Staphylococcus aureus lipoproteins – TLR2-mediated activation of innate and adaptive immunity

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Abbreviations

CARD	Aminoterminal caspase recruitment domain
CD	Cluster of differentiation
CFU	Colony forming unit
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
Erm	Erythromycin
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
H	Hours
IL	Interleukin
IFN	Interferon
IKK	Inhibitor of NF-κB kinase
IRF	IFN regulatory factor
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
Lpp	Lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
lgt	Gene encoding preprolipoprotein diacylglyceryl transferase

lnt	Gene encoding *N*-acyltransferase
lsp	Gene encoding lipoprotein signal peptidase II
MAPK	Mitogen-activated protein kinase
MDP	muramyl dipeptide
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
min	Minutes
MOI	Multiplicity of infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor κB
NOD	Nucleotide oligomerization domain
NLR	NOD-like receptor
OD	Optical density
ON	Overnight
Pam_2CSK_4	Palmitoyl-2-cysteine-serine-lysine
Pam_3CSK_4	Palmitoyl-3-cysteine-serine-lysine
PAMPs	Pathogen-associated molecular patterns
PE	Phycoerythrin
RIP	Receptor-interacting serine-threonine kinase
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
RT	Room temperature (about 23 °C)

S. aureus	*Staphylococcus aureus*
SD	Standard deviation
SEM	Standard error mean
Tat	Twin arginine translocation pathway
TCF	Tissue cage fluid
TCR	T cell receptor
T_H cell	T helper cell
TIR	Toll/IL-1-receptor
TIRAP	TIR-domain-containing adaptor protein
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Summary

Staphylococcus (*S.*) *aureus* is a very successful pathogen due to its immune evasion strategies. Besides toxins and adhesins, it expresses membrane lipoproteins (Lpp), which are bound by the pattern recognition receptor Toll-like receptor (TLR) 2 in the host. Recognition of Lpp activates the MyD88-signaling pathway, which allows mounting a strong inflammatory response. Interestingly, evolution did not select for *S. aureus* mutants deficient in lipid modification of proteins suggesting that Lpp, besides their signaling potential to the host, offer an advantage for *S. aureus.* The aim of this thesis was to identify the benefit of Lpp maturation for *S. aureus* and the contribution of Lpp-TLR2-signaling to the pathogenesis of staphylococcal disease in mouse infection models.

In the first part, we demonstrate the strong cytokine-activating potential of Lpp in murine macrophages, which was associated with the presence of TLR2 in the host. In a systemic infection model, Lpp-TLR2 activation was less contributing to inflammation and *S. aureus* killing than MyD88-signaling. This indicates that other receptors signaling to MyD88 participate in the antimicrobial response. We further showed in systemic infection that maturation of Lpp facilitates survival of *S. aureus* in organs due to improved iron acquisition. Studies on growth, uptake, and intracellular storage of iron in vitro confirmed the iron dependence of *S. aureus*. It is long known, that the immunocompetent host restricts iron in an infection. We show that Lpp enhanced *S. aureus* growth in the iron-overloaded immunocompetent host, while they were not required in the iron rich environment in the MyD88-deficient host. Interestingly, iron-

restricted *S. aureus* could not profit from Lpp for growth as long as the infected mice were fully immunocompetent. Only in mice deficient in MyD88-dependent inflammation iron-restricted *S. aureus* used Lpp for growth. In summary, the results in part 1 strongly suggest that Lpp confer a growth and survival advantage although allowing innate immune responses mediated through TLR2-MyD88-signaling.

In the second part, data are presented showing that Lpp released during growth activate TLR2-signaling but engulfment of *S. aureus* enhances cytokine production. Lpp enhanced phagocytosis by macrophages and intracellular survival of *S. aureus*. Moreover, Lpp-TLR2-signaling induced cathepsin B-mediated cytotoxicity in macrophages. An effect of Lpp on various interactions of *S. aureus* with PMN was not found in vitro and in vivo, whereas Lpp enhanced invasion of *S. aureus* in endothelial cells in vitro. These results point to an additional survival advantage by maturation of Lpp in *S. aureus* due to improved evasion from extracellular killing, better intracellular survival, and escape from the phagosome.

In the third part, we demonstrate that Lpp-TLR2-MyD88-signaling is important for activation of DCs to induce differentiation of naïve $CD4^+$ into IFN-γ- and IL-17-producing T cells in vitro. Induction of Lpp-TLR2-signaling was also required to promote IFN-γ release by naïve $CD8^+$ T cells. In systemic infection, restimulated spleen T cells produced MyD88-dependent IFN-γ and TLR2-MyD88-dependent IL-17. Surprisingly, the presence of B and T cells diminished eradication of *S. aureus* from organs during early sepsis. These data show that detection of invading *S. aureus* by DCs

leads to the development of adaptive immune responses, which are not always beneficial for eradication of *S. aureus*.

In the fourth part, the role of other pattern recognition receptors (PRRs) in staphylococcal infection was examined. TLR9 and NOD2, in contrast to IL-1R, had a positive effect on cytokine induction in macrophages and in systemic infection, whereas killing was not affected. In inflammation and bacterial killing during *S. aureus* infection, TLR2 and TLR9, which both require the MyD88-adaptor, were found to cooperate. These data suggest that concurrent activation of different PRRs elicit a strong antimicrobial defense in response to various molecules of *S. aureus*.

1 General Introduction

1.1 *Staphylococcus aureus*

1.1.1 Pathogenesis and epidemiology

Staphylococcus (*S.*) *aureus* is an extracellular Gram-positive pathogen colonizing the skin and mucosa of humans. About 20% of the population is persistently colonized in the nose, while another 50% are intermittent carriers. The comparison of disease-causing isolates with carriage isolates revealed that these isolates had a similar population structure (1). Therefore, carriage of *S. aureus* is an important risk factor for invasive diseases (2) and about 80% of blood infections originate from colonies in the nasal mucosa (3).

S. aureus can cause toxin-mediated diseases such as toxic shock syndrome. In addition, *S. aureus* can cause benign local infections such as furunculosis. If the local innate immune defense with polymorphonuclear lymphocytes (PMN) is insufficient, *S. aureus* can disseminate and finally cause severe organ diseases like osteomyelitis and lead to life-threatening infections like endocarditis and sepsis.

In the last decades, the incidence of staphylococcal infections has increased due to increased numbers of patients with risk factors such as implants and immunodeficiencies. Infections with resistant strains impose a threat to the society, which makes the development of novel therapies against *S. aureus* essential. However, identification of virulence factors is difficult due to horizontal gene transfer of mobile genetic elements (4) and further a lineage specific virulence in strains with close genetic relationship could

not be linked to the expression of specific virulence factors (5, 6). For *S. aureus*-host interactions, host factors such as polymorphisms in genes influence the pathogenesis of *S. aureus*. Therefore, identifying exactly which genes or gene combinations are necessary for infections or which ones could be targeted by new therapies remains difficult.

1.1.2 The staphylococcal cell envelope

The structural integrity and shape of *S. aureus* is maintained by the cell wall, a large mesh-like polymer, which surrounds the cell and is essential for viability (7, 8). The main constituent of the cell wall is peptidoglycan, a polymer made of muropeptides synthesized as pentapeptide chains linked to disaccharides composed of *N*-acetylmuramic acid and *N*-acetylglucosamine. This peptidoglycan layer contains different molecules including (lipo-) teichoic acids (LTA), and covalently and non-covalently associated proteins. Wall teichoic acids and lipoteichoic acids are composed of a polymer of glycerolphosphate. The wall teichoic acids are covalently linked to the peptidoglycan, whereas lipoteichoic acids are anchored in the outer leaflet of the cytoplasmic membrane by a glycolipid. Surface-associated proteins are transported through the bacterial membrane and further modified for attachment to the cell wall or anchoring in the bacterial membrane known as lipoproteins (Lpp).

1.1.3 Staphylococcal Lipoproteins

Lipoprotein biogenesis

The pathway for Lpp biogenesis is unique to bacteria and was first established in *Escherichia (E.) coli* (9). *S. aureus* Lpp are secreted by the general secretory (Sec) or twin arginine protein transport (Tat) pathway (Figure 1). The Lpp precursor is exported with a conserved motif L_{-3}-[A/S/T]$_{-2}$-[G/A]$_{-1}$-C_{+1} (lipobox) in which the conserved cysteine residue is required for lipidation (10). The phosphatidyl glycerol diacylglyceryl transferase (Lgt) adds a diacylglyceryl moiety to the cysteine via a thioether linkage (11) and the Lpp-specific type II signal peptidase (Lsp) cleaves the signal peptide form the pre-Lpp. In the final maturation step in Gram-negative bacteria, the N-acyltransferase (Lnt) attaches a third fatty acid to the lipid-modified cysteine yielding mature triacylated Lpp. While one group identified diacylated Lpp in *S. aureus* (12), which is in agreement with the lack of a *lnt*-gene homolog in *S. aureus* (11), Kurokawa *et al.* found a triacylated Lpp (13). They assumed that another acyltransferase further modifies diacylated Lpp in *S. aureus*. For that reason the lipid-linkage of staphylococcal Lpp remains unclear.

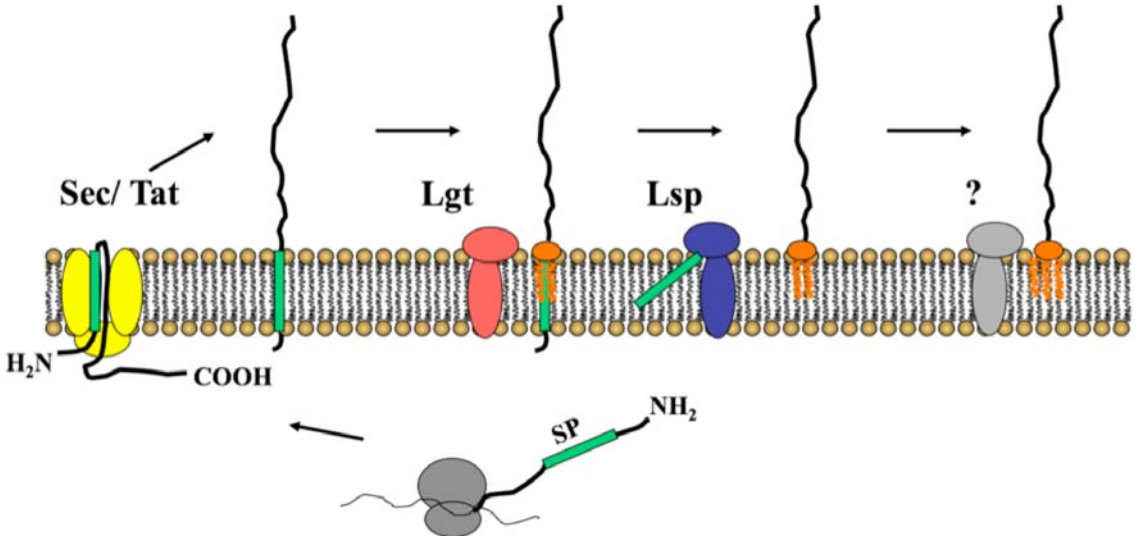

Figure 1. The lipoprotein biogenesis pathway in *S. aureus*. Lpp are translated with a conserved motif (the lipobox) and a N-terminal signal peptide (green). Precursor-Lpp are translocated through the membrane by the Sec or Tat system (yellow). The phosphatidyl glycerol diacylglyceryl transferase (Lgt, red) covalently binds a diacylglycerol group to the conserved cysteine in the lipobox, and the lipoprotein-specific type II signal peptidase (Lsp, blue) cleaves off the signal peptide. An acyltransferase (?, gray) might attach an additional lipid to the cysteine in *S. aureus*.

Functions of staphylococcal Lpp

Using bioinformatic approaches, about 50 genes encoding lipoproteins were predicted out of ~2500 open reading frames in the genome of *S. aureus* (14). Mature Lpp function within the subcellular region between the plasma membrane and the cell wall. About 40% of the Lpp genes encode for substrate binding proteins of ATP-binding cassette (ABC) transporter to import nutrients in Gram-positive bacteria (10). High substrate affinities of Lpp maintain the continuous import of molecules from the environment including sugars, siderophores, divalent metal ion, anions (such as phosphate and sulfate), amino acids,

oligopeptides, and nucleotides (10). In *S. aureus*, some Lpp are well characterized while other Lpp are only predicted to function in binding of oligopeptides (OppA), glycyl methionine (GmpC), glycine betaine/carnitine/choline (OpuCC), manganese (MntA), molybdate (ModA), and a beta-lactamase precursor (BlaZ) (11, 15, 16). A number of identified Lpp are required for siderophore or heme acquisition in *S. aureus* including FhuD1/FhuD2, SirA, SstD, SitC, HtsA, and IsdE (17-21). Other staphylococcal Lpp like the peptidyl-prolyl *cis/trans* isomerase (PrsA) and the thiol-disulfide oxidoreductase (DsbA) are involved in folding and formation of disulphide bonds of exported proteins, respectively (11, 15, 22). Nevertheless, most of the proteins containing the lipobox are hypothetical without known function (15).

1.2 Pattern recognition receptors

The immune system protects the organism against pathogens like bacteria, fungi, viruses, and parasites. The mammalian immune system consists of innate and adaptive immunity (23). Innate immunity is referred as first line defense mechanism to recognize the pathogen, induce antimicrobial defense and is crucial for establishing and modulating adaptive immune responses (23-25). Pattern recognition receptors (PRRs) are germ-line encoded and recognize conserved molecules on the bacterial surface, also called pathogen-associated molecular patterns (PAMPs). Among the PRRs, the signaling receptors including the Toll-like receptor (TLR) family and the nucleotide oligomerization domain (NOD) receptor family activate signal transduction (26). PRRs are expressed on various immune cells including macrophages, dendritic cells, B cells, specific types of T cells, as well as non-immune cells such as fibroblasts and epithelial

cells. PRRs basically serve three distinct functions: (i) sensing the presence of bacteria, (ii) inducing an immediate antimicrobial response, and (iii) promoting the development of a long-lasting adaptive response (27).

1.2.1 Signaling of Toll-like receptors in innate immunity against bacteria

The TLR family comprises 10 and 12 receptors in humans and mice, respectively (28, 29). While certain TLRs (TLR1, 2, 4, 5, and 6) are expressed on the cell surface and migrate to phagosomes after activation, others (TLR3, 7, 8, and 9) are found in intracellular compartments. Recognition of specific ligands induces the formation of heterodimers as reported for TLR2 together with TLR1 or TLR6, or homodimers as reported for other TLRs (30). The most extensively studied TLRs, TLR2 and TLR4 were found to recognize lipid structures of microbes. TLR2 together with TLR1 or TLR6 binds Lpp and LTA from bacteria as proposed by crystal structure analysis (30, 31). TLR4 is primarily responsible for inflammatory responses to bacterial lipopolysaccharide (LPS) from Gram-negative bacteria (32). Other TLRs sense proteins and nucleic acids. TLR5 detects flagellin that is required for bacterial motility (33). TLR9 recognizes CpG motifs in bacterial DNA (34, 35), whereas TLR7 seems to be responsible for RNA sensing (36). However, a single microbe is recognized by different TLRs simultaneously, which share and also differ in signaling pathways.

TLRs initiate signaling through a cytoplasmic domain called Toll/IL-1 receptor homology (TIR) domain. As extensively reviewed, signaling adaptor molecules also contain a TIR domain to bind to the receptor, including myeloid differentiation factor 88 (MyD88), TIR domain-containing adapter protein (TIRAP), TIR domain-containing

adapter-inducing interferon-β (TRIF), and TRIF-related adapter molecule (TRAM). The usage of these adapter molecules varies between TLRs and depending of the activated signal pathway, receptors are MyD88-dependent or MyD88-independent (Figure 2).

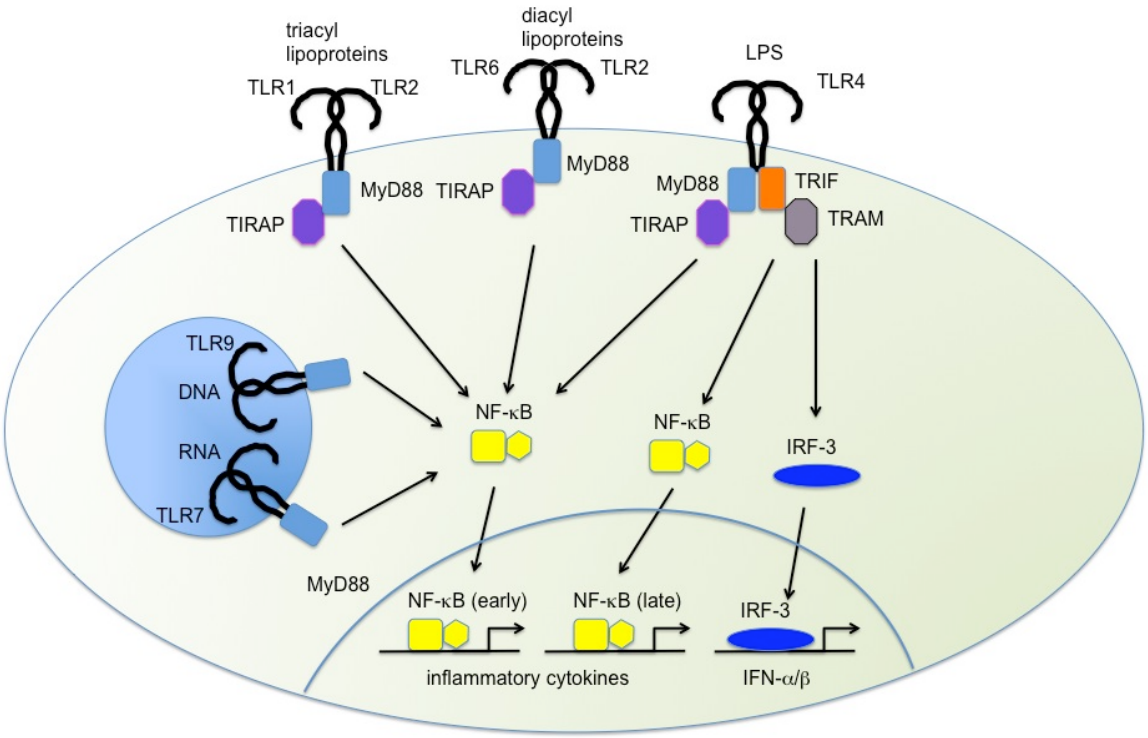

Figure 2. Model for Toll-like receptor signaling. Toll-like receptors (TLRs) involved in bacterial recognition and some of their representative bacterial ligands are shown. Bacterial Lpp, for example, can be recognized at the cell surface by TLR2 dimerized with either TLR1 or TLR6 through the TIR-containing adaptor MyD88 and TIRAP to activate cells through NF-κB and MAPKs (not shown). TLR4 homodimers recognize LPS and activate cells through MyD88 and TIRAP via the cell surface or TRAM and TRIF via the endosomal pathway. Recruiting of TRIF is required for the activation of IRF-3 resulting in the production of type I IFNs and late activation of NF-κB. TLR7 and 9 recognize nucleic acids in the endosome. TLR7/9-mediated signaling pathways are through TRIF and MyD88, respectively, resulting in the induction of proinflammatory cytokines and type I IFNs.

For example, bacterial Lpp activate TLR2 and signal by the MyD88-dependent pathway with MyD88 and TIRAP (also known as Mal) to activate the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) resulting in the production of proinflammatory cytokines, including tumor necrosis factor (TNF). LPS stimulation of TLR4 activates signaling pathways through TIRAP-MyD88 and TRAM-TRIF, which induce the production of proinflammatory cytokines and type I interferons (IFNs), respectively (37). TRIF and TRAM induce the phosphorylation and dimerization of the transcription factor IFN regulatory factor-3 (IRF3), leading to the production of type I IFNs and can later also activate NF-κB signaling (38-43).

1.2.2 Signaling of NOD receptors in innate immunity against bacteria

The NOD-like receptors (NLRs) fill a different niche than TLRs by sensing in the cytoplasm. NOD receptors, including NOD1 and NOD2 are signaling receptors involved in recognition of microorganisms (44-46). NOD1 recognizes the intracellular peptidoglycan fragment M-TriDAP (L-Ala-D-Glu-meso-diaminopimelic acid) from Gram-negative bacteria (47, 48). NOD2 detects the conserved structure within peptidoglycan from Gram-negative and Gram-positive bacterial cell walls called muramyl dipeptide (MDP) (Figure 3) (49, 50). Ligand binding by NOD1 or NOD2 leads to homo-oligomerization of the proteins, leading to the recruitment of caspase activation and recruitment domain (CARD)-containing adaptor molecules that are responsible for signaling. NOD1 and NOD2 interact with receptor-interacting protein 2 (RIP-2), which binds the inhibitor of NF-κB kinase γ (IKKγ) leading to the activation of proinflammatory responses mediated by NF-κB (51, 52). In addition to activation of NF-

κB, NOD2 activates the MAPK-signaling pathways via p38 and c-Jun N-terminal kinase (JNK).

