant mice. The antigen recognized by the F4/80 clone is expressed by several macrophage populations including Kupffer cells in the liver (324)**.** This suggests that the enhancement of RES activity associated with LPS tolerance induction originates at least partly from an increase in the numbers of liver macrophages. Independent evidence for this conclusion

derives from our macrophage depletion experiments using Cl_2MBP-liposomes that selectively accumulate in macrophages which are subsequently driven into apoptosis (316).

Our results that primarily cells of the innate immune system are involved in increased resistance of LPS-pretreated mice against *Salmonella typhimurium* infection are corroborated by the finding that athymic BALB/c mice, which lack functional T-cells and wild-type littermates equally benefit from LPS tolerance induction prior to bacterial infection (our unpublished data and (237)).

Besides antibody mediated phagocytosis, opsonization of bacteria by complement components facilitates receptor mediated uptake of bacteria by phagocytes. Published data are conflicting as to the activity of the complement system in endotoxin tolerance (217, 231). Since it is feasible that an increase in complement activity in the course of an acute phase response elicited by endotoxin administration could account for improved phagocytosis in our model, we determined complement activity (CH_{50} values) of sera from LPS-tolerant and control mice. In a modified rabbit erythrocyte lysis assay (325), no difference in total (classical + alternative) complement activity was detectable after LPS-pretreatment. Moreover, depletion of complement component C3 by administration of cobra venom factor (326), which efficiently abrogated complement mediated erythrocyte lysis, did not ablate improved reduction of bacteria in *S. typhimurium* infected LPS *i.v.* tolerant mice (unpublished results). Therefore, we consider this possibility unlikely.

In conclusion, this study provides evidence that induction of profound LPS tolerance, despite reducing cytokine production, improves host defense against infection with virulent *Salmonella typhimurium.* Several independent mechanisms contribute to enhanced resistance of LPS-pretreated mice by decreasing bacterial load at different stages of infection as shown by assessing the immunomodulation and blocking the respective alterations. Namely, local accumulation of leukocytes in the peritoneal cavity, improved recruitment of PMN during the course of infection and an increase in liver macrophage numbers account for the improved host defense. Although the data shown here derive from experiments with a Gram-negative, facultative intracellular bacterium, the protective effect of LPS tolerance induction applies for other models, using extracellular or Gram-positive bacteria as infectious agents, too. We could show that our pretreatment to induce LPS tolerance increased survival rates of mice lethally infected with *Staphylococcus aureus*, *Listeria monocytogenes* or a human stool suspension to induce a multi-germ peritonitis, which more closely mimicks the physiological

situation of the septic patient. Similar findings were reported recently for infection of LPS-tolerant mice with *Cryptococcus neoformans* (230).

The combination of two desirable effects, i.e. attenuation of systemic inflammatory responses and a concomitant fortification of host defense against infections, makes LPS tolerance a valuable model for sepsis prophylaxis.

5 Discussion

5.1 The LTA receptor

LTA from the cell membrane of Gram-positive bacteria is a potent activator of monocytes/macrophages, inducing cytokine release similar to LPS (10, 12, 327). Because of its amphiphilic structure and similar biologic activity LTA have been considered as the Gram-positive equivalent to LPS. Although LTA and LPS are both recognized by CD14 expressed on the surface of monocytes/macrophages (14, 15, 327), the question whether identical TLR mediate signaling in response to LPS and LTA has been discussed controversially (16, 17). The first reports on TLR involvement in LPS signaling derived from experiments using transfected cell lines identified expression of human TLR2 as sufficient for NF-κB translocation in response to LPS stimulation (120, 306, 328, 329). In contrast, inherently LPS hyporesponsive mice were found to have mutations in the tlr4 gene resulting in a nonfunctional receptor, which indicated that LPS signaling was mediated primarily via the TLR4 (199-201). In line, TLR4 knock-out mice displayed impaired LPS responsiveness, whereas no effect on LPS-induced cytokine expression was observed in mice lacking TLR2 (17, 202). Moreover, repurification of LPS resulted in a loss of cytokine production in TLR4-mutated C3H/HeJ mice, but not in the congenic C3H/HeN strain, suggesting that lipoprotein contamination of the commercial LPS preparation was responsible for macrophage activation in the absence of TLR4 (330). Our results substantiate the view that TLR4 is the major receptor responsible for LPS-induced cytokine production: Peritoneal macrophages from TLR4-mutated C3H/HeJ mice did not show TNF production up to concentrations of 1-10 ng/ml, whereas congenic C3H/HeN macrophages were already stimulated at 10 pg LPS /ml. In contrast, LPS stimulation of TLR2-deficient macrophages resulted in TNF release comparable to wild-type cells.