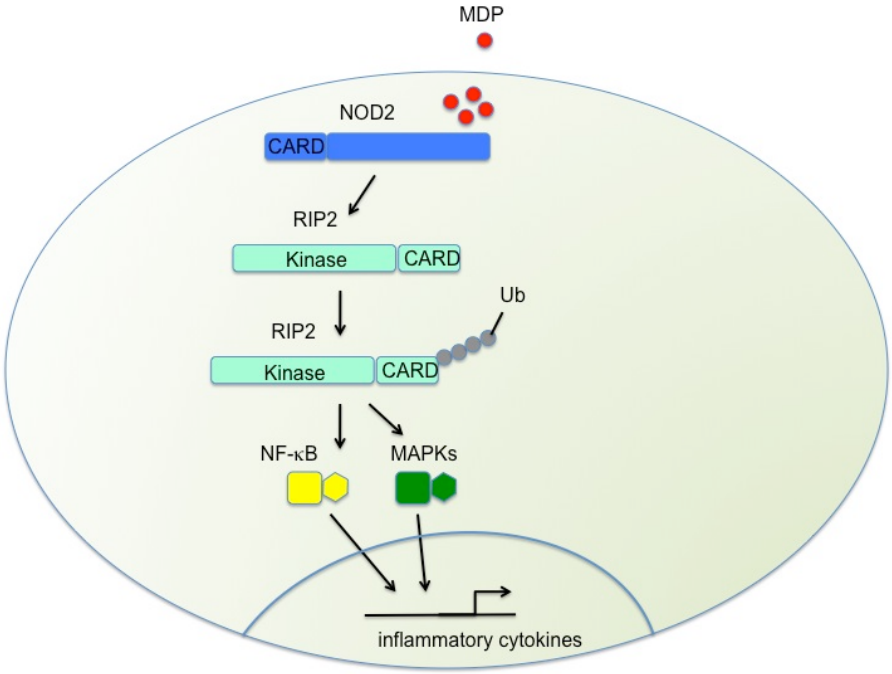

Figure 3. Pathway of NOD2 activation. NOD2 activates the canonical MDP activation pathway involving the kinase RIP2. RIP2 becomes ubiquitinated (Ub) and subsequently activates NF-κB and MAPK leading to inflammatory gene expression. (Adapted from (53)).

1.2.3 PRRs and adaptive immune responses against bacteria

Detection of infections by PRRs initiates the innate and the adaptive immune response. While innate immunity is rapid and not antigen-specific, adaptive immunity confers a highly specific and long-lasting immune response against invading pathogens. To efficiently eradicate pathogens both immune systems have to act together. Professional antigen-presenting cells, such as macrophages, dendritic cells (DCs), and B cells engulf bacteria and present bacterial antigens on Major Histocompatibility Complex (MHC)

class II molecules to $CD4^+$ T cells (signal 1). DCs are activated by PRRs and induce the upregulation of MHC molecules, costimulatory molecules (signal 2), and cytokines (signal 3) (54). $CD4^+$ T cells activate and regulate B cells, $CD8^+$ T cells, and other inflammatory cells. $CD4^+$ T cells differentiate into cells that produce a special set of cytokines, which specify them as T helper 1 (T_H1) cells, T_H2 cells, T_H17 cells, or regulatory T (T_{reg}) cells (Figure 4).

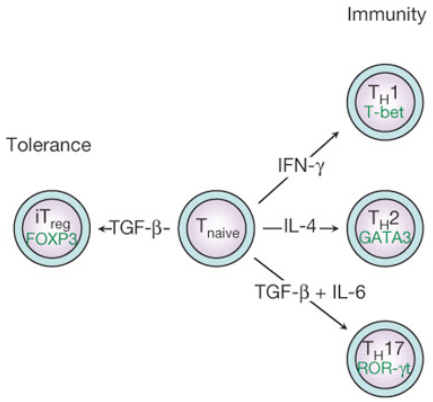

Figure 4. Subsets of T helper cells. Depending on the cytokine milieu present at the time of the initial engagement of the TCR and costimulatory receptors in the peripheral immune compartment, naïve $CD4^+$ T cells can differentiate into various subsets of T helper cells (T_H1, T_H2, and T_H17). However, in the presence of TGF-β, naïve T cells convert into FOXP3-expressing induced T_{reg} (iT_{reg}) cells. For each T helper cell differentiation program, specific transcription factors have been identified as master regulators (T-bet, GATA3, and ROR-γt). Terminally differentiated T helper cells are characterized by a specific combination of effector cytokines that orchestrate specific and distinct effector functions of the adaptive immune system. (adapted from (55)).

T_H1 cells are effective inducers of cellular immune responses, involving enhancement of antimicrobial activity of macrophages and consequently increased efficiency in lysing

microorganisms in intracellular compartments, whereas T_H2 cells are very effective in helping B cells to develop into antibody-producing plasma cells. T_H17 cells are required in defense against extracellular pathogens and T_{reg} cells are a specialized $CD4^+$ cell type that can suppress the responses of other T cells. T and B cells mount an adaptive, antigen-specific immune response, which is critical to control the infection and to eradicate the invasive bacterium.

1.2.4 Recognition of *S. aureus* by PRRs

Surface structures of *S. aureus* bind complement and antibodies, which allow phagocytosis of opsonized bacteria by complement and Fc receptors, respectively.

It has been shown in different infection models that several PRRs are involved in the host response against *S. aureus*. Scavenger receptors are required for recognition since $CD36^{-/-}$ mice are highly susceptible to *S. aureus* (56), whereas CD14 is not contributing in a murine brain abscess model (57). The lack of TLR2 increases the susceptibility of mice in systemic and subcutaneous infections and leads to increased numbers of *S. aureus* in a nasal carriage model (56, 58-60). TLR7 and TLR9 are involved in recognition of RNA and DNA from *S. aureus* (61). However, $TLR9^{-/-}$ mice did not display any difference compared to C57BL/6 mice in a corneal infection model (62). The role of TLR7 *in S. aureus* infection remains to be elucidated.

Like TLRs, IL-1R uses the common adaptor MyD88. IL-1R-deficiency leads to increased bacterial burden in systemic and subcutaneous infection (63, 64). In line with the results found for single receptor knock-outs, $MyD88^{-/-}$ mice are highly susceptible in murine infection models (58, 59, 62, 64, 65). Mice deficient in NOD2 are impaired in bacterial

killing, but not in cell recruitment (66, 67). In addition to these receptors, other known and yet unknown PRRs may be involved in recognition of *S. aureus*.

1.3 Competition for iron by *S. aureus* and the host

Iron availability is efficiently regulated in the host, which sequesters iron within cells or by proteins to avoid damage due to the high oxidative potential of iron. Most iron in the human body is bound as hemoglobin in erythrocytes. Iron for hemoglobin synthesis is recycled by phagocytosis of erythrocytes by macrophages in the spleen and the liver. Recycled iron is exported through ferroportin, binds to transferrin in blood circulation, and is delivered into bone marrow for hematopoiesis. Therefore, the amount of free iron is extremely low in serum (10^{-24} M). The challenge for *S. aureus* is to obtain sufficient quantities of iron (10^{-6} M) to maintain essential catalytic functions of enzymes mainly involved in the respiratory chain.

S. aureus circumvents iron limitation in the host by the release of iron-binding siderophores and uptake of those as well as direct uptake of heme by iron uptake ABC transporters (68). All uptake systems have common characteristics of ABC-transporters; a substrate binding protein anchored as Lpp in the membrane, a permease for translocation through the membrane, and an energy providing ATPase in the cytoplasm. These transporters are controlled by the ferric uptake regulator (fur) (69, 70), which represses transcription of genes in the presence of iron. Another sensor of intracellular iron is the aconitase, which can modulate the translation of mRNA under iron-limiting conditions (71).

Invading *S. aureus* are believed to gain access to iron by lysis of erythrocytes, which were recently also found in abscesses (72). The low iron availability in the host is known to upregulate iron uptake systems and cytotoxins such as hemolysins in *S. aureus* (Gordon Conference 2009). The host immediately responds with antimicrobial defense mechanisms including enhanced expression of transferrin receptor in liver and spleen cells to sequester iron (73) and release of lipocalin to bind siderophores (74). Inflammatory cytokines (e.g. IL-6) induce the release of hepcidin (75), which down-regulates ferroportin, the exporter of iron from cells into serum (76, 77). This leads to the reduction of free iron and together with erythropoesis results in iron-limiting conditions in the host. Thus, the host sustains iron levels at growth limiting concentrations for *S. aureus*. Therefore, the recognition by PRRs e.g. Lpp by TLR2 may be a handicap for *S. aureus* considering that host cells and other antimicrobial defense mechanisms are activated and iron is sequestered within host cells. On the other hand, *S. aureus* requires Lpp for the import of iron from heme, siderophores, and other iron-containing molecules as described above.

2 Aim of the thesis

Maturation of Lpp allows the host to mount a strong immune response by TLR2-signaling, which is generally viewed to be a drawback for *S. aureus*. However, no mutants deficient in Lpp maturation are found among staphylococci suggesting a benefit for *S. aureus* in keeping Lpp maturation. This might be due to the fact that most *S. aureus* Lpp are components of nutrient- and metal-uptake systems important for maintaining metabolism and growth. Therefore, the question arose how *S. aureus* profits from Lpp maturation in systemic infection.

The first aim was to understand the role of Lpp maturation in viable *S. aureus* for the activation of TLR2- and MyD88-signaling in macrophages. A special interest was to identify the contribution of Lpp and TLR2-MyD88-signaling in the pathogenesis of systemic staphylococcal disease in mice. In addition, the contribution of Lpp maturation in iron acquisition by *S. aureus* was investigated in the context of iron sequestration in inflammation.

Although regarded to be an extracellular pathogen, *S. aureus* is able to invade a variety of non-professional phagocytes and can also survive engulfment by professional phagocytes. In part 2, we therefore intended to investigate the role of Lpp maturation and TLR2-MyD88-signaling on phagocytosis, intracellular survival, and escape from phagosomes through induction of cell death. Further, the necessity of bacterial engulfment for triggering cytokines was examined.

The innate immunity “instructs” adaptive immune cells by providing signals for activation and proliferation. In part 3, we aimed to evaluate the requirements of Lpp-TLR2-signaling in DCs to promote the activation of T cells and more importantly, the differentiation of T cells into T_H1, T_H2, or T_H17 lineages. Finally, we ask for the effects of adaptive immune cells in *S. aureus* disease.

The aim in part 4 was to investigate which intracellular and extracellular PRRs – besides TLR2 – are involved in sensing of *S. aureus* or *S. aureus*-induced cytokines. Therefore, the function of TLR9, IL-1R, and NOD2 was studied in relation of cytokine induction and systemic infection. Moreover, the contribution of other MyD88-dependent receptors to cytokine responses and additive effects of TLR2 with TLR9 were investigated.

3 Results

3.1 Part 1 – Lipoproteins in *Staphylococcus aureus* mediate inflammation by TLR2 and iron-dependent growth in vivo

The Journal of Immunology

Lipoproteins in *Staphylococcus aureus* Mediate Inflammation by TLR2 and Iron-Dependent Growth In Vivo[1]

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Lipoproteins (Lpp) are ligands of TLR2 and signal by the adaptor MyD88. As part of the bacterial cell envelope, Lpp are mainly involved in nutrient acquisition for *Staphylococcus aureus*. The impact of Lpp on TLR2-MyD88 activation for *S. aureus* in systemic infection is unknown. *S. aureus* strain SA113 deficient in the enzyme encoded by the prolipoprotein diacylglyceryl transferase gene (Δ*lgt*), which attaches the lipid anchor to pro-Lpp, was used to study benefits and costs of Lpp maturation. Lpp in *S. aureus* induced early and strong cytokines by TLR2-MyD88 signaling in murine peritoneal macrophages. Lpp contributed via TLR2 to pathogenesis of sepsis in C57BL/6 mice with IL-1β, chemokine-mediated inflammation, and high bacterial numbers. In the absence of MyD88-mediated inflammation, Lpp allowed bacterial clearing from liver devoid of infiltrating cells, but still conferred a strong growth advantage in mice, which was shown to rely on iron uptake and storage in vitro and in vivo. With iron-restricted bacteria, the Lpp-related growth advantage was evident in infection of MyD88$^{-/-}$, but not of C57BL/6, mice. On the other hand, iron overload of the host restored the growth deficit of Δ*lgt* in MyD88$^{-/-}$, but not in immunocompetent C57BL/6 mice. These results indicate that iron acquisition is improved by Lpp of *S. aureus* but is counteracted by inflammation. Thus, lipid anchoring is an evolutionary advantage for *S. aureus* to retain essential proteins for better survival in infection. *The Journal of Immunology*, 2009, 182: 7110–7118.

Staphylococcus aureus is a frequent cause of life-threatening sepsis. It expresses multiple virulence factors, which contribute to its survival in the host and help its evasion from immune responses. Staphylococcal lipoproteins (Lpp)[3] comprise a large family of membrane-anchored proteins. In the *S. aureus* genome, >50 genes harbor the type II signal sequence typical for Lpp, 35 of which can be associated with a known or predicted function; many of them are part of ABC transporters and are involved in nutrient acquisition (1). Predominant Lpp are SitC (2), which is the binding protein of the staphylococcal iron transporter SitABC; PrsA, the peptidyl-prolyl *cis-/trans*-isomerase involved in protein folding; and OppA, the oligopeptide permease (1). Besides SitC, several characterized staphylococcal Lpp are involved in iron transport as siderophore binding parts of ABC transporters (FhuD1/FhuD2, SirA, SstD) (2–5) and as binding proteins for heme iron and transferrin iron (IsdE and HtsA) (6, 7).

Lpp are synthesized as pre-Lpp with an N-terminal signal sequence containing a conserved C-terminal lipobox. A diacylglyceryl moiety is transferred to the invariant C-terminal cysteine by the lipoprotein diacylglyceryl transferase (Lgt). The signal peptide of pro-Lpp is cleaved by the lipoprotein signal peptidase (LspA) (8). While in Gram-negative bacteria Lpp are further modified at the diacylglyceryl-cysteine by *N*-acyltransferase (Lnt), a homolog of Lnt was not identified in *S. aureus*, suggesting diacylated Lpp. The N-terminal part of an isolated staphylococcal Lpp was characterized as diacylated protein (9), whereas another study found evidence for triacylation of the Lpp SitC by a yet unknown enzyme (10).

Lpp purified from several pathogens, including *S. aureus*, as well as the synthetic lipopeptides $Pam_3CysSerLys_4$ (Pam_3Cys) (11, 12) and $Pam_2CysSerLys_4$ (Pam_2Cys), mirroring the tri- and diacylated Lpp of bacteria, are known to activate TLR2 (10, 13–18). The three lipid chains of Pam_3Cys mediate the heterodimerization of the TLR1 and TLR2 receptor (12, 19). Diacylated Lpp signal by TLR2/6 dimers, and crystallization modeling predicted binding of lipid chains to TLR2 and stabilization by TLR6 (19). Activation of TLR2 by synthetic lipopeptides leads to induction of cytokines, chemokines, adhesins, and nitrite in macrophages and epithelial cells (11). The complexity of TLR2 in recognition becomes obvious since staphylococcal lipoteichoic acids (LTA) have been shown to trigger the immune response (20–22). However, Lpp rather than LTA seem to be the dominant TLR2 stimuli in *S. aureus* strains (1, 10, 13, 18, 23).

Inactivation of Lpp maturation in *S. aureus*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, and *Bacillus subtilis* resulted in reduced growth under stress conditions or in the presence of phagocytes in vitro (1, 23–27). Moreover, *S. aureus* and group B streptococci *lgt* gene deletion mutants (Δ*lgt*) were found to release abundant pre-Lpp possibly by shedding precursor Lpp (1, 28). Lpp maturation is required for virulence of *M. tuberculosis* and *S. pneumoniae* in vivo (25, 26, 29). In contrast, Δ*lgt* mutants in *S. aureus* and group B streptococci were hypervirulent in vivo and induced less cytokines in vitro (1, 23, 28).

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[3] Abbreviations used in this paper: Lpp, lipoprotein; DIP, dipridyl; *lgt*, prolipoprotein diacylglyceryl transferase gene; Δ*lgt*, *lgt* gene deletion mutant; LTA, lipoteichoic acid; MHA, Mueller-Hinton agar; wt, wild type.

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TLR2-MyD88 signaling seems to play an important role in systemic infection with *S. aureus*, since TLR2$^{-/-}$ and MyD88$^{-/-}$ mice succumb to infection (30). A study described a hypervirulent phenotype of *S. aureus* Δ*lgt* in sepsis (23). However, it remains to be elucidated whether this phenotype is linked to impaired TLR2 signaling in the host.

Therefore, we investigated why Lpp maturation is an advantage for *S. aureus* even though this maturation leads to recognition by TLR2 resulting in a strong immune response and control of infection.

Here we show that on the one hand, Lpp maturation is important for induction of TLR2-MyD88-mediated inflammation in vitro and in systemic infection. However, enhanced inflammation caused by SA113 wild type (wt) is associated with a higher bacterial load in organs of infected C57BL/6, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice. We then reveal that on the other hand, Lpp maturation is fundamental for iron uptake and by this promotes growth of *S. aureus*. Thus Lpp-mediated growth and survival of *S. aureus* is counteracted by MyD88-mediated inflammation in vivo.

Materials and Methods

Mice

Mice were bred under specific pathogen-free conditions in the Animal House of the Department of Biomedicine, University Hospital Basel, according to the regulations of Swiss veterinary law. MyD88$^{-/-}$ mice were a kind gift from W. D. Hardt (ETH Zürich) and TLR2$^{-/-}$ from W. J. Rieflin (Tularik), all backcrossed on C57BL/6 background for 10 generations. Mice were euthanized by CO_2 or i.p. injection of 500 mg/kg Thiopenthal (Abbott Laboratories).

Bacterial strains and growth conditions

SA113 wt (American Type Culture Collection 35556), its isogenic mutants *lgt*::ermB (Δ*lgt*), and pRB*lgt*-complemented SA113 Δ*lgt*, described in detail previously (1), were used in this study. Newman wt (31) (own strain collection) and the Newman Δ*lgt* mutant, generated exactly as described for SA113 (1), were used for additional experiments.

Bacteria were grown in trypticase soy broth for 7 h and subcultured overnight under the same conditions. Overnight cultures were washed in 0.9% NaCl (Bichsel, Switzerland) and used for sepsis. Each inoculum was assessed by CFU/ml counting on Mueller-Hinton agar (MHA) plates. For NF-κB activation and cytokine induction, bacteria were further subcultured in trypticase soy broth to end-log phase. When appropriate, trypticase soy broth was supplemented with 10 μg/ml erythromycin.

Growth under iron-depleted conditions was done as previously published (32) with modifications. Briefly, bacteria were cultured in RPMI 1640 with 250 μM dipyridyl (DIP; Sigma-Aldrich) overnight at 37°C without shaking, then subcultured at 37°C with 120 rpm in RPMI treated for 1 h with 6% Chelex (Sigma-Aldrich) supplemented with divalent cations. Iron availability was restored with 20 μM $FeSO_4$, 20 μM $FeCl_3$, or 10% iron dextran (Serumwerk Bernburg). CFU/ml were assessed by plating serial dilutions on MHA plates.

For survival of *S. aureus* in murine mouse plasma, blood was collected by cardiac puncture in hirudin. Plasma was separated from contaminating cells by centrifugation and infected with *S. aureus* strains. Growth was assessed by plating serial dilutions on MHA plates.

Preparation of peritoneal macrophages

Mice were i.p. injected with 2 ml of 3% thioglycolate (BD Biosciences). Three to 4 days later, peritoneal exudate cells were collected from the peritoneal cavity by washing with 6 ml of cold RPMI 1640 complete (5% FBS, 2 mM glutamate, 1 mM sodium pyruvate, 1.5 mM HEPES, nonessential amino acids). Erythrocytes were lysed and 1×10^6 cells/ml were seeded either in 24-well plates for cytokine production or in 16-well chamber slides for NF-κB activation assays. Adherent macrophages were infected with 1×10^7 CFU/ml viable *S. aureus* strains in RPMI 1640 complete.

Macrophage cytokine assays

Supernatants were replaced 1 h after phagocytosis with RPMI 1640 complete containing 100 μg/ml gentamicin. Supernatants were collected at 2 and 18 h postinfection and TNF, IL-1β, IL-6, IL-10, and MIP-2 were determined by ELISA according to instructions from BD Biosciences and for MIP-2 from R&D Systems.

NF-κB translocation assay

Macrophages were stimulated with bacteria. After 20 and 120 min, extracellular bacteria were degraded by 20 U lysostaphin (Genmedics). Cells were washed with PBS and fixed in polylysin-paraformaldehyde solution (2.14 g/L periodate, 13.7 g/L lysine, 2% paraformaldehyde). Cells were incubated with polyclonal rabbit anti-mouse NF-κB p65 Ab (eBioscience) in PBS containing 2% normal goat serum, and further incubated with biotinylated goat-anti-rabbit Ab (Vector Laboratories). ABC kit solution (Vector Laboratories) was added and visualized with AEC solution (Dako). Nuclei were counterstained with hematoxylin, cells were mounted in Kaiser's glycerol gelatin (Merck), and nuclear localization of NF-κB was analyzed by light microscopy.

Sepsis model

Sepsis was induced in female C57BL/6, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice aged 6–8 wk. *S. aureus* (1×10^6 to 1×10^8 CFU) in 200 μl of 0.9% NaCl was injected into the lateral tail vein. For iron overloading, mice were i.p. injected with 2 mg of iron dextran (Serumwerk Bernburg) 3 h before infection. After infection, weight development was measured. Blood was collected by cardiac puncture in EDTA. Animals were perfused into the left ventricle with Ringer's solution; spleens, livers, both kidneys, and both knees were collected and homogenized in a 1 ml vol of 0.9% NaCl. Cytokines and chemokines were determined in plasma and spleen homogenates by ELISA (see "Macrophage cytokine assays"). Bacteria were assessed in blood and tissue homogenates by serial dilutions in 0.9% NaCl plated on MHA plates. Organs were fixed with paraformaldehyde, embedded in paraffin, and stained with H&E or Berlin blue to stain iron. Infiltrates, defined as accumulation of >20 leukocytes without central necrotic area, were enumerated by light microscopy at a magnification of ×10 in three randomly selected areas of H&E-stained organ sections. For cryosections, tissues were frozen and stained by immunohistochemistry as previously described (33) with anti-CD45, anti-CD11b, anti-BM8, or anti-Gr-1 Abs. Microscopic analysis was performed with CellP analysis software in an Olympus BX61 microscope.

Splenocyte stimulation in vitro

Splenocytes from infected mice were isolated 1 day after injection, mechanically disrupted (70-μm meshes), followed by lysis of erythrocytes and resuspension in RPMI 1640 complete. In 24-well plates, 1×10^6 cells were infected for 1 h with the same viable bacteria as used for infection at a ratio of bacteria/splenocytes of 1:1. For further 18 h of incubation, gentamicin was added to the cells to prevent bacterial growth. Supernatants were collected and cytokines were analyzed by ELISA as done for macrophages (see "Macrophage cytokine assays").

Streptonigrin assay and ^{55}Fe uptake

Bacteria from overnight cultures or ex vivo after homogenization of kidneys from infected C57BL/6 mice were exposed to 2 μg/ml of streptonigrin (34) at 37°C for 30 min. CFU/ml were assessed before and after exposure by plating serial dilutions on MHA plates.

For ^{55}Fe uptake experiments, bacteria were grown in Chelex-treated M9 minimal medium (1% casamino acids and 5% MEM vitamin solution). The medium was treated with 200 μM DIP. Bacteria were harvested at an OD_{578} of 0.8–1.0, washed twice with M9 transport medium (Chelex-treated 1× M9 salts, 1 ml of 1 mM $MgSO_4$, 0.1% glucose) at 4°C, and resuspended in M9 transport medium to OD_{578} of 1.0. After incubation at 37° C for 5 min with shaking, ^{55}Fe-reduced with ascorbate (100 μM (5.5 kBq/ml)/100 mM ascorbate) was diluted 100-fold in the bacterial suspension. Samples were taken at the indicated times, filtered on mixed cellulose GN-6 Metricel membrane filters (Pall), washed twice with 0.1 M LiCl solution, dried, and counted with a liquid scintillation counter.

Statistical analysis

In Kaplan-Meier plots, log rank test was used to compare sepsis survival among infected mice. A two-way ANOVA test was used for statistical analyses of cytokines and NF-κB in macrophages in vitro and for weight loss of mice after infection. CFU in organs, cytokines in plasma, or splenocyte restimulation assays were analyzed with Mann-Whitney. Statistical analysis was done with Prism 5.0a (GraphPad Software). A p value of <0.05 was considered statistically significant.

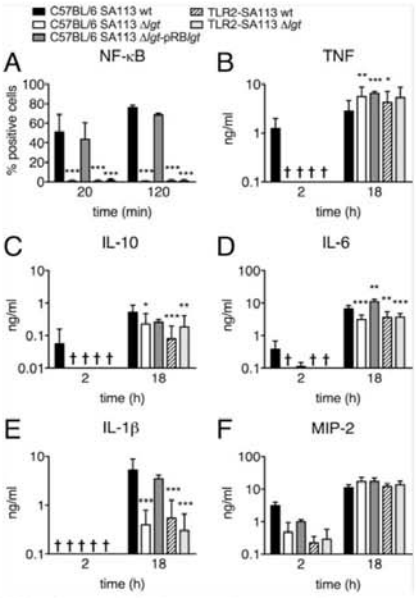

FIGURE 1. Lpp on viable *S. aureus* induce cytokines in macrophages via TLR2. Peritoneal macrophages from C57BL/6 and TLR2$^{-/-}$ mice were infected with SA113 wt (filled and striped bars, respectively), Δ*lgt* (open and dotted bars, respectively) and the complemented Δ*lgt*-pRB*lgt* (gray bars, only in C57BL/6) at multiplicity of infection of 10. *A*, Percentage of NF-κB-positive cells after 20 and 120 min of infection. *B–F*, Supernatants were analyzed by ELISA for (*B*) TNF, (*C*) IL-10, (*D*) IL-6, (*E*) IL-1β, and (*F*) MIP-2 after 2 and 18 h of infection. †, Not detectable. Data are means ± SD of three or more experiments. Significant differences of wt-infected C57BL/6 macrophages vs all other groups are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

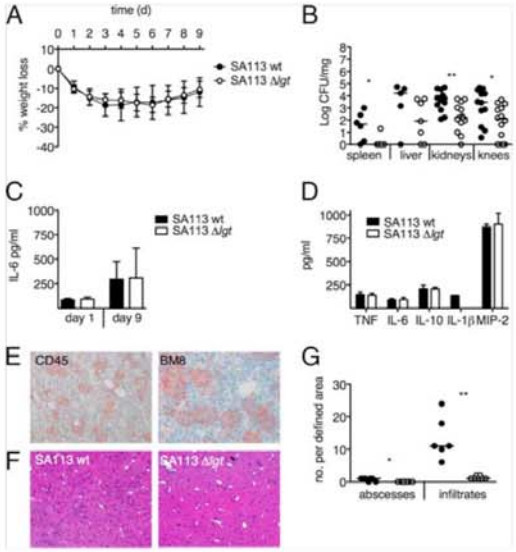

FIGURE 2. Lpp enhance virulence of *S. aureus* in sepsis. *A–F*, C57BL/6 mice infected with 1×10^7 CFU of SA113 wt (filled) and SA113 Δ*lgt* (open). *A*, Weight loss of mice during infection and (*B*) bacterial load in spleen, liver, both kidneys, and both knees on day 9. *C*, Plasma IL-6 level 1 and 9 days after infection. *D*, One day after infection, isolated splenocytes were restimulated with the corresponding strain for 24 h in vitro. TNF, IL-6, IL-10, IL-1β, and MIP-2 in supernatants were analyzed by ELISA. *E*, Anti-CD45 and anti-BM8 immunohistochemistry of frozen liver sections from mice infected with SA113 wt. *F* and *G*, Histological analysis of H&E-stained liver sections after infection with SA113 wt and Δ*lgt* on day 9 and quantification of abscesses and infiltrates by microscopy. Data are represented as means ± SD for weight loss and cytokines and median for CFU/mg and numbers in liver sections of at least six mice per group. Significant differences between wt and Δ*lgt* are indicated as follows: *, $p < 0.05$; **, $p < 0.01$.

Results

Lpp induce inflammation via TLR2 and MyD88 in vitro

We first investigated the effect of Lpp on induction of selected macrophage products, which are implicated in the pathogenesis of sepsis (35). Therefore, we infected peritoneal macrophages of C57BL/6 and TLR2$^{-/-}$ mice with viable *S. aureus* SA113 wt and Δ*lgt* to assess NF-κB activation, cytokines, and MIP-2 levels. SA113 wt but not Δ*lgt* induced NF-κB in C57BL/6 macrophages after 20 min, whereas infected TLR2$^{-/-}$ cells showed no activation until 2 h (Fig. 1*A*). TNF, IL-10, IL-6, and MIP-2 were induced by SA113 wt through TLR2 signaling early after infection (2 h). These cytokines were absent or less induced by SA113 Δ*lgt* and/or in the absence of TLR2 (Fig. 1, *B–D* and *F*). IL-1β was not detectable after 2 h (Fig. 1*E*). Late after infection (18 h), TNF levels remained high in the presence of Lpp; however, in their absence TNF was overshooting (Fig. 1*B*). IL-10, IL-6, and IL-1β remained partially Lpp-TLR2-dependent, while MIP-2 was independent (Fig. 1*C–F*). Results obtained with SA113 Δ*lgt*-pRB*lgt* in C57BL/6 cells were comparable to those of SA113 wt (Fig. 1).

Cytokine release required MyD88-mediated signals in macrophages, since none of the strains induced cytokines in MyD88$^{-/-}$ macrophages (data not shown). Taken together, these data show that Lpp of SA113 induce inflammatory mediators, which are relevant in sepsis, by TLR2-MyD88 signaling.

Staphylococcal Lpp increase bacterial growth and inflammatory response in vivo

Recently, Bubeck Wardenburg et al. showed better survival of C57BL/6 mice infected with 5×10^6 CFU of *S. aureus* Newman wt than with the Δ*lgt* mutant. The authors attributed this answer to a stronger immune response in *S. aureus* wt compared with Δ*lgt*-infected mice (23). In our survival studies with *S. aureus* SA113, 10^8 CFU led to similar death in 20% of wt-infected mice ($n = 9$) and 25% in Δ*lgt*-infected mice ($n = 9$) after 9 days.

We next examined the role of Lpp in *S. aureus* on growth in vivo. C57BL/6 mice were infected with 1×10^7 CFU of SA113 wt and Δ*lgt* and showed a similar weight loss during 9 days of infection (Fig. 2*A*). After 1 day, bacterial load of SA113 wt and Δ*lgt* was similar in all examined organs from C57BL/6 mice (data not shown). However, on day 9 after infection, bacterial load of SA113 wt in spleen, kidneys, and knees of C57BL/6 mice was significantly higher compared with Δ*lgt* (Fig. 2*B*), while bacterial load in liver was slightly, albeit not significantly, higher after infection with SA113 wt (Fig. 2*B*).

These data suggest that *S. aureus* Lpp are advantageous for bacterial growth in organs during a persistent infection.

We further studied whether bacterial growth was related to induction of inflammatory mediators by Lpp in vivo. Therefore, we

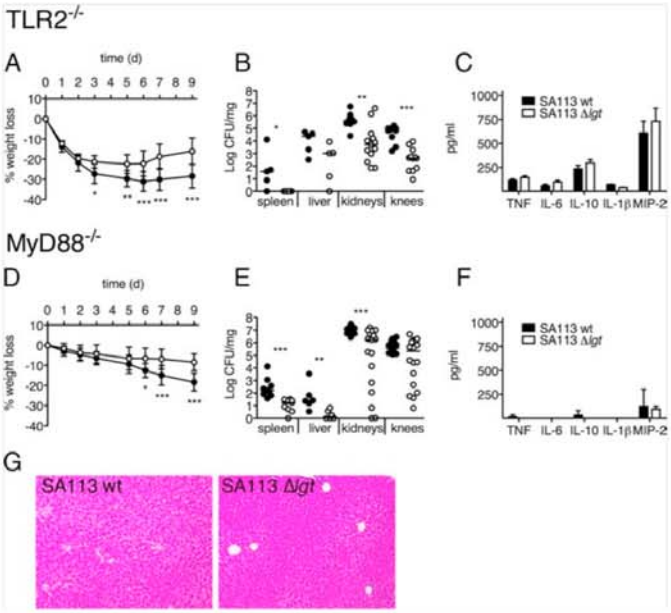

FIGURE 3. TLR2 sensing of Lpp has a minor effect on *S. aureus* in vivo. *A*, Weight loss and (*B*) bacterial load in spleen, liver, both kidneys, and both knees in TLR2$^{-/-}$ mice after infection with 1×10^7 SA113 wt (filled) and SA113 Δ*lgt* (open) on day 9. *C*, One day after infection, isolated splenocytes were restimulated with the corresponding strain for 24 h in vitro. TNF, IL-6, IL-10, IL-1β, and MIP-2 in supernatants were analyzed by ELISA. *D*, Weight loss and (*E*) bacterial load in spleen, liver, both kidneys, and both knees in MyD88$^{-/-}$ mice with 1×10^6 SA113 wt (filled) and SA113 Δ*lgt* (open) on day 9. *F*, One day after infection, isolated splenocytes were restimulated with the corresponding strain for 24 h in vitro. TNF, IL-6, IL-10, IL-1β, and MIP-2 in supernatants were analyzed by ELISA. *G*, Histological analysis of H&E-stained liver sections 9 days after infection. Data for TLR2$^{-/-}$ and MyD88$^{-/-}$ mice are represented as means ± SD for weight loss and cytokines and median for CFU/mg of five to six mice per group. Significant differences between wt and Δ*lgt* are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

determined the cytokines in plasma of C57BL/6 mice after infection with SA113 wt and Δ*lgt*. After 1 h of infection, IL-6 levels were higher with SA113 wt compared with Δ*lgt* (mean ± SD, 671 ± 251 and 95 ± 164 pg/ml; $p < 0.05$), but the difference was abolished 4 h after infection (SA113 wt 450 ± 431 pg/ml vs Δ*lgt* 234 ± 405 pg/ml). One day after infection, TNF, IL-10, IL-1β, and MIP-2 were not detectable in plasma (data not shown). IL-6 levels in plasma were comparable after infection with SA113 wt and Δ*lgt* and rose considerably to similar levels after 9 days (Fig. 2*C*), indicating no systemic cytokine regulation by staphylococcal Lpp in SA113. To examine organ-specific induction of cytokines mediated by Lpp, splenocytes were isolated 1 day after infection and restimulated for 24 h in vitro with the same strain as used in vivo. Both strains induced all mediators to a similar extent except IL-1β, which was exclusively produced in response to SA113 wt (Fig. 2*D*), suggesting that Lpp mediate a local regulation of cytokines in infected organs.

To assess the importance of staphylococcal Lpp in persistent tissue infection, we enumerated abscesses and infiltrates in liver sections from wt- and Δ*lgt*-infected C57BL/6 mice on day 9. Infiltrates after infection with either SA113 wt or Δ*lgt* were composed of CD45$^+$ leukocytes, BM8$^+$ macrophages (Fig. 2*E*), and CD11b$^+$ monocytes, whereas Gr-1$^+$ granulocytes were rarely recruited (data not shown). However, more infiltrates and abscesses were found after infection with SA113 wt than with Δ*lgt* (Fig. 2, *F* and *G*). Increased monocyte infiltrates in SA113 wt-infected livers correlated with increased levels of MIP-2 (81 ± 32 vs 41 ± 19 pg/ml, $p < 0.001$) and KC (968 ± 454 vs 617 ± 153 pg/ml, $p < 0.05$) in spleen after infection with SA113 wt compared with Δ*lgt* on day 9. In summary, mature Lpp in *S. aureus* allowed a better growth in organs and elicited an early stronger release of IL-1β and induced late chemokines accompanied by increased monocytic infiltration. The results indicate that Lpp support growth and survival of *S. aureus* despite eliciting an immune response.

Lpp have a TLR2- and MyD88-independent effect on bacterial growth in vivo

Our cytokine data revealed a role of TLR2 in sensing staphylococcal Lpp after stimulation of macrophages in vitro. To examine the contribution of TLR2 to the innate response in vivo, we infected TLR2$^{-/-}$ mice with SA113 wt and Δ*lgt*. SA113 wt induced a higher weight loss (28%) than did Δ*lgt* (16%) in infected mice (Fig. 3*A*). Bacterial load in TLR2$^{-/-}$ organs was higher after infection with SA113 wt than with Δ*lgt* (Fig. 3*B*). SA113 wt and Δ*lgt* did not induce TNF, IL-10, IL-1β, and MIP-2 in mouse plasma, and IL-6 was independent of Lpp (data not shown). In restimulated splenocytes of infected TLR2$^{-/-}$ mice, TNF, IL-6, IL-10, IL-1β, and MIP-2 were independent of staphylococcal Lpp and lower than in restimulated splenocytes from C57BL/6 mice (Fig. 3*C*). Compared with C57BL/6 mice (Fig. 2, *A* and *B*), TLR2$^{-/-}$ mice showed significantly more weight loss (Fig. 3*A*) and significantly higher bacterial load in kidneys and knees (Fig. 3*B*) after infection with SA113 wt ($p < 0.001$ and $p < 0.01$, respectively), but not after infection with Δ*lgt*.

MyD88$^{-/-}$ mice lack a macrophage inflammatory response and succumb to infections with staphylococci (30). Since *S. aureus* Lpp enhanced growth and inflammation in systemically infected C57BL/6 mice, we used MyD88$^{-/-}$ mice to avoid strong inflammation and analyzed the Lpp-mediated growth of *S. aureus*. Infected MyD88$^{-/-}$ mice slowly lost weight, but weight loss was more severe with SA113 wt than with Δ*lgt* (Fig. 3*D*). Bacterial load of mice infected with SA113 wt was higher in all organs than with Δ*lgt* (Fig. 3*E*). Interestingly, bacterial load in liver was dramatically reduced compared with C57BL/6 and TLR2$^{-/-}$ mice, while all other organs had increased bacterial numbers. Restimulated splenocytes from MyD88$^{-/-}$ mice induced no or very low levels of TNF, IL-6, IL-10, IL-1β, and MIP-2 independent on staphylococcal Lpp (Fig. 3*F*). Consistent with the lack of cytokines and chemokines (data not shown), livers had no granulomas

Table I. *Disease ranking to illustrate the influence of staphylococcal Lpp and iron on weight loss and bacterial load in C57BL/6, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice*[a]

		% Weight Loss		CFU/mg								Total Disease Rank (max. 15)
				Spleen		Liver		Kidneys		Knees		
	SA113 strain	Mean	Rank	Median	Rank	Median	Rank	Median	Rank	Median	Rank	
Untreated												
C57BL/6	wt	12	+	1.67	++	4.21	+++	3.72	+	3.49	+	8
	Δ*lgt*	11	+	0	−	1.92	++	2.21	−	2.09	−	3
TLR2$^{-/-}$	wt	29	+++	1.58	++	4.39	+++	5.63	++	4.86	++	12
	Δ*lgt*	16	++	0	−	3.03	+++	3.71	+	2.64	−	6
MyD88$^{-/-}$	wt	18	++	2.22	+++	1.39	+	7.15	+++	5.55	+++	12
	Δ*lgt*	8	−	1.25	+	0.13	−	6.23	++	5.34	++	5
Iron-depleted bacteria												
C57BL/6	wt	3	−	0	−	0.17	−	1.94	−	2.79	+	1
	Δ*lgt*	−1	−	0	−	0.29	−	2.42	−	0	−	0
MyD88$^{-/-}$	wt	12	+	1.85	++	1.40	+	7.32	+++	5.51	+++	10
	Δlgt	0	−	0.69	+	0	−	0	−	0	−	1
Iron dextran-overloaded mice												
C57BL/6	wt	27	+++	2.49	+++	4.59	+++	4.48	++	4.71	++	13
	Δ*lgt*	12	+	0	−	2.45	++	3.53	+	2.88	+	5
MyD88$^{-/-}$	wt	19	+++	1.93	+++	2.57	++	6.63	+++	6.34	+++	14
	Δ*lgt*	15	++	1.91	+++	1.25	+	6.71	+++	5.79	+++	12
	Median	12		1.41		1.66		4.10		4.10		
	Q1	9		0		0.53		2.70		2.68		
	Q3	18		1.89		2.91		6.53		5.47		

[a] Mean values of percentage weight loss and median values of $\log_{10}$ CFU/mg in spleen, liver, kidneys, and knees are shown in columns for C57BL/6, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice, which are grouped according to infection with either *S. aureus* wt or Δ*lgt* and according to the iron treatment. We introduced a ranking to facilitate comparison of all groups. Median, quartile 1 (Q1), and quartile 3 (Q3)were calculated from each column including all groups and ranks were assigned: −, $x \leq$ Q1; +, Q1 $< x \leq$ median; ++, median $< x \leq$ Q3; +++, $x >$ Q3. Total disease rank is the sum of ranks (+) per row, with a maximum of 15.

(Fig. 3*G*). Thus, the absence of inflammation was not only accompanied by an increase in bacterial load, but also by an atypical distribution of *S. aureus* into organs. However, as summarized in Table I, Lpp allow a better bacterial growth independently of host TLR2 and MyD88 signaling.

Maturation of Lpp is required for S. aureus *growth and iron uptake in vitro and ex vivo*

We showed that differences in growth of SA113 wt and Δ*lgt* in vivo were not dependent on Lpp-TLR2-mediated immune responses. Since many *S. aureus* Lpp are involved in iron transport (1, 36), we investigated the role of Lpp maturation in iron acquisition and growth in vitro. As expected, neither SA113 wt nor Δ*lgt* were growing within 48 h in iron-depleted RPMI 1640 (not shown). Supplementation of iron-depleted RPMI 1640 with $FeSO_4$, $FeCl_3$, and iron dextran permitted better growth of SA113 wt than Δ*lgt* (Fig. 4*A*). SA113 Δ*lgt* died in $FeSO_4$-supplemented RPMI 1640 and was growing less well than wt in the presence of $FeCl_3$ or iron dextran (Fig. 4*A*). This suggests a benefit of Lgt expression for growth and maximal acquisition of iron.

Since transferrin is saturated to 60% in mice (37) and serves besides heme as an iron source for *S. aureus* (7), we examined whether Lpp maturation improves utilization of transferrin-bound iron by using plasma as growth medium. We found no differences in growth of SA113 wt and Δ*lgt* in plasma (Fig. 4*B*).

To confirm that improved growth of SA113 wt was a consequence of better iron uptake, we exploited the iron-dependent antibiotic streptonigrin. SA113 wt grown under iron-limited conditions in RPMI-DIP were more sensitive to streptonigrin and showed a 2.5-fold higher ^{55}Fe uptake than Δ*lgt* or nonrestricted SA113 strains, indicating that Lpp improved iron uptake (Fig. 4, *C* and *D*). Lpp also affected iron uptake during infection since ex vivo SA113 wt from C57BL/6 kidneys was more sensitive to streptonigrin than Δ*lgt* (Fig. 4*E*). Taken together, our results indicate that Lpp maturation is a prerequisite for maximal iron acquisition of *S. aureus* in vitro and in vivo.