Several reports have been published recently also on the involvement of toll like receptors in signaling upon Gram-positive stimuli. Cells transfected with human or mouse TLR2 but not with TLR4 showed NF-kB translocation upon stimulation with peptidoglycan, lipoteichoic acid or whole Gram-positive bacteria (16, 304, 331). In line, macrophages from TLR2-deficient mice are hyporesponsive upon stimulation with *S. aureus* cell walls or peptidoglycan (17). As a consequence, these mice are highly susceptible to infection with viable *S. aureus*, although the effect is less pronounced than in MyD88-knock out mice, suggesting that other TLR can partially substitute for TLR2 in recognition of Gram-positive bacteria (332). The same authors stated that cytokine response by TLR4-deficient macrophages stimulated with

LTA was partially impaired, whereas normal cytokine release after administration of peptidoglycan or *S. aureus* cell walls was observed (17).

Our data obtained with C3H/HeJ mice lacking functional TLR4 show TNF induction in response to highly purified LTA independent of TLR4. In contrast, no TNF release upon LTA-stimulation of macrophages from TLR2-deficient mice was observed, whereas LPS-signaling was affected. These results strongly support the notion that TLR2 is the major signaling receptor not only for peptidoglycan and Gram-positive cell walls, but also for lipoteichoic acid.

5.2 Specificity of tolerance

The question whether early phase nonspecific tolerance is restricted to endotoxins as a class or whether it reflects a general state of altered macrophage activity resulting in diminished cytokine expression in response to non-endotoxin inflammatory stimuli as well has not been settled . The view that tolerance was restricted to endotoxins as a class originates from experiments performed by Greisman et al. demonstrated that rabbits rendered LPS-tolerant by infusion of endotoxin for several hours displayed normal fever reaction in response to pyrogenic non-endotoxin challenges such as influenza virus, old tuberculin and staphylococcal enterotoxin (47). Similarly, Roth et al. showed a lack of cross-reactivity between LPS and muramyl-dipeptides in terms of fever induction and production of TNF and IL-6 in guinea pigs (170). However, the experimental setting consisting of repeated injections of endotoxin over a period of 15 days with administration of muramyl-dipeptide 3 days after the last LPS injection may have been unsuitable to study the specificity of the early phase tolerance which is most prominent within the first 48 h and then starts to wane. Lack of cross-tolerance was reported also by Mathison et al. who failed to suppress TNF-release in response to *Staphylococcus aureus* by preexposure of rabbit macrophages to LPS (101). Similarly, LPS-tolerant Kupffer cells still produced TNF upon viral infection (171). However, differential suppression of TNF and IL-1 was reported by Wakabayashi et al. who showed that PBMC isolated from LPS-tolerant rabbits still produced TNF, but no IL-1, in response to *Staphylococcus epidermidis* (65), proposing differential regulation of these cytokines during hyporesponsiveness.

Further evidence that downregulation of macrophage/monocyte function after LPS-pretreatment is not restricted to restimulation with endotoxins was provided by Granowitz et al. (75). They demonstrated a reduction of cytokine release by human PBMC derived from

endotoxin pretreated volunteers restimulated ex vivo with LPS, IL-1 or TSST-1. In line, Cavaillon et al. reported suppression of TNF-release in response to zymosan, staphylococci and streptococci after exposure of human monocytes to LPS *in vitro* (76, 172). Similar results were obtained more recently by Karp et al. for downregulation of IL-12 production (130). In this work we demonstrate that pretreatment with LTA induced desensitization of macrophages and *in vivo* tolerance similarly to the effect of LPS pretreatment. In contrast to LPS, preexposure to LTA induced macrophage hyporesponsiveness in the absence of functional TLR4. Instead, preexposure to LTA failed to confer refractoriness to TLR2-deficient cells, whereas LPS-induced TNF suppression was unaffected in these cells. Thus, we can exclude that the observed suppression of TNF release in response to LTA were due to a possible endotoxin contamination of our LTA preparation.

Similar results on cross-tolerance induced by LPS and mycoplasmal lipopeptides via different TLR were published recently (123). These and our findings confirm and extend earlier reports on TNF suppression upon repeated exposure to whole *S. aureus* or synthetic lipopeptides (173). These findings suggest that tolerance and macrophage desensitization could represent a uniform mechanism induced by selected bacterial stimuli to prevent potentially harmful overshooting inflammation during sustained infection.