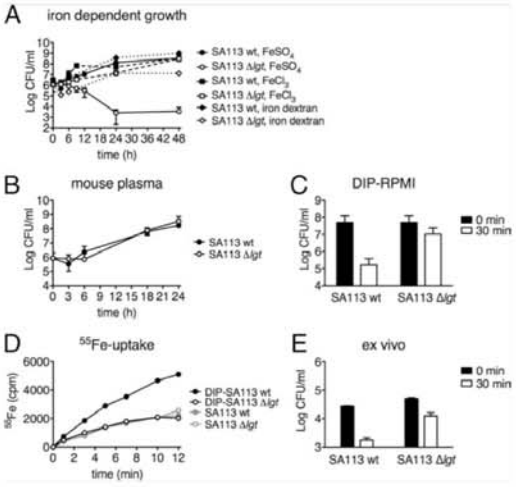

FIGURE 4. Lpp enhance iron acquisition in *S. aureus*. SA113 wt (filled) and Δ*lgt* (open) were grown in iron-depleted RPMI 1640 supplemented with (*A*) 20 μM $FeSO_4$ (circles), 20 μM $FeCl_3$ (squares), or 10% iron dextran (diamonds). *B*, Growth of SA113 wt (filled) and Δ*lgt* (open) in murine plasma from C57BL/6 mice. *C*, Streptonigrin sensitivity for 30 min of SA113 wt and Δ*lgt* grown overnight in RPMI 1640 with 250 μM DIP. *D*, ^{55}Fe uptake of SA113 wt (filled) and Δ*lgt* (open) in M9 medium with (black) or without (gray) 200 μM DIP. *E*, Streptonigrin sensitivity for 30 min of pooled SA113 wt and Δ*lgt* isolated from kidneys of infected C57BL/6 mice. Data show one representative out of three experiments.

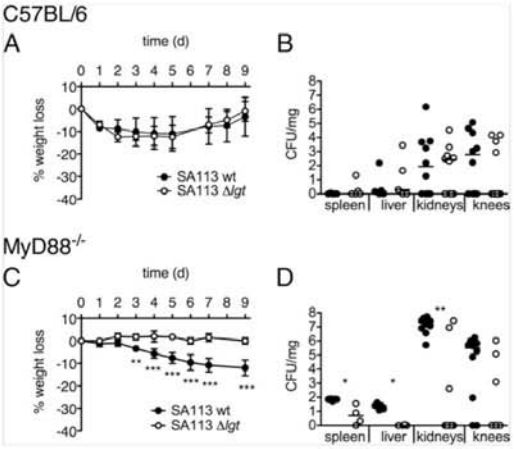

FIGURE 5. Iron depletion increases Lpp-mediated virulence of *S. aureus* in MyD88$^{-/-}$ mice. *A*, Weight loss and (*B*) bacterial load in spleen, liver, both kidneys, and both knees after infection of C57BL/6 mice with 4×10^7 CFU of iron-deprived SA113 wt (filled) and Δ*lgt* (open) on day 9. *C*, Weight loss and (*D*) bacterial load in spleen, liver, both kidneys, and both knees after infection of MyD88$^{-/-}$ mice with 1×10^6 CFU of iron-deprived SA113 wt (filled) and Δ*lgt* (open) on day 9. Data are represented as mean ± SD for weight loss and median for CFU/mg of at least four mice per group. Significant differences between wt and Δ*lgt* are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

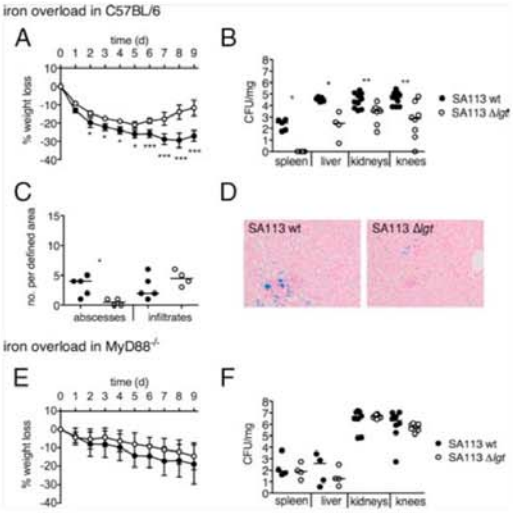

FIGURE 6. Iron overload increases virulence of *S. aureus* Δ*lgt* in MyD88$^{-/-}$ mice. *A–D*, Infection of iron-overloaded C57BL/6 mice with 4×10^7 CFU of SA113 wt (filled) and Δ*lgt* (open). *A*, Weight loss and (*B*) bacterial load in spleen, liver, both kidneys, and both knees on day 9. *C*, Number of abscesses and infiltrates of H&E-stained liver sections after infection with SA113 wt and Δ*lgt* on day 9 were evaluated by microscopy. *D*, Iron staining of liver sections after infection with SA113 wt and Δ*lgt* on day 9. *E* and *F*, Infection of iron-overloaded MyD88$^{-/-}$ mice with 1×10^6 CFU of SA113 wt (filled) and Δ*lgt* (open). *A*, Weight loss and (*B*) bacterial load in spleen, liver, both kidneys, and both knees on day 9 after infection. Data are represented as mean ± SD for weight loss and cytokines and median for CFU/mg and numbers in liver sections of at least four mice per group. Significant differences between wt and Δ*lgt* are indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Lpp allow bacterial growth of iron-restricted S. aureus *in MyD88$^{-/-}$ mice*

Lpp enhanced ^{55}Fe uptake of *S. aureus* after iron restriction in vitro, suggesting an up-regulation of Lpp under these conditions. Therefore, we expected a Lpp-mediated growth advantage of SA113 wt in vivo. We cultured SA113 wt and Δ*lgt* under iron-restricted conditions before infection. Iron restriction of SA113 wt and Δ*lgt* caused a transient weight loss of 10% and complete weight recovery of C57BL/6 mice on day 9 (Fig. 5*A*). The reduction of intrabacterial iron abolished the differences between SA113 wt and Δ*lgt* in bacterial load (Fig. 5*B*) as well as in liver infiltrates and abscesses (data not shown). This indicates that the Lpp-dependent advantage of SA113 wt in C57BL/6 mice (Fig. 2) was not due to iron acquisition in the immunocompetent host.

We hypothesized that the iron-restricted SA113 wt was unable to grow higher than Δ*lgt* in C57BL/6 mice due to iron restriction induced by strong MyD88-dependent inflammation. Indeed, bacterial load of iron-restricted SA113 wt was higher than Δ*lgt* after infection of MyD88$^{-/-}$ mice (Fig. 5*D*). MyD88$^{-/-}$ mice infected with SA113 wt showed a weight loss of 11%, and those infected with Δ*lgt* maintained weight (Fig. 5*C*). Infiltrates and abscesses in the liver were rare with both strains (not shown). These results are summarized in Table I. They suggest that for the growth-promoting effect of Lpp, *S. aureus* requires iron before infection to ensure immediate iron availability in the host, who efficiently sequesters iron by a MyD88-dependent mechanism.

Lpp are dispensable for bacterial growth in iron-overloaded MyD88$^{-/-}$ mice

We next investigated whether iron overload abrogates the growth defect of SA113 Δ*lgt* in the presence or absence of MyD88 signaling. Therefore, we injected iron dextran in C57BL/6 and MyD88$^{-/-}$ mice before infection with SA113 strains grown under normal conditions. During infection, SA113 wt caused 27% weight loss in iron-overloaded C57BL/6 mice, while Δ*lgt* caused a transient weight loss of 20% and a regain of weight after day 5 (Fig. 6*A*). Iron overload did not improve virulence of Δ*lgt* compared with wt as shown by lower bacterial load in all organs and less abscesses in liver after infection of C57BL/6 mice (Fig. 6, *B–D*). Although SA113 Δ*lgt* caused similar numbers of infiltrates as wt (Fig. 6*C*), the benefit of Lpp was observed by accumulation of iron in infiltrates after infection with SA113 wt (Fig. 6*D*).

In contrast, in iron-overloaded MyD88$^{-/-}$ mice, SA113 wt and Δ*lgt* caused a slow progression of weight loss to ~17% on day 9 (Fig. 6*E*). Bacterial numbers of SA113 Δ*lgt* attained similar levels as wt in all organs of infected mice (Fig. 6*E*). These results are summarized in Table I and show that under high iron levels, Lpp maturation is dispensable for growth in the MyD88-deficient host but is required for growth in organs of the immunocompetent host.

Lpp in strain Newman act in a proinflammatory manner and enhance virulence in MyD88$^{-/-}$ mice

Bubeck Wardenburg et al. observed a reduced virulence of *S. aureus* Newman wt compared with Δ*lgt*, which they explained by escape from host defense (23). We wanted to assess whether the growth-promoting and virulence-enhancing effect of Lpp, observed with SA113, was also seen with the staphylococcal enterotoxin B-producing Newman strain (38). With Newman wt and its

isogenic Δ*lgt* mutant, we found macrophages activated in a Lpp- and TLR2-dependent manner (data not shown). Results were similar to those observed with SA113 and as published earlier with heat-killed Newman wt and Δ*lgt* (23). Systemic infection with 1×10^8 CFU of the Newman wt and Δ*lgt* led to 100% and 20% death of C57BL/6 mice within 24 h, respectively; however, all Δ*lgt*-infected mice died within 32h (supplemental Fig. 1*A*).[4] With a nonlethal inoculum of 1×10^6 CFU per mouse, Newman wt caused a more severe weight loss than did Δ*lgt* (supplemental Fig. 1*B*). Newman wt caused a higher bacterial load in liver and kidneys and induced more IL-6 in mouse plasma than did Δ*lgt* (supplemental Fig. 1, *C* and *D*). In restimulated splenocytes isolated 1 day after infection of C57BL/6 mice, TNF, IL-6, IL-10, IL-1β, and MIP-2 were partially or completely dependent of staphylococcal Lpp (supplemental Fig. 1*E*).

To examine whether Lpp can enhance growth in the absence of inflammation, we infected MyD88$^{-/-}$ mice with Newman wt and Δ*lgt*. Newman wt-infected MyD88$^{-/-}$ mice died significantly earlier and surviving mice lost more weight than did Δ*lgt*-infected animals (supplemental Fig. 2, *F* and *G*). Additionally, after growth in iron-depleted medium we observed a higher streptonigrin susceptibility of Newman wt than Δ*lgt* in vitro (supplemental Fig. 1*H*), suggesting an improved iron acquisition by Lpp in ABC transporters as observed for SA113 strains. Our data show that Lpp of both *S. aureus* strains, Newman and SA113, induce not only a TLR2-mediated immune response but exert also a growth-promoting effect.

Discussion

We have evaluated the function of mature Lpp in staphylococcal immunopathogenesis. Using the low-virulent *S. aureus* strain SA113, we have shown that inflammation in vitro and in the immunocompetent host in vivo is dependent on Lpp. On the one hand, we show that mature Lpp elicit a strong cytokine and chemokine response by activating TLR2 early. Lpp activate other MyD88-signaling receptors late (e.g., via IL-1β and IL-1R), which is required for a potent antimicrobial defense of the host. On the other hand, our data show that mature Lpp allow better growth through iron acquisition. Iron availability before infection ensured Lpp-mediated growth advantage of *S. aureus* in the absence of MyD88 signaling, which was counteracted by inflammation in the immunocompetent C57BL/6 mouse. However, this advantage was abrogated when additional iron was available during infection, suggesting that the MyD88-deficient host could not efficiently restrict iron.

Our study shows that Lpp are responsible for early induction of cytokines by viable *S. aureus* and enhance late IL-1β-mediated inflammation in macrophages by TLR2 signaling in vitro. Our data are in agreement with previous reports showing that Lpp of viable SA113 wt and heat-killed Newman wt induce cytokines in a monocyte cell line and macrophages, respectively (1, 23). The strongest Lpp-TLR2 dependence was found for IL-1β, which is involved in neutrophil recruitment and consequently for killing of *S. aureus* in skin infection (39). Since we found macrophage activation to be completely MyD88-dependent, *S. aureus* uses TLR2 mainly as an early receptor, and other receptors such as IL-1R (40) and possibly TLR9 participate late (30, 41). Additionally, we confirmed the exclusive activation of TLR2 by Lpp and not LTA in viable SA113, which was already observed after purification of staphylococcal LTA and Lpp (10, 13, 18).

[4] The online version of this article contains supplemental material.

TNF, IL-6, IL-10, IL-1β, and MIP-2 are important mediators in sepsis (35) and were detectable in our systemic infection model. Using SA113 wt, we found that Lpp induced IL-1β production in spleen, consecutively late chemokines in spleen, and strong recruitment of monocytes into liver with formation of granulomas. IL-6 and other cytokines in plasma were independent of Lpp, except for a very early time point of 1 h after infection, indicating a local regulation of the inflammation by Lpp. A local regulation by Lpp may also explain the comparable disease course after infection with either SA113 wt or Δ*lgt*. Interestingly, the Lpp-related inflammation was associated with a higher bacterial load in organs, demonstrating that in SA113 Lpp maturation is involved in persistence of *S. aureus* infection. Lpp in strain Newman similarly promoted virulence, which was visible as stronger weight loss, pronounced inflammation in the spleen, acute phase reaction in plasma, and growth advantage in liver and kidneys. Our results cannot confirm the previously observed enhanced virulence of Δ*lgt* (23). The discrepancy may originate in the application route or the different generation of the Newman Δ*lgt* mutants.

To our knowledge, a study addressing the question whether a host response in systemic infection is mediated by staphylococcal Lpp and by TLR2 is missing. Using TLR2$^{-/-}$ mice, we show that recognition of staphylococcal Lpp by TLR2 allows inflammation, limits bacterial growth, and improves recovery of weight in SA113 wt-infected mice. Infection with SA113 Δ*lgt* was completely unaffected by TLR2. In agreement with Takeuchi et al. (30), we found that Lpp-TLR2 signaling restricted bacterial load only in kidneys, but not in spleen and liver. We propose an organ-specific TLR2-mediated response, but an overall moderate role of TLR2 signaling in *S. aureus* sepsis, as earlier published for staphylococcal skin infections (40).

Consistent with previous studies, *S. aureus* wt caused distinct clinical results in MyD88$^{-/-}$ mice with delayed appearance of severe symptoms and higher bacterial load compared with C57BL/6 mice (30, 40). Unexpectedly, in MyD88$^{-/-}$ mice SA113 and Newman Δ*lgt* still induced a milder disease than wt, excluding the contribution of other Lpp-TLR2-independent cytokines to the lower bacterial load of Δ*lgt*. While in most organs the lack of MyD88 allowed bacterial growth, bacterial numbers of SA113 were low and granulomas were absent in the liver of MyD88$^{-/-}$ mice. Failure of chemokine induction and leukocyte infiltration possibly prevented bacterial hiding in MyD88$^{-/-}$ mice and enabled bacterial killing in livers. In contrast, lack of MyD88 allowed a much stronger bacterial growth in kidneys and knees, which suggests that MyD88-induced effects protect the host in the kidney, but not in the liver.

However, bacterial numbers of SA113 wt in liver and other organs of MyD88$^{-/-}$ mice were higher than those of Δ*lgt*, which points toward an Lpp-mediated growth advantage of SA113 wt in an inflammation-impaired host. The liver is the primary organ for iron storage and heme recycling. *S. aureus* preferentially utilizes iron from heme by the Hts and Isd transporters, which contain Lpp (7). In our SA113 Δ*lgt* mutant, these transporters consist of nonmature Lpp HtsA and IsdE, which might be impaired in function. Therefore, it can be expected that nonmature HtsA and IsdE contribute to the reduced bacterial load in the liver of MyD88$^{-/-}$ mice. Accordingly, a deletion mutant in the *htsB* gene was previously shown to be associated with reduced abscess formation and bacterial numbers in the liver (7). In line with these findings, we experimentally confirmed that Lpp deficiency slowed growth of *S. aureus* Δ*lgt* mutants under iron-restricted conditions in vitro and also diminished streptonigrin susceptibility in vitro and ex vivo due to decreased iron (^{55}Fe) acquisition. Our finding is strengthened by the fact that Lpp maturation improves iron uptake in *S.*

pneumoniae, as suggested by higher streptonigrin sensitivity of Δ*lspA* than wt (26). Reduced iron uptake likely also explained the enhanced streptonigrin sensitivity that we observed with Newman Δ*lgt* and may explain the growth defect of a Newman Δ*lgt* mutant in whole blood and macrophages described earlier (23).

From these findings we conclude that the growth deficiency of *S. aureus* Δ*lgt* in the organs of C57BL/6, TLR2$^{-/-}$, and especially in inflammation-impaired MyD88$^{-/-}$ mice was due to reduced iron uptake and storage. Support for our hypothesis was provided by the demonstration that iron overload in MyD88$^{-/-}$ mice reversed the growth defect of Δ*lgt*. Both SA113 wt and Δ*lgt* became more virulent, but the bacterial load increased only in Δ*lgt* infection above the level reached in MyD88$^{-/-}$ mice under normal diet. However, we cannot exclude that the immunocompetent host may affect growth of Δ*lgt* by reducing availability of other nutrients such as oligopeptides, Cu, or Zn (42). Also, Lpp-related uptake of other nutrients and metals (1) may contribute to growth of Δ*lgt* in MyD88$^{-/-}$ mice in vivo.

We previously found precursor Lpp accumulated in the supernatant of SA113 Δ*lgt* in vitro (1), indicating less strong anchoring or LspA-independent processing of precursor Lpp in SA113 Δ*lgt*. High iron concentrations probably saturated the iron uptake systems of Δ*lgt* before release of pro-Lpp and thus allowed better growth of Δ*lgt* in the MyD88-deficient host.

Immunocompetent C57BL/6 mice can utilize many pathways to sequester iron in macrophages upon infection. Cytokines enhance iron storage by inducing ferritin synthesis and erythrophagocytosis due to up-regulation of iron importer and reducing iron exporter molecules (43). Additional inflammation-dependent restriction of iron is achieved by increase of transferrin saturation, as well as by PMN granule release of lipocalin and lactoferrin (43, 44). All these mechanisms may explain that only *S. aureus* wt, but not Δ*lgt*, was able to profit from the iron overload in C57BL/6 mice. The competition for iron between host and pathogen was further demonstrated by infection with iron-restricted *S. aureus*. SA113 wt without intracellular stored iron did not gain advantage from Lpp for growth in organs after infection of the immunocompetent host. This suggests that *S. aureus* has to store iron prior to infection to grow in organs and to resist PMN killing in vivo. However, in the absence of MyD88, iron-deprived SA113 wt won the race for iron over the host, and Lpp conferred a growth advantage during infection. In the absence of inflammation, cellular iron retention was probably failing; for example, hepcidin, the iron retention molecule in the liver, was low, as published for *S. pneumoniae* (45). Under these circumstances, Lpp permitted growth of *S. aureus* during infection.

In conclusion, we have demonstrated that, on the one hand, recognition of Lpp by TLR2-MyD88 signaling controls systemic infection and organ-specific early immunity in the host. On the other hand, as summarized in Table I by the total disease ranks, we showed that Lpp of SA113 wt enhance growth 1) in untreated C57BL/6, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice, 2) of iron-depleted staphylococci in the absence of MyD88-mediated inflammation, and 3) in iron-overloaded immunocompetent C57BL/6 mice. Therefore, evolution may select for *S. aureus* with lipidated Lpp.

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Disclosures

The authors have no financial conflicts of interest.

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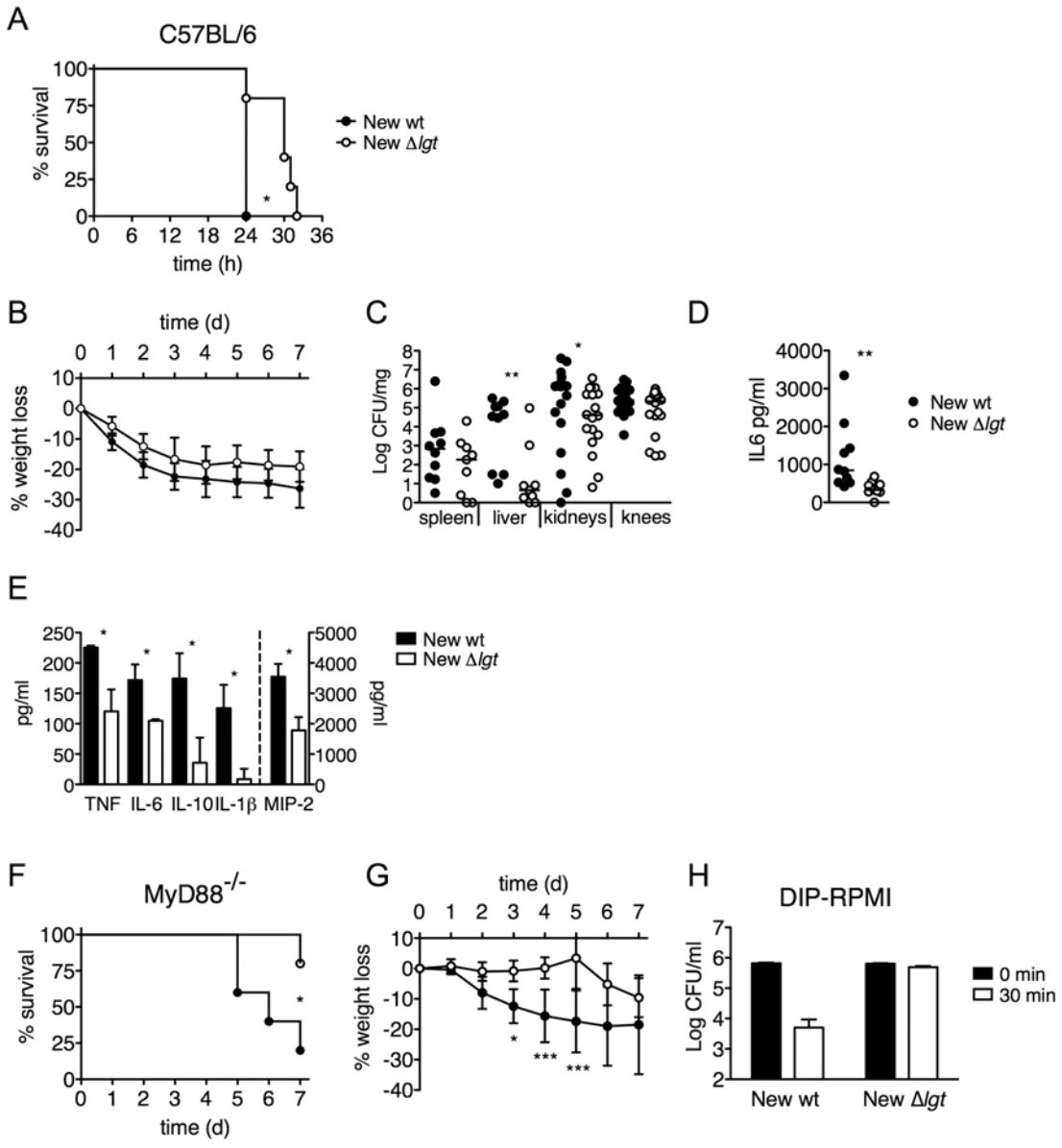

Supplemental Fig. 1. Lpp enhance virulence of *S. aureus* Newman in sepsis.

(A) Survival of C57BL/6 mice infected with 1 x 10^8 CFU of Newman wt (filled, n = 5) and Δ*lgt* (open, n = 5). (B-G) Mice were infected with 1 x 10^6 CFU of Newman wt and Δ*lgt*. (B) Weight loss of C57BL/6 mice during infection, (C) bacterial load in spleen, liver, both kidneys, and both knees on day 7 and (D) plasma IL-6 level on day 7. Data are represented as mean ± SD for weight

loss and cytokines and median for CFU/mg of at least 9 mice per group. (E) One day after infection (n = 5 per group), isolated splenocytes were restimulated with the corresponding strain for 24 h *in vitro*. TNF, IL-6, IL-10, IL-1β, and MIP-2 in supernatants were analyzed by ELISA. (F) Survival and (G) weight loss of MyD88$^{-/-}$ mice infected with 1 x 10^6 CFU of Newman wt (filled, n = 5) and Δ*lgt* (open, n = 5). (H) Streptonigrin sensitivity for 30 min of Newman wt and Δ*lgt* grown overnight in RPMI with 250 µM Dipyridyl. Data show one representative out of three experiments. Significant differences between wt and Δ*lgt* are indicated by *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

3.2 Part 2 – Staphylococcal lipoproteins and their role in bacterial survival in mice

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Mini Review

Staphylococcal lipoproteins and their role in bacterial survival in mice

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ABSTRACT

Staphylococcus aureus expresses about 50 lipoproteins (Lpp), which are lipid-anchored in the membrane. The processing of the precursor to the mature Lpp is catalyzed by the phosphatidyl glycerol diacylglyceryl transferase (Lgt) and the lipoprotein-specific type II signal peptidase (LspA) leading to diacylated Lpp. Possibly another acyltransferase attaches a third fatty acid leading to triacylated Lpp. Lpp function as binding proteins for transport of nutrients across the microbial membrane and are involved in processing of other proteins, but most Lpp remain of predicted or unknown function. The di- or triacylated lipid structure is sensed by host pattern recognition receptor TLR2 and induces innate immune responses in professional and non-professional phagocytes. In the host, maturation of Lpp confers optimal metal ion – particularly iron – acquisition, it enhances staphylococcal invasion and phagocytosis, intracellular survival and persistence of infections. However, the advantages of Lpp maturation are counterbalanced by the capability to induce inflammation. In this review, we summarize the current knowledge about the role of Lpp in iron acquisition and TLR2 recognition in the host and describe the consequences of Lpp maturation for survival of *S. aureus* in the host.

Staphylococcal lipoproteins

Most of the surface-associated proteins in *Staphylococcus aureus* are secreted by the general secretory (Sec) or twin arginine protein transport (Tat) pathway and are further modified for attachment to the cell wall or anchoring in the cytoplasmic membrane. The lipoprotein precursor (pre-Lpp) contains an N-terminal signal sequence for recognition and translocation by the Sec pathway. Translocated pre-Lpp undergo maturation by two or three modification steps in *S. aureus*. In the initial step, the phosphatidyl glycerol diacylglyceryl transferase (Lgt) transfers a diacylglyceryl group from phosphatidylglycerol onto the conserved lipobox cysteine via a thioether linkage (Sankaran et al., 1995). This step appears to be a prerequisite for recognition and cleavage of the signal peptide catalyzed by the lipoprotein-specific type II signal peptidase (LspA). In Gram-negative bacteria, Lpp are further modified by N-acyltransferase (Lnt) transferring an N-acyl group to the diacyl-glyceryl cysteine yielding mature triacylated Lpp. However, Lnt was not found in the genome of *S. aureus* (Stoll et al., 2005) suggesting that staphylococcal Lpp are diacylated. Mass spectroscopy of an Lpp supported this assumption (Tawaratsumida et al., 2009), but was contradicted by another report identifying a different Lpp, which was triacylated possibly by a distinct unknown acyltransferase (Kurokawa et al., 2009).

Functions of staphylococcal Lpp

Sequence analysis in different *S. aureus* strains revealed that Lpp represent 2% or more of the staphylococcal proteome (in DOLOP (Babu et al., 2006)). Lpp fulfill their function at the interface between the membrane and the cell wall, mainly involved in protein maturation or as substrate-binding proteins of ABC transporter uptake systems.

The Lpp oligopeptide permease (OppA) and peptidyl-prolyl *cis/trans* isomerase (PrsA), which assist in protein folding, were described, but not functionally analyzed in *S. aureus* (Stoll et al., 2005). An Lpp with thiol-disulfide oxidoreductase activity (DsbA) with similarities to zinc-binding protein AdcA was identified (Dumoulin et al., 2005; Sibbald et al., 2006). The Lpp GmpC was crystallized and found part of an ABC transporter binding glycyl methionine (Williams et al., 2004). In addition to the above-described proteins, Sibbald et al. (2006) predicted a binding protein of glycine betaine/carnitine/choline ABC (OpuCC), a manganese-binding protein (MntA), a molybdate-binding protein (ModA), and a beta-lactamase precursor (BlaZ) by homology search. Nevertheless, most of the proteins containing the lipobox are hypothetical without known function.

Six operons encoding Lpp are iron-regulated via the negative repressor proteins Fur (Maresso and Schneewind, 2006) or SirR

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(Hill et al., 1998), respectively. Each operon encodes a lipoprotein component (FhuD1/FhuD2, SirA, SstD, SitC, HtsA, IsdE), which transports iron bound to siderophores or heme through the cytoplasmic membrane, a permease and an ATPase (Cockayne et al., 1998; Dale et al., 2004; Mazmanian et al., 2003; Morrissey et al., 2000; Sebulsky and Heinrichs, 2001). Deletion mutants of these Lpp are compromised in growth in iron-restricted media. FhuD1 and FhuD2 are Lpp that function as high-affinity receptors in a ferric hydroxamate uptake (fhu) system (Sebulsky and Heinrichs, 2001; Sebulsky et al., 2000). HtsA and SirA mediate transport of iron complexed to staphyloferrin A and B, respectively (Beasley et al., 2009; Dale et al., 2004). For the other Lpp, SitC and SstD, the transported iron substrate is unknown (Cockayne et al., 1998; Morrissey et al., 2000). The *sstD* mutant was investigated in vivo and it did not present a phenotype different from wild-type *S. aureus*. The Lpp IsdE was identified as binding protein for heme and transferrin-iron (Grigg et al., 2007). Heme represents the largest iron storage pool in the host and seems to be the preferred iron source for *S. aureus* (Skaar et al., 2004). Siderophore-mediated iron acquisition is central for the utilization of transferrin-bound iron, while the Fur-regulated broad-spectrum adhesins IsdA and FrpA bind to, but do not take up transferrin-bound iron (Clarke et al., 2004; Morrissey et al., 2002; Park et al., 2005).

Genes encoding Lpp for iron uptake are upregulated under iron-restricted conditions in vitro and in vivo underscoring their importance for bacterial growth and survival (Allard et al., 2006; Skaar et al., 2004). In line, under similar conditions several Lpp are strongly expressed in vitro and in vivo (Dale et al., 2004; Morrissey et al., 2000; Sebulsky et al., 2004). These observations allow the conclusion that maturation of Lpp improves uptake of nutrients and iron. However, high intrabacterial iron concentrations lead to the generation of hydroxyl radicals, which are toxic to *S. aureus*. Therefore iron import is carefully balanced by the Fur regulon and by heme concentrations itself (Friedman et al., 2006), and *S. aureus* is able to export excessive iron through the HrtAB transport system regulated by the HssRS two-component signal transduction system (Stauff et al., 2007; Torres et al., 2007).

Recognition of *S. aureus* by pattern recognition receptors

S. aureus colonizes mucosal and dermal surfaces of the human host. After invasion, professional phagocytes including macrophages, neutrophils, and dendritic cells recognize *S. aureus*. Complement and antibodies in serum opsonize bacteria, which then allow phagocytosis by complement and Fc receptors. Pattern recognition receptors (PRRs) of the Toll-like receptor (TLR)/IL-1 receptor family recognize conserved molecules on the bacterial surface (Akira et al., 2006; Medzhitov, 2007). TLR 1, 2, 6 and 9 contribute to the recognition of *S. aureus*.

An important breakthrough in staphylococcal recognition was the purification of native staphylococcal Lpp, including SitC, which were shown to induce cytokines through the TLR2-MyD88 signaling pathway (Kurokawa et al., 2009; Tawaratsumida et al., 2009). The use of *S. aureus* mutants deficient in maturation of lipoproteins (Δ*lgt*) and improved Lpp purification methods give evidence that TLR2 is exclusively activated by Lpp (Bubeck Wardenburg et al., 2006; Stoll et al., 2005). These tools revealed that all the other postulated ligands such as lipoteichoic acid (LTA) and soluble peptidoglycan were contaminated with Lpp (Hashimoto et al., 2006a, b; Zahringer et al., 2008).

Studies with synthetic lipopeptide indicate that mature staphylococcal Lpp possibly induce association of TLR2 with TLR1 and TLR6 to signal by MyD88 and NF-κB inducing the production of cytokines (Jin et al., 2007; Takeda et al., 2002; Takeuchi et al., 2001, 2002). The three lipid chains of the synthetic lipopeptide Pam_3CSK_4 mediate the heterodimerization of TLR1 and TLR2 (Jin et al., 2007). The two ester-bound lipid chains are inserted into a pocket in TLR2, while the amide-bound lipid chain is inserted into a hydrophobic channel in TLR1 (Jin et al., 2007). Diacylated Lpp signal by TLR2/6 dimers, and models predicted binding of lipid chains to TLR2 and stabilization by TLR6. Accessory molecules facilitate TLR2 signaling, e.g. the scavenger receptor CD36 participates in recognition of diacylated but not triacylated lipopeptides (Hoebe et al., 2005). CD14 is partially involved in recognition of Lpp by TLR2 (Jiang et al., 2005). Additionally, the serum protein vitronectin binds Lpp and mediates TLR2 activation though the integrin β3 receptor in human monocytes (Gerold et al., 2008).

Since it is known that staphylococcal Lpp are the major ligand for TLR2, it became evident that Lpp-TLR2 signaling must play a role in innate immune response. Different murine infection models show that TLR2 deficiency leads to a more severe disease course, higher bacterial loads in tissue and/or reduced inflammation (Kielian et al., 2005; Miller et al., 2006; Schmaler et al., 2009; Sun et al., 2006; Takeuchi et al., 2000). TLR2 signaling requires the recruitment of MyD88. Accordingly, $MyD88^{-/-}$ mice are highly susceptible in murine infection models with reduced cytokines and leukocyte attraction leading to a higher bacterial burden (Miller et al., 2006; Schmaler et al., 2009; Sun et al., 2006; Takeuchi et al., 2000). In these studies, the comparison of $TLR2^{-/-}$ and $MyD88^{-/-}$ mice gives evidence for the participation of other MyD88-dependent receptors in defense against *S. aureus*. Hultgren et al. (2002) and Miller et al. (2006) showed the contribution of IL-1R to efficient killing of *S. aureus* in systemic and subcutaneous infection, respectively. The latter model revealed an instrumental role of IL-1R to promote chemokine induction by resident cells (Miller et al., 2006). Staphylococcal DNA might be recognized by TLR9; however, results regarding the contribution of TLR9 to defense against *S. aureus* are controversial (Kapetanovic et al., 2007; Sun et al., 2006; Zhu et al., 2008). Thus in conclusion, the relative contribution of each of these PRRs in staphylococcal recognition remains elusive (Deshmukh et al., 2009).

Lpp in phagocytosis and survival in macrophages

On the one hand, bacterial Lpp are exposed on the surface and on the other hand, some are possibly required for maturation and correct folding of non-Lpp proteins (see above). Therefore, Lpp might also play a role in recognition and phagocytosis. The first indication for this assumption was obtained by the growth attenuation of an *S. aureus* Newman Δ*lgt* mutant in whole human blood and in the presence of activated macrophages (Bubeck Wardenburg et al., 2006). In that report, phagocytosis and killing of Newman wild type and the Δ*lgt* mutant were not affected in macrophages. In contrast, our own data with peritoneal macrophages revealed that the maturation of Lpp increases phagocytosis of SA113 wild type compared with the Δ*lgt* mutant (Fig. 1A). Similar results were obtained with *S. aureus* Newman wild type and the Δ*lgt* mutant (data not shown). We further found that this Lpp-dependent phagocytosis of *S. aureus* strains did not require TLR2 or TLR9 (data not shown), but MyD88 (Fig. 1A). Neither serum nor scavenger and mannose receptors were involved, since the Lpp-enhanced phagocytosis was maintained in serum-free medium, in scavenger $receptor^{-/-}$ cells and in mannan-pretreated macrophages (unpublished observation). Altogether, the results suggest that Lpp maturation might contribute to the recognition of *S. aureus* by additional MyD88-regulated macrophage receptors as known for CD14 (Landmann et al., 1991).

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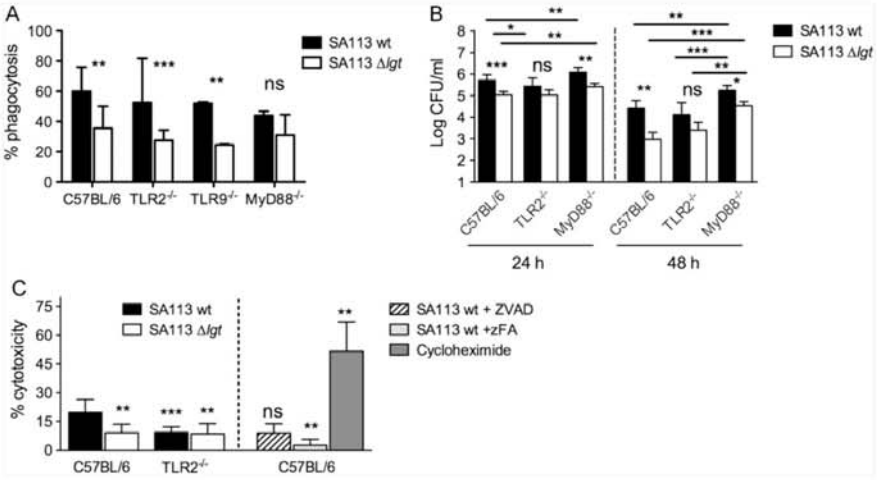

Fig. 1. Peritoneal macrophages from C57BL/6, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice were infected with SA113 wild type and the Δ*lgt* mutant at a bacteria:macrophage ratio of 10. After 1 h of phagocytosis intracellular bacteria were quantified (A) and after further incubation for 24 and 48 h intracellular survival of *S. aureus* was assessed by plating for colony-forming units (B). Significant differences of wild type- and Δ*lgt*-infected macrophages as well as to other infected mouse strains are indicated by *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$. (C) LDH release expressed as percentage of cytotoxicity in macrophages from C57BL/6 and TLR2$^{-/-}$ mice induced by intracellularly surviving SA113 wild type and the Δ*lgt* mutant. SA113 wild type-infected cells were incubated in the presence of zVAD-fmk and zFA-fmk. Cycloheximide was used as a positive control. Significant differences of wild type-infected C57BL/6 macrophages versus all other groups are indicated by **: $p < 0.01$, and ***: $p < 0.001$.

Although *S. aureus* is generally viewed as an extracellular pathogen, recurrent infections of patients suggest persistence in host cells (Clement et al., 2005). This observation was underlined by several studies showing that *S. aureus* is not only able to invade but also to persist in mammalian non-professional phagocytes and moreover survive engulfment by professional phagocytes such as neutrophils and monocytes as reviewed by Garzoni and Kelley (2009). In a recent study with macrophages, *S. aureus* persisted in vacuoles before escaping into the cytoplasm and causing host cell lysis (Kubica et al., 2008).

As outlined before, staphylococcal Lpp contribute to the uptake of nutrients that might be important for survival in the host. So far, the role of Lpp in intracellular survival and growth has been studied in *Listeria monocytogenes* and *Mycobacterium tuberculosis* Δ*lgt* or Δ*lsp* mutants, which were growth attenuated (Baumgartner et al., 2007; Sander et al., 2004). Similar studies addressing the role of staphylococcal Lpp are still missing. We therefore investigated intracellular survival of phagocytosed *S. aureus* wild type and Δ*lgt* mutant after 24 and 48 h (Fig. 1B). Intracellular *S. aureus* strain SA113 expressing mature Lpp survived better than the Δ*lgt* mutant or than SA113 wild type in TLR2$^{-/-}$ macrophages. This suggests that intracellularly, Lpp either improve survival or suppress a TLR2-dependent killing mechanism possibly by activating JNK as previously published (Watanabe et al., 2007). Interestingly, Lpp enhanced intracellular survival of *S. aureus* similarly in C57BL/6 and MyD88$^{-/-}$ cells indicating that the Lpp-dependent survival does not require MyD88 signaling. These observations support our recent data showing an enhanced survival mediated by staphylococcal Lpp in a sepsis model, which was also MyD88-independent (Schmaler et al., 2009).

To know the contribution of Lpp-TLR2 interaction for intracellular survival, we assessed the toxicity of viable intracellular *S. aureus* for macrophages by LDH release. Interestingly, the recognition of Lpp by TLR2 was a prerequisite for cell damage (Fig. 1C). Blocking of cathepsin B with zFA-fmk diminished cell death, whereas inhibition of pan-caspases with zVAD-fmk had no effect (Fig. 1C). This suggests that TLR2 mediates a caspase-independent, cathepsin-dependent apoptosis; the molecular players in this reaction remain to be determined (Conus and Simon, 2008).

Preliminary data suggest that Lpp also improve escape from the intracellular compartment of macrophages since we found higher numbers of released *S. aureus* wild type compared with the Δ*lgt* mutant one day after macrophage infection. These data suggest that Lpp are not only required for *S. aureus* phagocytosis and survival in cells, but also for escape from the compartments with antimicrobial activity.