5.3 Mechanisms of macrophage desensitization

In the last years, our understanding of the molecular mechanisms underlying desensitization of macrophages by exposure to LPS has increased considerably. Expression of CD14 is unaffected or even increased following LPS-stimulation, thus it is highly unlikely that tolerance is mediated via expression of this LPS receptor (95, 109). Refractoriness in response to LPS preexposure has been shown to be associated with altered G-protein content (112, 113), phospholipase D and phosphatidylinositol-3 kinase expression (114). West et al. reported compromised protein kinase C (PKC) activation in LPS desensitized cells (115) and receptor independent stimulation of the PKC by phorbol myristate acetate could overcome the suppression of cytokine production associated with refractoriness. Others described suppressed signal transduction via both the MAP kinase cascade (98, 116-118) and I-κB kinases, resulting in impaired transcription of NF-κB- and AP-1-regulated genes (85, 116). An alternative mechanism for suppression of NF-kB-dependent gene expression was suggested by Ziegler-Heitbrock et al.. They used a human monocyte cell line (Mono Mac-6) to demonstrate an upregulation of the p50 subunit of NF-kB in LPS refractory cells, leading to

a predominance of transactivation-inactive p50/p50 homodimers. These homodimers bind to NF-κB motifs in several promotors and thereby inhibit the transcription of genes such as TNF (94, 110). Support for this hypothesis originates from experiments with p50 deficient mice that are resistant to tolerance induction by LPS (100). Inhibition of gene transcription in response to a second LPS stimulus via the formation of a specific nuclear suppressor of LPS-induced gene transcription was also suggested by others (84, 86). LaRue et al. provided evidence that decreased LPS-induced transcription of IL-1β in LPS-desensitized THP-1 cells was regulated by a labile repressor which required constant protein synthesis and suggested I-κB-α as a potential candidate, although by then a contribution of p50 had not been studied.
In contrast, recent data showing decreased surface expression of TLR4 on LPS-tolerized cells (111) and suppression of IL-1 receptor-associated kinase (IRAK) activation and association with myeloid differentiation protein (MyD88) (119), support the notion that already very early steps in LPS-signaling upstream of NF-kB are altered after LPS-exposure. Further evidence for this was provided by Medvedev et al. (116) who re-evaluated *in vitro* desensitization by IL-1 and TNF, showing induction of cross-tolerance to LPS via the IL-1 receptor but not the TNF-receptor. Intriguingly, signal transduction of the IL-1R, the LPS-receptor TLR4, and TLR2 employ similar signaling molecules (120-122). In this thesis it was demonstrated that preexposure to lipoteichoic acid inducing signaling via TLR2 results in hyporesponsiveness to TLR4-mediated LPS-signaling and vice versa. This finding adds further indirect evidence for a suppression of common signaling molecules shared by TLR2/4 and IL-1R, i.e. MyD88, IRAK, TNF receptor-activated factor 6 (TRAF6) or NF-κB-inducing kinase (NIK) in desensitized macrophages. As demonstrated by Nomura et al., LPS induced macrophage desensitization was associated with decreased surface expression of TLR4. As LPS tolerance induced cross-desensitization to the TLR2 ligand LTA, it is mandatory to study whether surface expression of this receptor (and also of the IL-1RI) is affected in the same way. First data on TLR2 mRNA published by Medvedev et al. suggest an inverse regulation of TLR2 and TLR4 after exposure of macrophages to LPS. However, as shown by Nomura, the effects on TLR surface expression were not associated with significant downregulation of TLR4 mRNA. Thus, unless antibodies directed against the extracellular domain of TLR2 are available this issue can not be settled satisfactorily. Recent data published by Sato et al. on cross-desensitization by mycoplasmal lipopeptide and LPS via TLR2 and TLR4, respectively, indicate that in contrast to LPS-induced LPS tolerance, macrophage desensitization in response to MALP-2 exposure was not associated with reduced surface expression of TLR4.

Despite the large number of studies dealing with macrophage hyporesponsiveness in response to LPS pretreatment, the exact mechanism of suppression of cytokine production has not been identified yet. Since there is sound evidence for a contribution of various of the aforementioned factors, it is feasible that i) macrophage desensitization is the result of the orchestrated action of multiple factors activated by the primary LPS stimulus or ii) depending on the model employed to study tolerance (species, cell type, experimental settings) varying distinct mechanisms account for refractoriness in response to inflammatory bacterial components.

5.4 Mediators of tolerance

In our model system of macrophage desensitization by LPS versus LTA we also studied whether hyporesponsiveness could be transferred by soluble mediators to cells inherently inert to the desensitizing agent due to TLR deficiencies. The idea that autocrine mechanisms were involved in tolerance induction was suggested shortly after the finding that most of the effects of endotoxin were mediated by cytokines and arachidonic acid metabolites released.

5.4.1 *In vitro* desensitization

As outlined before, LPS-pretreatment of cultured macrophages results in hyporesponsiveness to cytokine release in response to a subsequent LPS stimulus. It has been shown that several cytokines could substitute for LPS as the desensitizing stimulus. Cavaillon et al. demonstrated that incubation of human PBMC with recombinant cytokines prior to restimulation with LPS partially suppressed production of TNF to a different extent. Whereas preexposure to TGFβ or IL-10 reduced TNF release by nearly 60% as compared to saline pretreated cells, IL-4 and IL-1 were less effective (35% and 30% inhibition, respectively) and no inhibition at all was found after administration of TNF, IL-6, IL-8 or leukaemia inhibitory factor (76). The differential role of TNF and IL-1β in desensitization of macrophages in vitro was confirmed by recent studies from Medvedev et al. who showed that exposure of murine macrophages to LPS or IL-1, but not to TNF resulted in inhibition of transcription factor activation and suppressed transcription of GM-CSF and several chemokines in response to a second LPS stimulus (116). Unfortunately, no information on the regulation of TNF mRNA and protein was given in this study. In contrast, our unpublished data demonstrate only a minor reduction in LPS/LTA-induced TNF release by IL-1β pretreated murine macrophages. Convincing data