Lpp in cytokine induction in macrophages

Lpp are major activators of human myeloid cells, since viable *S. aureus* SA113 wild type but not the Δ*lgt* mutant induces TNF and IL-10 release in the MonoMac6 cell line (Stoll et al., 2005). We have recently described the early and strong cytokine and chemokine response upon *S. aureus* infection with Lpp-TLR2-MyD88 signaling in murine peritoneal macrophages (Schmaler et al., 2009). In that study and the present review, we were able to support the general idea that TLR2 is an early receptor of the innate immune system since 2 h after infection Lpp-TLR2 dependence was found complete for NF-κB, for TNF, IL-10 and IL-6 (Fig. 2). Later after infection TNF was suppressed by Lpp and TLR2, while IL-6, IL-10 and IL-1β remained partially Lpp-dependent (Fig. 2; Schmaler et al., 2009). The cellular effect of mature Lpp was not strain dependent, since it was visible with viable SA113 and Newman (Schmaler et al., 2009) and heat-killed Newman (Bubeck Wardenburg et al., 2006). During growth cell wall components including Lpp are released (Stoll et al., 2005) and sensed by immune cells; therefore supernatants from *S. aureus* wild type and the Δ*lgt* mutant were tested for their potential to induce the same proinflammatory cytokines. Supernatants activated NF-κB (Fig. 2A) and induced early TNF in an Lpp-

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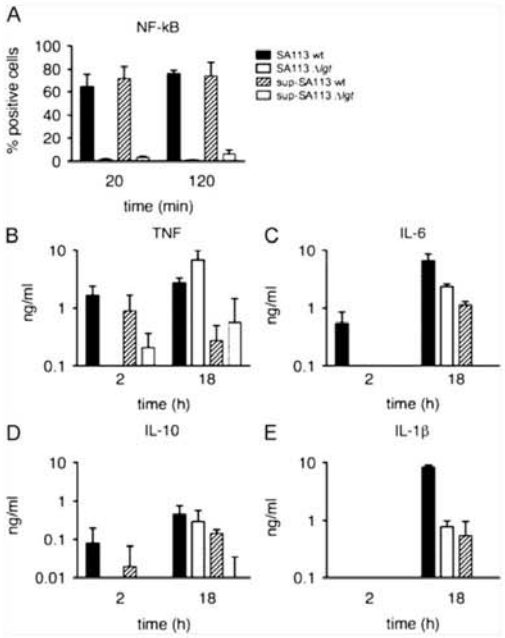

Fig. 2. Peritoneal macrophages from C57BL/6 mice were stimulated with viable *S. aureus* wild type and the *Δlgt* mutant or steril-filtered (0.22 μm) bacterial supernatants after overnight growth in complete RPMI. (A) Percentage of NF-κB-positive cells after 20 and 120 min. Supernatants of stimulated macrophages were analyzed by ELISA for (B) TNF, (C) IL-6, (D) IL-10, (E) IL-1β after 2 and 18 h of infection. If no bars are shown, no cytokines were detectable. Data are mean ± SD of ≥3 independent experiments.

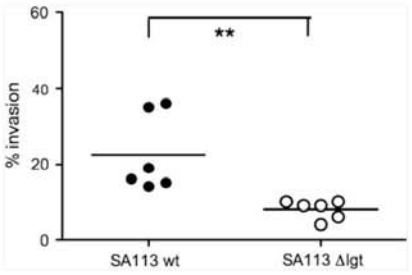

Fig. 3. HUVECs were infected with SA113 wild type and the Δ*lgt* mutant at a bacteria:cell ratio of 10. After 1 h of invasion intracellular bacteria were quantified. Data are mean ± SD of ≥3 independent experiments. Significant differences are indicated by **: $p < 0.01$.

dependent manner (Fig. 2B). Early induction of IL-6, IL-10, and IL-1β was not detectable with supernatants (Fig. 2C–E). Supernatants contributed moderately to the late Lpp-dependent induction of cytokines. TLR2-deficient macrophages displayed the Δ*lgt* mutant phenotype indicating that released Lpp are recognized by extracellular TLR2 (data not shown). Results with cytochalasin D-treated macrophages were similar to those obtained with the supernatants (data not shown).

Released staphylococcal Lpp are recognized by TLR2 on macrophages and activate the production of inflammatory cytokines by MyD88 and NF-κB. The excessive secretion of these cytokines might be additionally enhanced by the damage of cells after engulfment and the release of alarmins, which together with the microbe-associated molecular patterns (MAMPs) act as danger-associated molecular patterns (DAMPs) (Rubartelli and Lotze, 2007). Such a dramatic increase in systemic infections would lead to an uncontrolled inflammatory response.

Lpp and polymorphonuclear neutrophils (PMN)

PMN are the first cells recruited to the site of staphylococcal infection in blood, in abscesses and implants. Growth of *S. aureus* at a very low multiplicity of infection in whole blood needed the maturation of Lpp in Newman strain (Bubeck Wardenburg et al., 2006). This finding is not convincingly supported by the literature, since *S. aureus* is rapidly killed by human PMN. Our own data showed similar phagocytosis of *S. aureus* SA113 wild type and the Δ*lgt* mutant by mouse and human blood PMN and no differences in intracellular survival in these cells (unpublished data). Consistent with this finding, growth of *S. aureus* wild type and the Δ*lgt* mutant in an implant infection model and the recruitment of PMN into the tissue cage were not affected by Lpp maturation. Furthermore, in vitro chemotaxis of PMN and the recruitment of cells to peritoneum by sterile-filtered supernatants from *S. aureus* wild type and the Δ*lgt* mutant indicated no chemotactic function of Lpp (data not shown). All our findings point to a negligible effect of Lpp in interaction with PMN, although TLR2 is expressed in 50% of uninfected PMN (Letiembre et al., 2005).

Lpp and non-professional phagocytes

On the mucosal surface, epithelial cells provide a barrier for invasion of *S. aureus* and thereby prevent colonization of other tissues by activating phagocytic cells. In A549 pulmonary type II cells adhesion and invasion was not dependent on Lpp maturation (Stoll et al., 2005). To colonize new tissues and organs in the host, *S. aureus* has to adhere to and transcytose endothelial cells. Thus, we compared adhesion and invasion of *S. aureus* wild type and the Δ*lgt* mutant in primary endothelial HUVECs and found similar adhesion (not shown), but significantly more intracellular bacteria after infection with *S. aureus* wild type than with the *Δlgt* mutant (Fig. 3). The unaltered adhesion, but Lpp-mediated invasion may be related to improved recognition by surface molecules on the endothelial cells or increased survival of intracellular *S. aureus* wild type.

A requirement of Lpp maturation in *S. aureus* to induce cytokine release from A549 epithelial cells was found using SA113 wild type and the Δ*lgt* mutant (Stoll et al., 2005). The involvement of alarmins in induction of cytokine levels from dying cells after SA113 wild type infection was excluded by viability staining. Further studies confirmed Lpp-TLR2-dependent effects in epithelial cells including NF-κB and MAP kinases leading to induction of cytokines as well as the antimicrobial molecules iNOS and Mn-SOD (Li et al., 2008).

Lpp requirements during infection of the host in vivo

The reviewed results indicate an important role of Lpp in growth and survival in *S. aureus*, but sensing of them enhances TLR2-mediated defense mechanisms. The deletion of the *lgt* gene is therefore the most promising model to investigate the maturation of Lpp and consequences for *S. aureus* during infection. An *S. aureus* Newman Δ*lgt* mutant was hypervirulent in a murine sepsis model (Bubeck Wardenburg et al., 2006). The phenotype of the Δ*lgt* mutant was associated with higher bacterial load in

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organs and impaired leukocyte recruitment. However, we recently showed that Lpp conferred virulence to Newman and SA113 in systemic infection (Schmaler et al., 2009) as indicated by higher bacterial numbers in organs with Newman and SA113 wild types compared to the Δ*lgt* mutants. Impaired monocyte attraction was confirmed in liver sections of Δ*lgt*-infected mice (Schmaler et al., 2009). In line with our observation, incomplete maturation of Lpp by deletion of the *lsp* gene resulted in reduced virulence of *S. aureus* Newman in systemic infection and of transposon mutants of RN6390 in different infection models (Bubeck Wardenburg et al., 2006; Coulter et al., 1998; Mei et al., 1997). The contradictory in vivo effects of staphylococcal Lpp maturation reported by different groups might be due to variations in the route of infection, the generation of the deletion mutant, and possibly the time of infection. In early infection, recognition of *S. aureus* is essential for establishing a proinflammatory immune response that leads to protection, opposed by the fact that later in infection, Lpp are more important for the acquisition of nutrients and ions that are essential for survival within the host.

Our group was interested to link the Lpp-mediated phenotype with TLR2 recognition by systemic infection of TLR2$^{-/-}$ mice with *S. aureus* wild type and the Δ*lgt* mutant. While cytokines in spleen were low and no longer different in TLR2$^{-/-}$ mice infected with either strain, weight loss and bacterial numbers in organs were still higher after SA113 wild type than after Δ*lgt* infection. This suggested that *S. aureus* wild type had a growth advantage in the organs of infected mice. These data were strengthened by the fact that also in MyD88$^{-/-}$ mice SA113 wild type infection caused a higher bacterial load than the Δ*lgt* mutant and raised the question whether maturation of Lpp affects the function of proteins involved e.g. in iron uptake.

Staphylococcal Lpp in iron uptake

In agreement with the known Lpp function as binding proteins in iron uptake systems of *S. aureus*, we found reduced growth of the Δ*lgt* mutant in iron-depleted media supplemented with $FeCl_3$ or iron-dextran as sole iron sources (Schmaler et al., 2009). This was associated with a reduced accumulation of ^{55}Fe (Schmaler et al., 2009). We found similar growth of wild type and the Δ*lgt* mutant in plasma, where iron is mainly in transferrin-bound form. This was surprising, considering that the Lpp siderophore binding proteins (SirA and HtsA) are required for iron uptake from transferrin. It may indicate that other non-Lpp siderophore binding proteins exist. Furthermore, our study using SA113 and Newman Δ*lgt* mutants in systemic infection models showed an Lpp-related growth advantage, which we could relate to enhanced iron uptake. Indeed in MyD88$^{-/-}$ mice (Schmaler et al., 2009), where we observed a higher bacterial load of wild type compared to the Δ*lgt* mutant in organs, iron overload restored the growth deficit of the mutant. However, in immunocompetent mice iron overload did not enhance growth of SA113 Δ*lgt*. This suggests that Lpp are required for maximal iron uptake under conditions, where the host opposes with a functional iron uptake system.

Iron availability in the host is limited by transferrin in plasma and by ferritin within cells. During infections and inflammation, bacteria may become iron-starved due to increased transferrin saturation. In addition, elevated hepcidin levels cause a decrease in serum iron levels by blocking the export of iron by ferroportin in hepatocytes and macrophages (Ganz and Nemeth, 2006). Extracellular iron is additionally reduced by continuing erythropoiesis within hours (Ganz, 2008; Ganz and Nemeth, 2006) and myeloid cell receptors such as CD163 and lipocalin. Therefore *S. aureus* has to overcome iron starvation in the inflamed host possibly with the need of Lpp.

Conclusions

The studies reviewed here serve to illustrate the complexity of Lpp function. On the one hand Lpp confer an advantage for the bacterium by improving the nutrient/iron uptake. On the other hand Lpp are strong inducers of inflammatory responses. Both functions might be beneficial for *S. aureus* in colonization of mucosal membranes, where Lpp may be required for nutrient uptake, and the induction of inflammation might help to restrict competing commensals. Further, Lpp contribute to uptake and invasion in phagocytic and non-phagocytic cells. Within phagocytes Lpp enable better survival and later escape by inducing cell death, as shown in our study. Escaped extracellular *S. aureus* may survive iron restriction by the infected host due to Lpp.

In conclusion, *S. aureus* "accept" TLR2 recognition of mature Lpp for improved nutrient acquisition and therefore evolution did not select for Lpp-deficient bacteria.

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3.2.1 Summary of additionally published results

In this review, additional results concerning the function of Lpp in *S. aureus* are published. Firstly, maturation of Lpp increases phagocytosis of *S. aureus* by macrophages depending on the presence of MyD88 but not TLR2. Furthermore, Lpp confer an advantage for survival and escape of *S. aureus* after engulfment by macrophages and Lpp-TLR2-mediated cell death by activation of cathepsin B, respectively. Both mechanisms might improve the escape from intracellular organelles into the cytoplasm. Secondly, we demonstrate that released Lpp induce TLR2-signaling and production of cytokines. Internalization of *S. aureus* enhances the total amount of cytokines suggesting that Lpp are mainly detected by TLR2 in the phagosome. Thirdly, in contrast to macrophages, Lpp have no effect on phagocytosis or survival of *S. aureus* in PMN in vitro or in a tissue cage model, but augment the capacity of *S. aureus* to invade endothelial cells. In summary, we extend the knowledge about maturation of staphylococcal Lpp in infection by elucidating its function in engulfment, intracellular survival, and cell death in macrophages.

3.2.2 Material and Methods

Stimulation of macrophages with bacterial supernatants

Macrophages from C57BL/6, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR9$^{-/-}$, and SR$^{-/-}$ mice were prepared as described in Part 1 Material and Methods.

Adherent macrophages were stimulated with sterile-filtered (0.22 µm) bacterial supernatants from *S. aureus* wt and Δ*lgt* grown ON in RPMI 1640 complete. At indicated time points, NF-κB translocation and cytokines were analyzed as described in Part 1.

Phagocytosis and killing assay

Macrophages were infected with viable *S. aureus* strains and their isogenic mutants in RPMI 1640 complete (MOI 10). To test the contribution of complement and mannose receptors, phagocytosis was also performed in serum-free RPMI complete and in presence of 10 mg/ml mannan (Sigma). After 1h of phagocytosis, extracellular bacteria were degraded by 20 U lysostaphin. Intracellular bacteria were determined after lysis of macrophages with water (pH 11) by plating serial dilutions on MHA plates. For killing assays, supernatants were replaced by medium containing 100 µg/ml gentamicin. After 24 and 48h intracellular bacteria were determined as above. Additionally, lactate dehydrogenase (LDH) in the supernatants of infected macrophages with or without 50 nM zVAD-fmk or zFA-fmk was determined after 18h of infection with Cytotox 96® (Promega) according to the manufacture's instructions. 10 µg/mL cycloheximide (CHX, Sigma) was used as positive control.

Adhesion and Invasion assay

Human umbilical vein endothelial cells (HUVECs) were seeded at 3 x 10^4 cells/well into 24-well plates. HUVECs were infected with SA113 wt and Δ*lgt* (MOI 10) at 37°C and 5% CO_2. After 1h, cells were washed with PBS and trypsinized to detach. To determine total bacteria, cells were lysed with water (pH 11) and serial dilutions were plated on MHA to assess CFU. For invasion, remaining extracellular bacteria were killed by 50 U lysostaphin for 10 min before lysis and CFU plating. The number of adherent bacteria was calculated as the difference of total minus intracellular bacteria.

Mice and tissue cage model

C57BL/6 and TLR2$^{-/-}$ female mice (12–14 weeks old) were anesthetized via i.p. injection of 100 mg/kg ketamine (Ketalar) and 20 mg/kg xylazinium and sterile Teflon tissue cages were implanted s.c. in the back, as described previously (1). Mice were treated with buprenophinum (2 mg/kg s.c.) after implantation. Two weeks after surgery, the sterility of tissue cages was verified. Mice were infected with indicated CFU of SA113 wt and Δ*lgt* in 200 µl 0.9% NaCl. Mice were anesthetized by isofluorane (Minrad Inc., Bethlehem, PA, USA), and 100 µl tissue cage fluid was percutaneously collected with EDTA at day 2, 5, and 8. Number of infiltrating cells was determined (Coulter counter) and growth of bacteria was assessed by plating serial dilutions on MHA plates.

Preparation of peripheral blood-derived PMN

Murine and human blood PMN were isolated by density gradient centrifugation on a discontinuous Percoll (Pharmacia Biotech AB, Uppsala, Schweden) gradient with 59%

(murine) or 53% (human) and 67% Percoll in PBS as described previously (2). The interface, which contains the PMN, was collected and contaminating erythrocytes were removed by hypotonic lysis in water. By morphologic criteria, the final cell preparation contained >95% PMN and was used for chemotaxis assays, phagocytosis, and killing assays. The viability of the cells was >95% as assessed by trypan blue exclusion.

PMN phagocytosis and killing assay

Murine and human blood PMN were resuspended in Dulbecco's PBS (10% pooled mouse plasma and 10% pooled human serum, respectively) and infected with SA113 wt or Δ*lgt* at MOI 10 for phagocytosis and MOI 1 for killing (GFP-expressing strains were used for phagocytosis) at 37°C, 200 rpm. Phagocytosis was determined as the percentage of GFP-positive cells by flow cytometry. For killing assay, PMN were lysed in water (pH 11) and serial dilutions were plated on MHA to enumerate surviving bacteria.

Chemotaxis assay

Chemotaxis of 1 x 10^5 murine blood PMN attracted by staphylococcal supernatants or 10^{-7} M synthetic formylated peptide (fMLP) was determined by using a 48 well camber as described recently (3, 4). Supernatants from ON cultures of SA113 wt and Δ*lgt* were sterile-filtered (0.22 μm) and either directly used for stimulation, or washed twice with PBS in a 10 kDa concentrator (Amicon) and resuspended in equal volume TSB. PMNs migrated through a 3 μm polycarbonate filter membrane (Costar) for 30 min at 37°C in 5% CO_2, in humidified air. After incubation, filters were removed, fixed, and stained with

Wright-Giemsa. Cells that migrated through the pores of the filter were counted by light microscopy (63x).

Chemotaxis in peritonitis model

Bacterial supernatants from ON cultures of SA113 wt and Δ*lgt* grown in IMDM (Invitrogen) were sterile-filtered (0.22 μm) and 2 ml were injected i.p. After 6 h, infiltrating cells were collected by peritoneal lavage with 6 ml RPMI-1640 complete medium, counted (Coulter counter) and spun on glass slides to differentiate recruited cells.

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3.3 Part 3 – *S. aureus* Lpp activate dendritic cells by TLR2-MyD88 signaling and promote production of IFN-gamma and IL-17 by T helper cells

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Keywords: *Staphylococcus aureus*, lipoproteins, TLR2, MyD88, dendritic cells, T_H1 and T_H17 response

(Manuscript in preparation)

Abstract

S. aureus infection activates through its lipoproteins (Lpp) toll-like receptor 2 (TLR2) signaling to MyD88; the subsequent innate immune response improves bacterial clearing and outcome. The impact of this activation upon dendritic cell (DC) and T cell function is unclear. Here, we used *S. aureus* Newman wild type and an isogenic mutant deficient in mature lipoproteins *(*Δ*lgt)* for DC infection in vitro. We show that Lpp contribute to DC activation by modest upregulation of surface CD40, CD80, and CD86 and by a strong secretion of inflammatory cytokines, including TNF, IL-6, IL-10, IL-1β, and IL-12p70. The enhancing effect of Lpp in DCs was dependent on sensing of *S. aureus* by TLR2 and on signaling via myeloid differentiation factor 88 (MyD88). Interleukin-1 receptor- (IL-1R) or TLR9-signaling did not mediate the remarkable reduction of cytokine levels found in the absence of MyD88. Lpp and TLR2 also markedly contributed to IFN-γ and IL-17 production in $CD4^+$ T cells and to IFN-γ production in $CD8^+$ T cells. In systemic infection with *S. aureus*, spleen cells from $TLR2^{-/-}$ mice produced similar amounts of IFN-γ and IL-10, but reduced levels of IL-17 compared to C57BL/6 mice. $MyD88^{-/-}$ mice were highly impaired in the production of all cytokines. The importance of the adaptive immune response after 6 days of infection was found to be negligible in systemic *S. aureus* infection, since $Rag2^{-/-}$ mice had less bacterial load in kidneys and knees and less plasma IL-6 than C57BL/6 mice. Our data indicate that *S. aureus* is able to activate innate and adaptive immunity in a TLR2-MyD88 modulated manner; the host however does not profit from early adaptive immunity.

Introduction

A successful immune defense against pathogens results from an immediate innate and a long-lasting specific response, both processes are tightly connected and controlled. Dendritic cells (DCs) are indispensable for initiation and orchestration of adaptive immunity (1, 2). In peripheral organs, residing immature DCs phagocytose invading pathogens and get concurrently activated through pattern recognition receptors (PRRs) that sense conserved patterns on the microorganism. These pathogens associated molecular patterns (PAMPs) induce maturation of DCs including upregulation of MHC class II and costimulatory surface molecules, switch of chemokine receptors as well as production of inflammatory and anti-inflammatory cytokines (3). DCs migrate to the lymphoid tissue for presentation of Ag to lymphocytes (4), which get activated by direct contact with the DC. This interaction promotes the differentiation of $CD4^+$ T cells into T_H1, T_H2, and T_H17 cells.

Like many other cells, DCs express PRRs, including all Toll-like receptors (TLRs), to sense PAMPs (5). After recognition of PAMPs, TLRs elicit signaling pathways through the myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF) proteins, leading to activation of nuclear factor (NF)-κB and other transcription factors (6) with subsequent upregulation of surface molecule and production of mediators.

Staphylococcus (*S.*) *aureus* is one of the most important pathogens causing severe systemic infections like endocarditis or sepsis. Several staphylococcal molecules are known to act as PAMPs for TLRs. Infections of mice and macrophages with *S. aureus*

revealed that lipoproteins (Lpp) activate TLR2, which is required for early activation of the innate immune system (7-11), whereas TLR9, which allows DNA-induced responses, has no effect on innate immune responses or bacterial killing (7, 8, 10). TLR2 and TLR9 signal exclusively through MyD88 and macrophages lacking MyD88 produce no or low levels of cytokines after recognition of *S. aureus* (9, 11). The failure of inflammatory defense is responsible for the increased susceptibility of these mice to staphylococcal infections (9-12). The results further indicate that other receptors beyond TLR2 and TLR9, which also signal through MyD88, participate in an effective immune response against *S. aureus.* Indeed, IL-1 receptor (IL-1R) and IL-18R were found to have an important role (12, 13).

There is increasing evidence that additional endosomal TLRs or cytosolic PRR play a role in the innate defense and mediate an interferon (IFN) response to RNA or DNA of Gram-positive bacteria (14). Indeed, it was shown that plasmacytoid DCs (pDCs) are rather stimulated by staphylococcal DNA or RNA and TLR9 or TLR7 respectively (16). It remains however to be explored whether – as shown for group B streptococci – (14), staphylococcal RNA interacts with TLR7 in phagosomes of myeloid cell-derived DCs. Additionally, it remains to be investigated, whether staphylococcal ligands – similar to those from *Listeria* – induce in the cytosol NOD2-dependent IFN-β (15).