on the contribution of soluble mediators in desensitization of macrophages derived from experiments with human PBMC (79). Randow et al. demonstrated that a combination of recombinant human IL-10 and TGFβ was as effective as low dose LPS pretreatment in terms of reduction of TNF release upon subsequent high dose LPS stimulus, whereas preexposure to either cytokine alone only partially suppressed the release of TNF. In the same setting, addition of neutralizing antibodies to IL-10 and TGFβ inhibited desensitization in response to the first LPS stimulus, providing direct proof for a contribution of these two anti-inflammatory cytokines in LPS-induced monocyte/macrophage refractoriness *in vitro* (79). The critical role of IL-10 and TGFβ in downregulation of TNF production was confirmed by Karp et al., whereas inhibition of IL-12 production in LPS-pretreated human monocytes occurred independently of these cytokines (130).

The production of a yet unidentified suppressor of TNF formation not identical with IL-1, IL-10 or TGFβ during endotoxin tolerance was reported by Schade et al. and Fahmi et al. (132-134). They showed that addition of culture supernatants of LPS-stimulated peritoneal murine macrophages from endotoxin pretreated mice suppressed TNF release by naive macrophages. The idea of a negatively acting autocrine mediator in macrophage desensitization was extended by Baer et al. who demonstrated the production of a yet unidentified "TNF-inhibiting factor" (TIF) in supernatants of a LPS-stimulated macrophage cell line. Downregulation of cytokine release by TIF was specific for TNF, as the production of IL-1, IL-6 and MCP-1 was accelerated or enhanced. Inhibition of TNF-alpha expression by macrophage conditioned medium was associated with selective induction of the NF-κB p50 subunit which selectively inhibited a TNF-promoter reporter construct (135). Since a contribution of IL-4, IL-10 and TGFβ was excluded, these findings provide evidence for LPS induction of a novel cytokine with selective TNF-inhibitory potential participating in endotoxin desensitization (135).

Although there is evidence for a contribution of autocrine mediators in desensitization of macrophages to cytokine induction by repeated LPS stimuli, our data derived from co-culture experiments argue against a major role of soluble mediators in acquired hyporesponsiveness. Cross-desensitization induced by pre-exposure to LPS or LTA in wild-type macrophages was not transferred to co-cultured macrophages from mice lacking functional TLR2 or TLR4 as evidenced by sustained TNF release upon rechallenge with the other stimulus. Although in our setting we cannot rule out the possibility that autocrine factors requiring the presence of functional TLR2 or TLR4 (i.e. yet unknown ligands of TLR2/TLR4) are involved in

desensitization, our data strongly support the view that direct TLR2/TLR4 activation is a prerequisite for subsequent macrophage refractoriness. However, as we did not perform any neutralization experiments, we cannot rule out that besides ligand-TLR interaction additional signals provided by soluble mediators were required for desensitization.

5.4.2 *In vivo* tolerance

As pointed out for macrophage desensitization in vitro, the involvement of soluble mediators in establishing LPS tolerance *in vivo* has also been discussed controversially. Attempts to induce tolerance to the pyrogenicity of subsequent endotoxin injection by repeated administration of endogenous pyrogen (EP) were not successful (140). In contrast, pretreatment of rabbits with IL-1 partially abolished hypotension and TNF release in response to subsequent endotoxin challenge (141). When mice were treated with recombinant TNF or interleukin-1α, neither cytokine alone was able to mimic LPS for induction of tolerance. However, the two cytokines synergized to induce features of early endotoxin tolerance such as alterations of the monocyte/macrophage bone marrow pool and suppression of CSF release upon subsequent LPS challenge (142). In addition, suppression of CSF release associated with LPS-tolerance was partially reversed by administration of recombinant IL-1 receptor antagonist (IL-1ra) during LPS-pretreatment (143). Administration of IL-1a or TNF but not of IL-6 to mice for four days partially inhibited the production of IL-6 and TNF in response to a subsequent LPS challenge, although to a lesser extent than LPS (62). In line, TNF infusion in rats resulted in reduced capacity of isolated bone marrow cells to produce TNF, IL-6 or PGE_2 upon LPS stimulation in vitro (144). In contrast, Mathison et al. failed to suppress the production of TNF in response to LPS by pretreating rabbits with TNF infusions (145). Pretreatment with IL-1 conferred protection to subsequent high dose LPS challenge (146, 147) and sepsis induced by CLP (148) as well as *E. coli* induced peritonitis (149). Similar results were obtained for TNF, which induced tolerance to the lethality of subsequent LPS challenge (146, 150). We used the model of LPS/LTA induced liver damage in GalN-sensitized mice to demonstrate induction of cross-tolerance by LPS and LTA *in vivo*. It has been shown that in this model of inflammatory liver damage pretreatment with TNF or IL-1 was equally protective as LPS in reducing the extent of liver damage and lethality although neither of the cytokines suppressed TNF production (151, 152). Moreover, administration of IL-1, TNF or LPS induced tolerance to the toxicity of TNF injection itself , as shown for the metabolic changes, weight loss, temperature increase and lethality in response to high dose

TNF injection (only TNF pretreatment) (150, 154) as well as for low dose TNF-induced liver failure in GalN-sensitized mice (TNF or IL-1 pretreatment) (151-153). Since enhanced clearance or neutralization of TNF in LPS- or cytokine-pretreated animals was excluded (150, 154) hyporesponsiveness of target cells to TNF activity itself, e.g. by downregulation of TNF receptors and by the production of acute phase proteins or anti-apoptotic factors was suggested as an additional mechanism contributing to LPS tolerance (151-153, 155).

Thus, the protection afforded by LTA pretreatment in the GalN/LPS or GalN/LTA model is likely to be mediated by two independent mechanisms differing in their requirement of endogenously produced cytokines. On the one hand, the reduction of TNF levels in mice pretreated with LPS/LTA suggests macrophage hyporesponsiveness similar to *in vitro* desensitization. As demonstrated in the first part of this work, macrophage desensitization *in vitro* depends on engagement of a functional TLR stimulated by its ligand and is not mediated by soluble mediators. This view is substantiated by our unpublished results showing suppression of TNF release in TNFR1 deficient mice in response to repeated LPS injections. On the other hand, it is likely that TNF and IL-1 produced upon LTA pretreatment induced hyporesponsiveness of hepatocytes to TNF activity itself as an additional mechanism of protection. This has to be elucidated by studying the resistance of LTA-pretreated mice challenged with GalN/TNF.

As outlined before, several *in vitro* studies suggested that LPS-induced desensitization of macrophages was mediated via formation of IL-10. In line, administration of IL-10 protected mice against a lethal endotoxin challenge (125). However, a major role of the antiinflammatory cytokine IL-10 in mediating LPS-tolerance *in vivo* was excluded by Berg et al. using IL-10 deficient mice. Although these mice were LPS-hyperresponsive in terms of TNF-production and lethality, tolerance after an initial sublethal LPS dose developed normally as determined by decreased lethality and diminished levels of TNF and IL-6 after subsequent high dose LPS challenge. In addition, infusion of recombinant IL-10 could not substitute for the initial desensitizing dose of LPS (126).

In conclusion, although evidence has been provided that cytokines such as TNF or IL-1 have the potential to mimick some of the beneficial effects of LPS pretreatment *in vivo* the actual role of these cytokines in LPS-induced macrophage desensitization still has to be characterized.

5.5 Nonspecific resistance

It has been known for a long time that LPS is a potent stimulator of immune functions including comlement activation, cytokine induction by macrophages or stimulation of antibody production. However, the repeated administration of LPS is associated with attenuation of inflammatory responses required to mount an efficient immune defense against invading pathogens. It was thus suggested that induction of LPS tolerance rendered the host more susceptible to subsequent infection with virulent Gram-negative pathogens as reported for inherently LPS hyporesponsive mouse strains. In contrast, LPS-tolerant mice were more resistant to subsequent infection with *Salmonella typhimurium.* This is in line with a plethora of studies demonstrating increased nonspecific resistance to infection as one of the beneficial effects of endotoxin administration. Several mechanisms were suggested to account for the enhanced antibacterial resistance in response to LPS injection. We tried to establish the relative contribution of various mechanisms in enhancement of antibacterial defense within one infection model.

5.5.1 Humoral factors

Several studies demonstrated that enhanced resistance after LPS injection was associated with increased bactericidal activity of serum towards certain Gram-negative bacteria. Since evidence was provided that LPS administration enhanced serum bactericidal activity only towards properdin-sensitive organisms, increased serum properdin levels were suggested to be a major mechanism of LPS-induced resistance (231). This view was questioned later by findings that LPS pretreatment afforded protection also to properdin-insensitive organisms such as Gram-positive bacteria. Moreover, alterations in host resistance against bacterial infection were not always paralleled by serum properdin levels (232). In this work we provided further evidence against a major role of the complement system in LPS-induced increased resistance by demonstrating LPS-induced nonspecific resistance to *S. typhimurium* and *L. monocytogenes* in the absence of any changes in complement activity as determined in a sheep erythrocyte lysate assay. Furthermore, depletion of the central C3 protein of the complement cascade by administration of cobra venom factor did not abolish the protective effect of LPS-pretreatment on *S. typhimurium* infection.