Besides the unknown spectrum of *S. aureus*-activated PRR in DC, data on the skewing of an *S. aureus*-induced T_H cell response are controversial. While one group found a T_H2 response after stimulation with enterotoxin B through a TLR2-dependent recognition (17), other studies reported a beneficial effect of T_H1 responses by using antibodies or several knock-out mice (18-20). Additionally, DCs activated by *S. aureus* PGN promote

IL-17 production in memory T_H cells possibly by amplification of TLR2-induced IL-23 and IL-1 by NOD2 (21). In the absence of IL-17, mice were more often colonized with *S. aureus* (22). Nevertheless, IL-17 might be not only beneficial for the host, since it is known to recruit neutrophils (22) and *S. aureus* is able to reside in infected organs by attraction of neutrophils (23). Under these conditions, *S. aureus* survives although the adaptive immune response with IFN-γ production was elicited.

In the current study, we examined the capacity of *S. aureus* to activate DCs and whether these are able to promote differentiation of naïve T cells into T_H1, T_H2, and T_H17 cells. We used *S. aureus* strain Newman and an isogenic mutant deficient in mature Lpp in order to evaluate their contribution to TLR2 activation. Our results demonstrate that Lpp enhance cytokine production in *S. aureus*-infected DCs by TLR2 and MyD88. Lpp-TLR2-MyD88-signaling was required to initiate the production of IFN-γ and IL-17 in naïve T cells in vitro, while only MyD88 was required for cytokine production during systemic infection. We further demonstrate that T cells are the major producers of IFN-γ and IL-17 in vivo. However, both T nor B cells positively affect early sepsis as in their absence disease course was less severe and bacterial killing was improved.

Material and Methods

Mice

Wild-type inbred C57BL/6, TLR2$^{-/-}$, MyD88$^{-/-}$, IL-1R$^{-/-}$, TLR9$^{-/-}$, Rag2$^{-/-}$, MuMt$^{-/-}$, and CD3$^{-/-}$ mice were bred in the Animal House of the Department of Biomedicine, University Hospital Basel according to the regulations of Swiss veterinary law. MyD88$^{-/-}$ and TLR9$^{-/-}$ mice were a kind gift from W. D. Hardt (ETH Zürich, Switzerland) and TLR2$^{-/-}$ mice from W. J. Rieflin (Tularik, South San Francisco, CA), MuMt$^{-/-}$ and CD3$^{-/-}$ mice from Antonius Rolink (DBM, University Hospital Basel, Switzerland), all backcrossed on C57BL/6 background for 10 generations.

Antibodies and reagents

Monoclonal antibodies recognizing CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11c (N418), CD40 (1C10), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL-1), and MHCII (M5/114.15.2) were purchased from Biolegend, eBioscience or Pharmingen. Streptavidin-phycoerythrin and Streptavidin-FITC were purchased from CALTAG laboratories. Normal rabbit serum was provided by the Animal Care Unit at the University Hospital Basel. Specificity of staining was confirmed with isotype-matched control antibodies.

Pam_2CSK_4 and Pam_3CSK_4 were purchased from EMC microcollections (Germany) and smooth LPS from *Streptococcus equi* abortus was kindly provided by Marina Freudenberg (Max-Plank Institute for Immunobiology, Freiburg, Germany). Sandwich ELISAs were purchased from BD Biosciences and R&D Systems.

Bacterial strains and growth conditions

In this study, we used Newman wt (24) and SA113 wt strains and their isogenic *lgt*::ermB (Δ*lgt*) mutants (9, 25). Bacteria were grown in TSB for 7h and subcultured overnight under the same conditions. Overnight cultures were washed in 0.9% NaCl (Bichsel, Switzerland) and used for sepsis. Each inoculum was assessed by CFU/ml counting on Mueller-Hinton agar (MHA) plates. For infection of DC, bacteria were further subcultured in TSB to end-log phase. When appropriate, TSB was supplemented with 10 μg/ml erythromycin.

Generation of DC

DCs were generated as described (26, 27). Briefly, Bone marrow cells from tibia and femur were flushed with RPMI medium. Red blood cells were lysed and $CD11b^+$ BMDCs (DCs) were generated by plating of bone marrow progenitors in RPMI 1640 supplemented with gentamicin, 2-mercaptoethanol (all from Invitrogen), 10% (vol/vol) heat-inactivated FBS (Gibco) and 10% conditioned medium from GM-CSF transduced X63 (28). After 3 days, non-adherent cells were removed and attached cells were further cultured in supplemented RPMI medium. On day 7 and 8, cells were harvested and analyzed for $CD11c^+$ expression which was routinely 80-90% positive.

Stimulation of DC

For analysis of cytokine production in supernatants and surface marker expression, 1 x 10^6 DCs per well were cultured in 24 well plates in 1 ml RPMI 1640 containing GM-CSF. DCs were stimulated with smooth lipopolysaccharide (1 μg/ml), Pam_2CSK_4 (10

μg/ml), or Pam_3CSK_4 (10μg/ml). When stimulated with *S. aureus*, DCs were infected with viable *S. aureus* strains at a multiplicity of infection (MOI) of 10 bacteria per DC for 1h. After phagocytosis, supernatants were replaced with RPMI 1640 containing 100 μg/ml gentamicin and cells were further incubated. Supernatants were collected at 2 and 18h post-infection or incubation with various stimuli. Cytokines in the supernatants were analyzed by sandwich ELISA per manufacture's instructions (BD Biosciences).

Phagocytosis of S. aureus *by DC*

DCs (1×10^6 cells) were infected for 1h with *S. aureus* at a MOI of 10. For phagocytosis, extracellular bacteria were killed by 50 U lysostaphin and cells were lysed with water (pH 11) to assess intracellular bacteria by plating serial dilutions on MHA plates.

Flow cytometry

Cell suspensions were stained in ice-cold PBS supplemented with 0.04% (vol/vol) FCS and 25 mM sodium azide for surface staining. Data were acquired on a CyAN Flow cytometer (Dako) and were analyzed with FlowJo software (TreeStar).

In vitro T cell activation and differentiation

$CD4^+$ or $CD8^+$ T cells from spleen of C57BL/6 background were purified by negative selection by MACS beads (Miltenyi Biotec). DCs (1×10^4) were infected with *S. aureus* at a MOI of 10, bacteria were killed by Pen/Strep and gentamicin after 1h of infection. DCs were further co-cultured with purified T cells (5×10^4) for 72 h in RPMI 1640

without GM-CSF. Supernatants were analyzed by sandwich ELISA and T cells were analyzed by flow cytometry for expression of CD69.

Systemic infection

Mice were infected intravenously with 1 x 10^6 CFU of *S. aureus* Newman and SA113. Weight loss was monitored over the time of infection. On days 6 or day 28 after infection, mice were killed, bacterial load determined in organs by CFU plating. Cytokines in plasma were analyzed by ELISA.

In vitro stimulation of splenocytes

Spleen cells from infected mice were isolated 6 days after injection, mechanically disrupted (70 µm meshes) followed by lysis of erythrocytes and resuspension in RPMI 1640. In 96-well plates, 2 x 10^5 cells were infected for 1h with the same viable bacteria as used for infection at a MOI of 1. Bacteria were killed with Pen/Strep and gentamicin to prevent bacterial growth. Supernatants were collected after 48h and cytokines were analyzed by sandwich ELISA.

Statistical analysis

Statistical analyses of data with Mann-Whitney t tests and two-way Anova were performed using Prism (GraphPad Software, Inc.). Values of $p<0.05$ are shown when differences in the compared data sets are significant.

Results

S. aureus *enhances maturation of mDC*

We were interested to know whether *S. aureus* supports maturation of DCs in a Lpp-TLR2-MyD88-dependent manner. GM-CSF stimulation of bone-marrow cells resulted in the generation of 80-90% of immature $CD11c^{+}$ BMDCs (DCs, data not shown). Infection with *S. aureus* Newman wt led to similar upregulation of surface MHC class II, but to a slightly higher CD80, CD86, and CD40 in DC in C57BL/6 compared to $TLR2^{-/-}$ and $MyD88^{-/-}$ mice (Figure 1A). In addition, infection of C57BL/6 DCs with Newman Δ*lgt* led to comparable induction of MHCII (data not shown), and a slightly but not significantly lower upregulation of CD80, CD86, and CD40 than observed with Newman wt (Figure 1A). The synthetic lipopeptides Pam_2CSK_4 and Pam_3CSK_4 induced DC maturation dependent on TLR2 and MyD88 (data not shown) confirming that lipopeptides act as agonists of TLR2. The TLR4 agonist LPS, elicited strong maturation of DCs from C57BL/6, $TLR2^{-/-}$, and $MyD88^{-/-}$ mice. These controls indicate that LPS uses the TRIF pathway for TLR4-modulated expression of comolecules in DCs (Figure 1A). Taken together, Lpp-TLR2-signaling by MyD88 in DCs weakly enhances induction of maturation markers after infection with viable *S. aureus*.

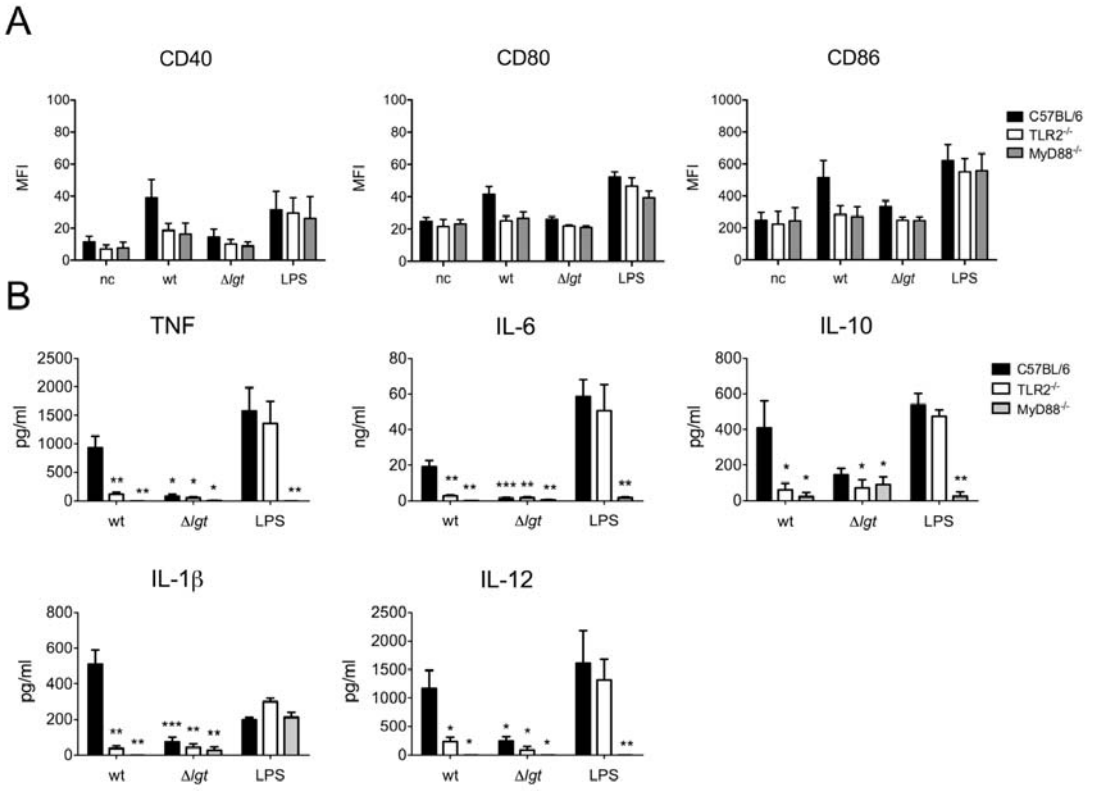

Figure 1. Staphylococcal Lpp enhance upregulation of comolecules and cytokines by TLR2-MyD88-signaling. DCs from C57BL/6 (black), TLR2$^{-/-}$ (white), and MyD88$^{-/-}$ mice (gray) were incubated with medium (nc), *S. aureus* wt and Δ*lgt* (MOI 10) for 1h. Fresh medium with gentamicin was added and DCs were further incubated for 18h. As a positive control, DCs were stimulated with 1 µg/ml of smooth LPS for 18h. (A) Expression of CD40, CD80, and CD86 on the surface of CD11c^{+} DCs was determined by flow cytometry. Data show mean ± SD of mean fluorescence intensity (MFI) of at least three independent experiments. (B) TNF, IL-6, IL-10, IL-1β, and IL-12 levels in the supernatants of DCs were measured by ELISA. Data are represented as mean ± SEM of 3-5 independent experiments. C57BL/6-DC infected with wt vs. all other groups, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ (Mann-Whitney test).

Lpp-TLR2-MyD88-signaling is required for cytokine release in DCs

DCs produce cytokines in response to synthetic or purified TLR agonists (29). The response to live bacteria may differ from that to individual TLR ligands as pathogens

express several PAMP and thus trigger combinations of TLRs, which may mitigate the individual pathways and lead to synergistic effects. Therefore, we investigated the production of cytokines in *S. aureus*-infected DCs to see results of a composite TLR activation by these bacteria.

18h after infection, Newman wt induced higher levels of TNF, IL-6, IL-10, IL-1β, and IL-12p70 in C57BL/6 DCs than in TLR2$^{-/-}$ DCs (Figure 1B). DCs from MyD88$^{-/-}$ mice were highly impaired in the production all cytokines compared to C57BL/6 cells after infection (Figure 1B). The TLR2-dependent cytokine response with Newman was mainly mediated by Lpp since Newman Δ*lgt* induced similar quantities as Newman wt in TLR2$^{-/-}$ DCs (Figure 1B and data not shown). Cytokine induction by LPS was unimpaired in TLR2$^{-/-}$ DCs; it was nearly absent in MyD88$^{-/-}$ DCs, except for IL-1β which was completely unaffected by MyD88, as had been shown before (30). In contrast, Pam_2CSK_4- and Pam_3CSK_4-induced cytokine production was dependent on TLR2-MyD88-signaling (data not shown). At the very early time point of 2h after infection, IL-6 was induced by Newman wt in C57BL/6 DCs, but not by its isogenic Δ*lgt* strain or in TLR2$^{-/-}$ and MyD88$^{-/-}$ DCs, whereas TNF, IL-1β, and IL-12 were not detectable (data not shown). IL-10 was similarly induced by Newman wt and Δ*lgt* in C57BL/6 and TLR2$^{-/-}$ DCs after 2h (data not shown).

We conclude that staphylococcal Lpp selectively trigger the TLR2-MyD88-signaling pathway in DCs leading to induction of cytokines. However, our data indicate that in *S. aureus* infection, cytokine production by DCs is also mediated by other MyD88-dependent signaling receptors.

To examine the role of other MyD88-dependent receptors, we generated DCs from IL-1R$^{-/-}$ and TLR9$^{-/-}$ mice. Neither the deficiency in IL-1R nor in TLR9 affected the expression of surface markers on DCs (data not shown). Cytokine responses were as well not affected by the absence of these receptors (Figure 2) indicating that other MyD88-dependent receptors or synergistic/additive stimulation is required for induction of cytokines.

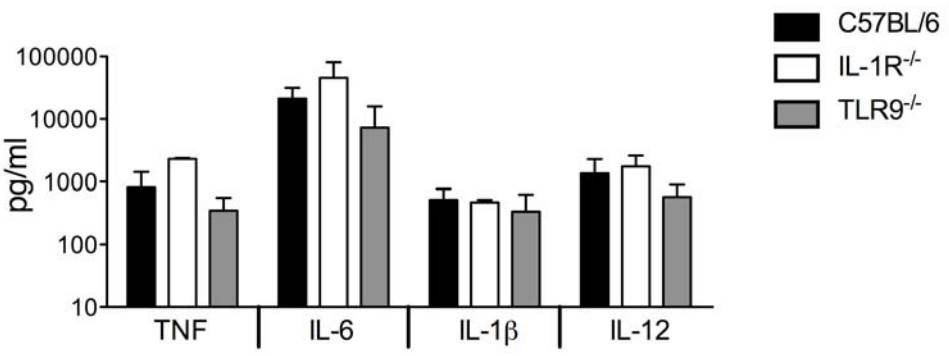

Figure 2. MyD88-signaling is independent of *S. aureus* recognition by IL-1R and TLR9. DCs from C57BL/6 (black), IL-1R$^{-/-}$ (white), and TLR9$^{-/-}$ mice (gray) were infected with *S. aureus* Newman wt for 1h. Fresh medium with gentamicin was added and DCs further incubated for 18h. TNF, IL-6, IL-1β, and IL-12 levels in the supernatants of DCs were measured by ELISA. Data are represented as mean ± SEM of 3-5 independent experiments.

Phagocytosis of S. aureus *by DCs is independent of TLR2-MyD88*

We next wanted to exclude that the amount of engulfed bacteria is responsible for the enhanced activation of DC in the presence of Lpp, TLR2 or MyD88. However, phagocytosis of Newman wt and Δ*lgt* was similar in DCs from C57BL/6 mice (Figure 3). The absence of TLR2 and MyD88 did not influence phagocytosis of both Newman wt

and Δ*lgt* suggesting that activation of DCs is enhanced by Lpp-TLR2-MyD88-signaling and not by the amount of ingested bacteria.

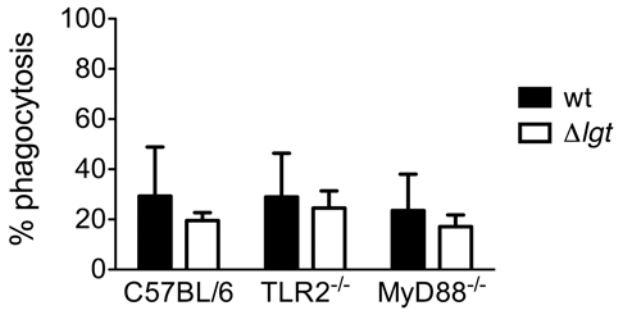

Figure 3. Phagocytosis of DCs is unaffected by *S. aureus* Lpp and TLR2-MyD88-signaling. DCs from C57BL/6, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice were infected with Newman wt (filled) and Δ*lgt* (open) for 1h. Extracellular bacteria were killed with lysostaphin and intracellular CFU were assessed by plating serial dilutions on MHA plates. Data are mean ± SD of three independent experiments.

Lpp-TLR2-MyD88-signaling programs DCs to enhance IFN-γ and IL-17 secretion in naïve CD4$^+$ T cells

S. aureus primes DCs to promote production of IFN-γ and IL-17 in T_H cells (21). We tested whether Lpp on viable *S. aureus* Newman are capable of modulating T_H1 and T_H17-cytokines via TLR2 and MyD88. Newman wt-infected C57BL/6 DCs induced more IFN-γ and IL-17 in naïve CD4$^+$ T cells than Δ*lgt*-infected DCs (Figure 4A). Experiments with DCs from TLR2$^{-/-}$ and MyD88$^{-/-}$ mice revealed that the capacity of DCs to enhance IFN-γ and IL-17-production in naïve CD4$^+$ T cells resulted from DC activation by Lpp, TLR2, and MyD88 (Figure 4A). Infected DCs from all tested mice did not induce IL-4 production in naïve CD4$^+$ T cells (data not shown) underscoring the importance of T_H1 and T_H17 cells in this bacterial infection. LPS stimulated IFN-γ and

IL-17 in a MyD88- but not TLR2-dependent manner (Figure 4A). Infected DCs alone or T cells alone did not produce any cytokines after *S. aureus* infection (data not shown). Intracellular staining of IFN-γ and IL-17 in naïve T cells will elucidate the proportion of T_H1 and T_H17 primed cells (work in progress). Furthermore, DCs from MHC class $II^{-/-}$ mice will be used to examine the antigen-specific activation of *S. aureus* and to what extend the T cell cytokine production after *S. aureus* infection in vivo reflects MHC class II and TCR engagement (work in progress).

In addition, we investigated the expression of CD69, an early activation marker of T cells. DCs from C57BL/6, $TLR2^{-/-}$ and $MyD88^{-/-}$ mice infected with *S. aureus* Newman wt or Δ*lgt* did not cause upregulation of CD69 on naïve $CD4^+$ T cells after 24 and 72h (data not shown).