5.5.2 Macrophages

On the cellular level of host defense LPS injection is associated with a transient depression of RES activity, followed by a longer lasting period of enhanced clearance of carbon particles, radioactive LPS , labelled chromium phosphate and viable or heat-killed bacteria by the RES (233, 236). Detailed studies by Chedid's group demonstrated that irradiation and cyclophosphamide-resistant cells mediated improved survival, enhanced RES phagocytic activity and reduced bacterial burden associated with LPS pretreatment of mice subsequently submitted to an otherwise lethal *Klebsiella pneumoniae* infection (226, 236, 237). Thus, although definite protection of irradiated mice by LPS injection additionally depended on a further, bone-marrow derived cell type not identical with T lymphocytes, their experiments strongly supported the notion that activation of RES macrophages was a major mechanism of LPS-induced host defense against *Klebsiella pneumoniae* (236, 237).

Our results that enhanced hepatic phagocytosis of bacteria in LPS-pretreated mice was associated with increased numbers of Kupffer cells extend previous findings from LPS-tolerant rats (238). Direct evidence for a contribution of Kupffer cells in LPS-stimulated clearance of bacteria derive from our depletion experiments using chlodronate-liposomes. Ruggiero et al. used isolated perfused rat livers to demonstrate increased hepatic uptake of *Escherichia coli* after *in vivo* LPS pretreatment due to enhanced phagocytic activity of the liver and improved opsonization by the serum (234). Besides an increase in Kupffer cell numbers, enhanced phagocytic activity of individual liver macrophages could account for improved hepatic clearance after LPS-treatment, as demonstrated by Hafenrichter et al. for isolated Kupffer cells from LPS-pretreated rats (239). Although an enhancement of oxidative burst activity was reported (147) (240) for LPS-tolerant murine peritoneal macrophages, Kupffer cells from LPS-pretreated rats displayed decreased generation of superoxide anions (238). In contrast, our unpublished data indicate improved antibacterial activity of Kupffer cells from endotoxin-tolerant mice.

5.5.3 Neutrophilic granulocytes

LPS induces a plethora of chemokines leading to accumulation of leukocytes, consisting mainly of neutrophilic granulocytes, at the site of LPS administration. This is of importance when bacteria are injected at the site of previous LPS administration, since the microorganisms are confronted immediately with a large number of phagocytes absent in the naive host. Here we demonstrated that intraperitoneal accumulation of leukocytes associated

with enhanced inactivation of intraperitoneally injected *Salmonella typhimurium* during the first hours postinfection was strictly dependent upon the route of LPS-pretreatment. Similar results were obtained by Astiz et al. who studied the therapeutic value of administration of monophosphoryl lipid A, (MPL) a detoxified LPS derivative, to mice prior to induction of peritonitis by cecal ligation and puncture (CLP). In their setting, intraperitoneal injections of MPL were more effective in reducing mortality than intravenous MPL administration (241).

It has long been known that endotoxin is a potent stimulator of hematopoesis. Post-endotoxin serum was shown to have potent colony-stimulating factor (CSF) activity in vitro as well as in vivo, when transferred to naive animals (242-244). Intensive studies on radioprotection by previous administration of endotoxin suggested an important role of accelerated hematopoiesis, as reviewed by Nowotny et al. (214). Administration of LPS resulted in increased white blood cell numbers (233, 245), neutrophilia (60), (233) and augmented numbers of monocyte/macrophage precursors in the bone marrow (57, 246). In this thesis we demonstrated that endotoxin-pretreated mice displayed elevated numbers of circulating neutrophils throughout the course of *Salmonella* infection, indicating improved recruitment from the bone marrow and/or decreased rate of apoptosis of these cells after LPS treatment (233). A critical role of diminished neutrophil apoptosis for the survival benefit associated with endotoxin pretreatment prior to induction of multi-germ peritonitis was suggested recently (247). This is in line with previous findings by Yamamoto, showing a delay of neutrophil apoptosis by LPS and LPS-induced cytokines *in vivo* and *in vitro* (248).

Besides an increase in overall PMN numbers, the individual phagocytes isolated from LPS-tolerant mice in our model displayed an increased oxidative burst response upon stimulation *ex vivo* (data not shown). The importance of the NADPH-oxidase system for inactivation of *Salmonella typhimurium* and survival of infected animals was demonstrated recently in gp91phox-deficient mice (333, 334). The view that neutrophils play a decisive role in LPS-mediated resistance to infection has been substantiated by our findings that PMN depletion partially abrogated the survival benefit of LPS-pretreated mice infected with *Salmonella typhimurium*.

5.5.4 Lymphocytes

Activation of lymphocytes by LPS or LPS-induced mediators is well documented, and Galelli et al. demonstrated that definite protection by LPS treatment of irradiated mice required bone-marrow derived radiosensitive cells (236). However, the adaptive immune system seems to be

of minor importance for the establishment of the early phase of LPS-induced nonspecific resistance as suggested by experiments performed with athymic or SCID-mice which showed protection in spite of lacking functional T-and B-lymphocytes (236, our own results).

5.6 Mediators of nonspecific resistance

Many of the effects of endotoxin are mediated by endogenous mediators such as cytokines, arachidonic acid metabolites or reactive oxygen or nitrogen radicals. Although a major role of autocrine mediators in the process of inducing or maintaining macrophage refractoriness *in vitro* has been ruled out in this thesis, there is evidence that LPS-enhanced nonspecific resistance is the result of the biological activity of several cytokines produced in response to LPS injection. As pointed out for the model of inflammatory liver damage of galactosamine-sensitized mice, pretreatment with several proinflammatory cytokines is associated also with protection against subsequent bacterial infection. Injection of IL-1 improved survival of mice infected subsequently with *Listeria monocytogenes* (249), *Pseudomonas aeruginosa* (249-253), *Klebsiella pneumoniae* (249, 250, 253), *Escherichia coli* (149) and in the sepsis model of CLP (146), (254). Furthermore, the combination of IL-1 and TNF reduced mortality and bacterial load of C3H/HeJ mice infected with *E. coli* at 20-fold the $LD_{50\ (255)}$. Pretreatment with IL-1, GM-CSF or G-CSF improved survival after aerosol pneumococcal challenge (256-258). This effect could be due to enhanced microbicidal activity of alveolar macrophages and improved clearance of blood-borne pathogens of cytokine-pretreated mice (259). The beneficial effect of G-CSF treatment prior to induction of bacterial peritonitis or *L. monocytogenes* infection is probably mediated via the recruitment or activation of PMN (224-226). Extensive studies on the beneficial effect of cytokine pretreatment on resolution of infection were performed in the model of *Salmonella typhimurium* infection of mice employed also in our studies. It has been shown that administration of TNF resulted in improved survival of otherwise lethal bacterial challenge (30, 35). In line, protection against salmonella infection was conferred also by pretreatment with IFNγ (30), IL-18 (2) or TGFβ (264). Morrissey et al. demonstrated that injection of IL-1 or a combination of IL-1 and TNF protected mice against an otherwise lethal challenge with *Salmonella typhimurium* (37, 265). However, protection was restricted to Ity^r mice, whereas animals carrying the Ity^s or the LPS^d allele (LPS nonresponder mice) did not benefit from the pretreatment with exogenous cytokines (37, 38, 265). The same hold true for administration of GM-CSF (39).

Since LPS administration induces the formation of all of these mediators it is feasible that nonspecific resistance is conferred via endogenous formation of these cytokines. However, direct proof for this hypothesis has not been provided yet. Studies on the role of LPS-induced cytokines in enhancing resistance to infection are hampered by the fact that normal host defense initiated by the pathogen itself also depends on an intact cytokine response. Thus, cytokine-deficient mice are unsuitable and the use of cytokine-specific antibodies requires detailed titration experiments in order to selectively neutralize only LPS-induced cytokines during the pretreatment phase but not during infection. Furthermore, because of the plethora of cytokines with similar protective effect, it is unlikely that neutralization of single mediators will abrogate the beneficial effect of LPS-pretreatment.

5.7 Conclusion

Taken together, administration of endotoxin is associated with nonspecific resistance to bacterial, fungal and even some viral infections. Development of tolerance to LPS toxicity, despite of suppressing cytokine production in response to virulent bacteria, does not impair antibacterial defenses. Although the phenomenon itself had been studied for more than a century, the mechanism of protection had not been identified yet. Our data demonstrating a contribution of different immune cells at different stages of infection indicate that enhanced host defense following the administration of endotoxin is the result of the orchestrated action of multiple factors corresponding to the innate immune system. Our findings substantiate the view that LPS tolerance represents a physiological adaptation to sustained infection via prevention of potentially autodestructive systemic inflammation albeit maintaining improved defense capability against virulent pathogens. These beneficial effects suggest the controlled induction of LPS tolerance as an promising tool in the prophylaxis of post-operative sepsis.

6 Summary

Lipopolysaccharide (LPS) is a potent immune stimulator which induces many of the pathological sequelae observed during systemic Gram-negative infection. The term endotoxin tolerance describes the phenomenon that the toxicity of LPS is attenuated upon repeated LPS-stimulation. Selective downregulation of certain macrophage activities is considered one of the mechanisms underlying LPS tolerance. In the present thesis it was investigated whether lipoteichoic acid, a component of Gram-positive bacteria, induced similar tolerance *in vivo* and macrophage refractoriness *in vitro*. In the second part of the thesis the effect of acquired LPS tolerance on host defense against virulent bacteria was studied.

1. TNF release by murine macrophages in response to lipoteichoic acid (LTA) is independent of Toll like receptor (TLR) 4 but requires functional TLR2.
2. LTA induced macrophage refractoriness similar to LPS *in vitro* and *in vivo*.
3. LTA and LPS induced cross-desensitization of macrophages *in vitro* and cross-tolerance in galactosamine-sensitized mice *in vivo*.
4. Paracrine mediators did not suffice to induce cross-desensitization of macrophages *in vitro*.
5. Cytokine production in response to viable *Salmonella typhimurium* was attenuated in LPS-tolerant mice.
6. Induction of LPS tolerance prior to *Salmonella typhimurium* infection was associated with decreased bacterial load and a prolongal of survival.
7. Early but not late phase reduction of bacteria in LPS-tolerant mice depended on the accumulation of PMN in the peritoneal cavity in response to intraperitoneal LPS injections.
8. LPS-tolerance was associated with increased PMN counts in blood and tissues and enhanced oxidative burst capacity of the individual PMN. Depletion of PMN prior to infection partially abrogated the survival benefit associated with LPS-tolerance.
9. LPS-tolerant mice displayed improved clearance of systemically injected *Salmonella typhimurium* and enhanced phagocytic activity of the liver. LPS-tolerant mice had three-fold increased numbers of Kupffer cells and depletion of Kupffer cells completely reversed the enhanced hepatic phagocytosis and the improved clearance of *Salmonella typhimurium* in LPS-tolerant mice.

7 Zusammenfassung

Lipopolysaccharid (LPS) ist ein potenter Immunstimulus und viele Symptome einer systemischen bakteriellen Entzündung können durch LPS-Gabe experimentell induziert werden. Diese Symptome sind stark vermindert, wenn mit einer niedrigen LPS-Dosis vorbehandelt wurde. Dieses Phänomen der Desensitivierung gegenüber der immunstimulatorischen Aktivität von LPS durch wiederholte Gabe wird als "Endotoxintoleranz" bezeichnet. Eine selektive Modulierung der Makrophagenaktivität mit verminderter Produktion proinflammatorischer Zytokine wird als ein zentraler Mechanismus der Endotoxintoleranz postuliert. In der vorliegenden Arbeit wurde untersucht, ob ähnliche Formen der Desensitivierung oder Toleranz auch durch Bestandteile Gram-positiver Bakterien wie Lipoteichonsäure (LTA) induziert werden können. Im zweiten Teil der Arbeit sollte der Einfluss verminderter Zytokinproduktion nach Induktion von LPS-Toleranz auf den Immunstatus in einem zytokinabhängigen bakteriellen Infektionsmodell charakterisiert werden.

1. Die TNF-Freisetzung aus murinen Makrophagen nach Stimulation mit LTA erforderte die Beteiligung von Toll like receptor (TLR) 2, nicht aber von TLR4.
2. Wiederholte Gabe von LTA induzierte eine Desensitivierung von Makrophagen *in vitro* und *in vivo*.
3. LTA und LPS verursachten eine Kreuzdesensitivierung von Makrophagen *in vitro* und von Galactosamin-sensitivierten Mäusen *in vivo*.
4. Die Kreuzdesensitivierung von Makrophagen *in vitro* wurde nicht von parakrinen Mediatoren verursacht.
5. Die Zyokinfreisetzung nach Infektion mit *Salmonella typhimurium* war in LPS-toleranten Mäusen reduziert.
6. *Salmonella typhimurium*-infizierte LPS-tolerante Mäuse wiesen eine geringere Bakterienlast auf und zeigten verlängertes Überleben.
7. Die verbesserte Inaktivierung von Bakterien in LPS-toleranten Mäusen in der Frühphase der Infektion war von der Akkumulation neutrophiler Granulozyten während der intraperitonealen LPS-Vorbehandlung abhängig, während die Reduktion der Bakterienzahlen zu späten Zeitpunkten auch bei intravenöser LPS-Vorbehandlung auftrat.
8. LPS-tolerante Mäuse zeigten erhöhte Zahlen von neutrophilen Granulozyten in Blut, Leber und Milz, sowie einer gesteigerten Fähigkeit der einzelnen Neutrophilen zum

oxidativen Burst. Durch Depletion der Neutrophilen vor der Infektion konnte der Überlebensvorteil partiell aufgehoben werden.

9. LPS-tolerante Mäuse wiesen eine stark verbesserte Clearance systemisch applizierter Bakterien und eine erhöhte Phagozytoseleistung der Leber auf. Dieser Effekt war assoziiert mit einer ca. dreifachen Erhöhung der Kupfferzellen. Durch Depletion der Kupfferzellen mittels Chlodronatliposomen konnten sowohl die erhöhte Leberphagozytose als auch die verbesserte Clearance systemischer *Salmonella typhimurium* in LPS-toleranten Mäusen aufgehoben werden.

8 References

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