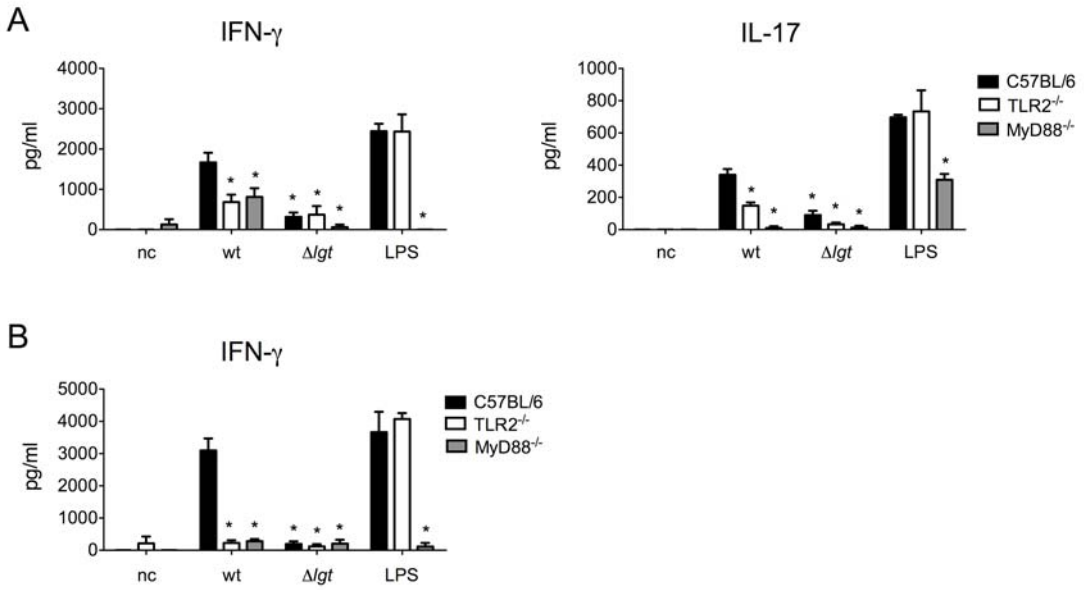

Figure 4. DCs infected with *S. aureus* promote differentiation of naïve T cells to T_H1 and T_H17 cells by Lpp-TLR2-MyD88-signaling. (A, B) DCs from C57BL/6 (black), $TLR2^{-/-}$ (white), and $MyD88^{-/-}$ mice (gray) were infected with *S. aureus* Newman wt and Δ*lgt* for 1h. Extracellular bacteria were killed with gentamicin and penicillin/streptomycin and negatively

selected (A) $CD4^+$ T cells or (B) $CD8^+$ T cells were added. Non-infected DCs (nc), and T cells only (data not shown) were used as negative controls. LPS was added to non-infected DCs as a positive control. Cytokines in the supernatants of cocultures were determined by ELISA after 72h of coculture. Data represent mean ± SEM of quadruplicates of one out of two independent experiments. C57BL/6-DC infected with wt vs. all other groups*: $p<0.05$ (Mann-Whitney test).

Lpp-TLR2-MyD88-signaling enhances IFN-γ in $CD8^+$ T cells

S. aureus is known to survive in phagocytes by phagosomal escape (31) and in addition it constantly releases proteins during growth (25), which could be taken up by micropinocytosis in DCs. Therefore, *S. aureus* might be able to activate naïve $CD8^+$ T cells by cross-presentation of peptides on MHC class I. Indeed, *S. aureus* wt-primed DCs from C57BL/6 mice were able to induce the production of IFN-γ in naïve $CD8^+$ T cells (Figure 4B). In co-cultures of *S. aureus* wt-infected C57BL/6 DCs with isolated $CD8^+$ T cells, IFN-γ was higher than in cocultures using DC from $TLR2^{-/-}$ and $MyD88^{-/-}$ mice or with Δ*lgt*-infected C57BL/6 DCs (Figure 4B). DCs of MHC class $I^{-/-}$ mice and $CD4^+$ T cells from transgenic OT-I mice will be used as a control for antigen-specific activation of $CD8^+$ T cells (work in progress). The data suggest that Lpp-TLR2 interaction and MyD88-signaling are required for the activation of $CD8^+$ T cells in vitro, possibly by cross-presentation of staphylococcal peptides on MHC class I.

T cells produce IFN-γ during systemic infection

To assess whether *S. aureus*-infected DCs are able to induce T cell activation in vivo, we systemically infected C57BL/6, $TLR2^{-/-}$, and $MyD88^{-/-}$ mice with *S. aureus*. After 6 days of infection, spleen cells were isolated, restimulated with Newman wt and compared to

non-stimulated cells in vitro. Restimulated spleen cells from infected C57BL/6 and TLR2$^{-/-}$ mice produced high levels of IFN-γ, and IL-10, but no IL-4, IL-17 was significantly lower in TLR2$^{-/-}$ compared to C57BL/6 cells (Figure 5A). Since we previously observed high mortality of Newman wt-infected MyD88$^{-/-}$ mice (9), we used the less virulent SA113 wt to investigate spleen cytokine production of infected MyD88$^{-/-}$ mice. In agreement with results obtained with Newman, SA113 wt induced similar quantities of IFN-γ in restimulated spleen cells of C57BL/6 and TLR2$^{-/-}$ mice; and it induced only low amounts in MyD88$^{-/-}$ mice (Figure 5B). IL-10 production after stimulation with SA113 wt was partially dependent on TLR2 and completely on MyD88. IL-4 production was not detectable (Figure 5B). Intracellular staining revealed that numbers of IFN-γ- and IL-17-producing CD4^{+} T cells were 2- to 3-fold higher in infected C57BL/6 mice than in non-infected mice (data not shown).

In summary, after in vivo infection, the effect of Lpp-TLR2 activation upon T cell cytokines was not detectable in non-separated spleen cells; it was overridden by other signals to MyD88, which strongly contribute to IFN-γ and IL-10.

We sought to examine whether T cells are the major IFN-γ-producing cell type of the adaptive immune system during systemic infection with *S. aureus*. Therefore, we first infected CD3$^{-/-}$ and Rag2$^{-/-}$ mice with *S. aureus* Newman wt to assess the role of B and T cells. In restimulated spleen cells from infected CD3$^{-/-}$ and Rag2$^{-/-}$ mice, Newman wt induced less IFN-γ, IL-17, and IL-10 than in C57BL/6 spleen cells (Figure 5C). IL-4 was only detectable at background level. The data suggest that primarily T cells are responsible to mount a strong cytokine response against *S. aureus*. Next, we used MuMt$^{-/-}$ mice to evaluate the contribution of B versus T cells in *S. aureus* infection. Compared to

C57BL/6 spleen cells, restimulated spleen cells of MuMt$^{-/-}$ mice produced 6-, 7- and 17-fold more IFN-γ, IL-17, and IL-10 in response to Newman wt, respectively (Figure 6C). Taken together, the results indicate that T cells are the major IFN-γ-producing cell type and that B cells regulate the production of the cytokines by T cells. The direct or indirect control of IFN-γ production by B cells will be investigated by measuring IFN-γ in infected spleen cells from MuMt$^{-/-}$ mice after costimulation with purified C57BL/6 B cells (work in progress). It will be further evaluated whether the interaction of B with T cells needs cell-cell contact and leads to transcriptional downregulation of IFN-γ and IL-17 mRNA.

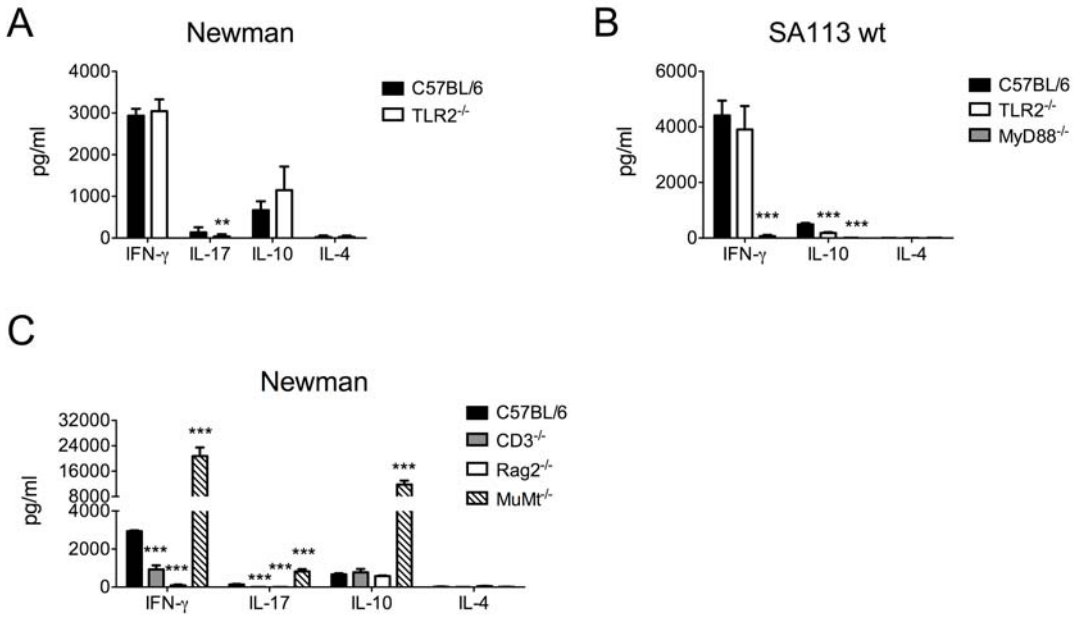

Figure 5. T cells are produce IFN-γ and IL-17 during *S. aureus* infection. (A, B) C57BL/6 (black), TLR2$^{-/-}$ (white), and MyD88$^{-/-}$ mice (gray) were i.v. infected with *S. aureus* Newman or SA113 wt. (C) C57BL/6 (black), CD3$^{-/-}$ (gray), Rag2$^{-/-}$ (white), and MuMt$^{-/-}$ mice (striped) were i.v. infected with *S. aureus* Newman wt. (A–C) After 6 days of infection, spleen cells were isolated and restimulated with Newman wt or SA113 wt (MOI 1) for 1h. Bacteria were killed with gentamicin and penicillin/streptomycin and cells were further incubated for 48h. Cytokines

in the supernatant were analyzed by ELISA. Data are represented as mean ± SEM of quadruplicates of 4 mice per group. C57BL/6 vs. all other groups, *: $p<0.05$, ***: $p<0.001$ (Mann-Whitney test).

Adaptive immune cells do not contribute to killing of S. aureus *in early sepsis*

We aimed to elucidate whether T and/or B cells are beneficial for outcome and contribute to the reduction of the bacterial load in systemic infection. Surprisingly, the survival of $CD3^{-/-}$, $Rag2^{-/-}$ and $MuMt^{-/-}$ mice infected with an inoculum of Newman wt, which was sublethal in C57BL/6 mice (9), was not affected in these mice (data not shown). Weight loss of $MuMt^{-/-}$ mice was similar to that of C57/BL/6 mice. In contrast, weight loss in $Rag2^{-/-}$ and $CD3^{-/-}$ mice was significantly lower than in C57BL/6 mice after 2 and 6 days of infection (Figure 6A). Bacterial load in spleen and knees of $CD3^{-/-}$, $Rag2^{-/-}$, and $MuMt^{-/-}$ mice was lower than in C57BL/6 mice; also in liver and kidneys bacterial numbers were slightly, but not significantly lower in the immunocompromised compared to C57BL/6 mice (Figure 6B). Plasma IL-6 levels were significantly lower on day 6 and kidney abscesses were absent in $CD3^{-/-}$, $Rag2^{-/-}$, and $MuMt^{-/-}$ mice compared to C57BL/6 mice (Figure 6C). These data indicate that the innate immune response is required for staphylococcal defense, whereas the adaptive immune response mediated by T cells and B cells is not beneficial for eradication of *S. aureus* in early murine sepsis.

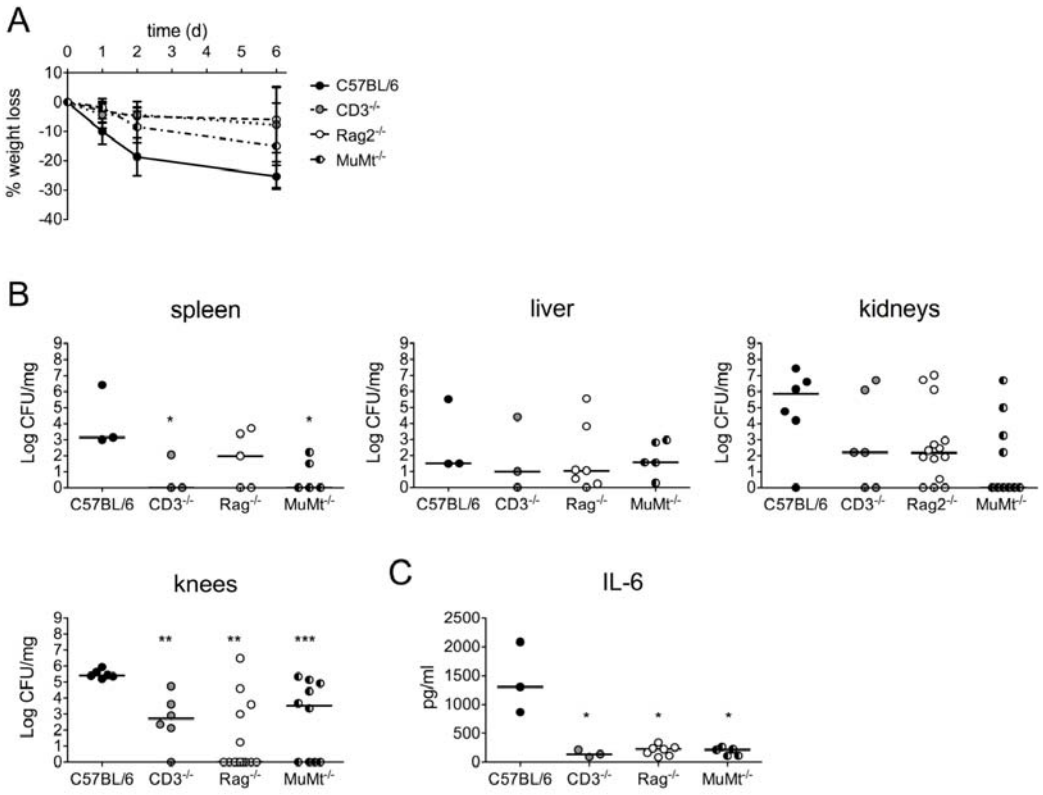

Figure 6. Absence of adaptive immune cells enhances killing of *S. aureus* in mice. C57BL/6 (black, solid line), Rag2$^{-/-}$ mice (white, dashed line), CD3$^{-/-}$ (gray, dotted line), and MuMt$^{-/-}$ mice (half filled, dash-dotted line) were infected with Newman wt. (A) Weight loss, (B) bacterial load in spleen, liver, both kidneys, and both knees, and (C) plasma IL-6 in mice on day 6. Data for mice are represented as mean ± SD for weight loss and cytokines and median for CFU/mg of 3-7 mice per group. Significant differences between mice are indicated by *: $p<0.05$, **: $p<0.01$, and ***: $p<0.001$.

In the above studies, we selected a sublethal inoculum and followed sepsis during 7 days. During this acute phase, we found T and/or B cells disadvantageous. Next, we were interested into their role in persistence. To that aim, we examined the ability of innate and adaptive immune cells to eradicate *S. aureus* in chronic infection. Thus, we monitored mice for 28 days after infection with the low-virulent SA113 wt strain in C57BL/6 (Figure 7) and Rag2$^{-/-}$ mice (work in progress). Notably, although infected C57BL/6 mice

regained weight (Figure 7A), not all were able to clear *S. aureus* SA113 from liver, kidneys, and knees (Figure 7B).

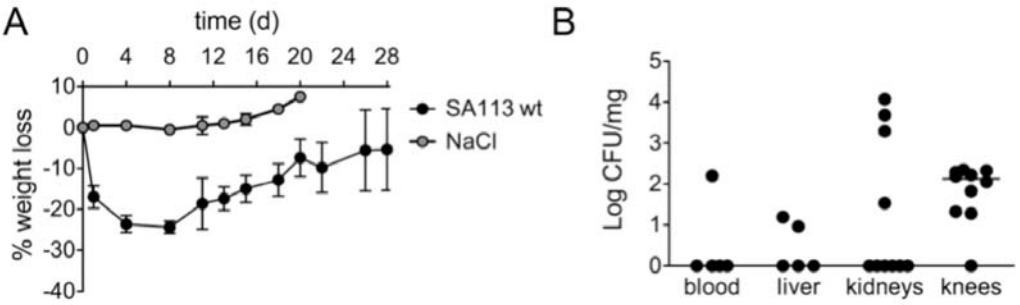

Figure 7. Innate and adaptive immune cells do not eradiate *S. aureus* from organs. C57BL/6 mice were infected with SA113 wt (black) for 28 days. NaCl was injected in control mice (gray). (A) Weight loss, (B) bacterial load in blood, liver, both kidneys, and both knees on day 28. Data for mice are represented as mean ± SD for weight loss and cytokines and median for CFU/mg of 5 mice.

Discussion

In this report, we show for the first time that staphylococcal Lpp expressed on viable *S. aureus* enhance the activation of DCs by TLR2 and thereby promote the differentiation of naïve $CD4^+$ T cells into T_H1 and T_H17 cells in vitro. We further demonstrate that in vivo in whole spleen cells the contribution of Lpp-TLR2 to IFN-γ production is mitigated by other MyD88-signaling receptors, while Lpp-TLR2-dependence remains detectable for IL-10 and IL-17. We then provide evidence that beside T_H cell differentiation, *S. aureus*-infected DCs that receive Lpp-TLR2 signals are competent to activate $CD8^+$ T cells to produce IFN-γ. Finally, we show that T cells are the major producers of IFN-γ, IL-17, and IL-10 after systemic *S. aureus* infection, and B cells negatively regulate these T cell cytokines. Surprisingly, one week after infection, T and/or B cells do not improve outcome, but impair the killing of *S. aureus* from the organs.

The innate immune response to *S. aureus* is primary during acute invasive infections. However, it is well known that *S. aureus* also activates the adaptive immune response with its superantigens, yet the incidence of superantigen-related toxic shock syndrome is low. This is possibly due to the fact that *S. aureus* downmodulates the T cell response to these superantigens (32), which was recently shown to be related to the release of cell wall components that preferentially activate the anti-inflammatory axis of the adaptive immune system (33). The contribution of superantigens to sepsis is controversial and mostly believed to be due to general inflammation. However, the regulation of the antigen-specific T cell polarization and on B cell responses as well as the effect of T and B cells upon outcome in acute *S. aureus* sepsis is not clear yet. Previous studies on the activation of T cells have used purified staphylococcal molecules, but not viable *S.*

aureus. To close the gap, we evaluated the requirements for *S. aureus* and for the host to promote the activation of T cells and more importantly, the differentiation of T cells into T_H1, T_H2, or T_H17 lineages.

The expression of surface markers e.g. CD40 is required for contact of DCs with T cells to induce inflammatory cytokines. It has been shown that TLR2 on DCs increased expression of MHCII and CD80 after stimulation with staphylococcal PGN (34). We now demonstrate that Lpp present in viable *S. aureus* are modestly contributing through the activation of the TLR2 pathway to induce the upregulation of the surface marker CD40, CD80, and CD86. In contrast, the MHC class II molecule was strongly upregulated after infection with *S. aureus*, but independent of MyD88-signaling as demonstrated repeatedly for LPS (own data and (35)). There is however controversy whether signals from TLRs can regulate phagosome maturation and hence phagosomal processing and presentation. Blander and Medhzitov demonstrated that apoptotic cells or particulate Ags are only presented efficiently by MHC class II in DCs when they are associated with a TLR ligand (36). In contrast, Russell and coworkers demonstrated that TLR2- or TLR4-agonists did not affect phagosome maturation (37).

DCs infected with viable *S. aureus* released TNF, IL-6, IL-10, IL-1β, and IL-12. Surprisingly, although viable *S. aureus* are recognized by various PRRs, in our experiments the activation of DCs was mostly dependent on Lpp-TLR2-signaling and not on other MyD88-dependent receptors. Nevertheless, some cytokines were induced by *S. aureus* in the absence of TLR2 but not MyD88. In contrast, TLR2 was essential only for the response to purified streptococcal components, while whole *Streptococcus gordonii* elicited similar levels in C57BL/6 and $TLR2^{-/-}$ cells (38). However in line with our

results, cytokine induction by live streptococci and by its purified PAMPs was dependent on functional MyD88. In our study, the recognition of staphylococcal DNA by TLR9 and the sensing of IL-1 by its receptor IL-1R had no impact on the released cytokines. Therefore, it remains to be elucidated, whether other MyD88-dependent signaling receptors, like the bacterial RNA-sensing TLR7 (14), or TLR4, which activates DCs after exposure to staphylococcal leukocidin, are involved (39). Alternatively, the number of engulfed bacteria can affect the cytokine production in cells, which can be excluded since DCs without TLR2 and MyD88 phagocytosed similar numbers of *S. aureus*.

TLR stimulation of DCs induces T_H1, T_H2, or T_H17 responses depending on the expression of cytokines. IL-12 mediates the differentiation of naïve T cells into IFN-γ producing T_H1 cells. Indeed, the high levels of IL-12 released in a Lpp-TLR2-dependent fashion in DCs may have contributed together with MHC-TCR interaction to IFN-γ production by naïve T cells. IL-4 was absent, which may be related to the suppressive effect of IFN-γ on T_H2 development (41). Low levels of IL-17 were detected. This finding is surprising, first, because IFN-γ inhibits the differentiation of T_H17 cells and alters the cytokine profile of DCs to reduce IL-17 by limiting IL-23 release (42, 43, 44). Second, the Lpp-TLR2-MyD88 pathway activated by *S. aureus* was found associated with a reduced proportion of IL-17 secreting $CD4^+$ T cells, at least in immune cells associated with a brain abscess (45). Yet, the production of IL-17 may have been supported by high quantities of the proinflammatory cytokine IL-6, possibly together with TGF-β and amplified by high levels of TNF and IL-1β in DCs (46).

We found high levels of IFN-γ produced by C57BL/6 $CD4^+$ T cells after *S. aureus* wt infection. Superantigens were unlikely contributing to this effect, since infection of

TLR2$^{-/-}$ and MyD88$^{-/-}$ cells with the same superantigens-expressing *S. aureus* strain resulted in a reduced IFN-γ response. However, it remains to be documented that the β-chain of the TCR is unaltered in TLR2$^{-/-}$ and MyD88$^{-/-}$ cells before participation of superantigens in IFN-γ production can be ruled out.

We found IFN-γ production by CD8^{+} T cells upregulated by Lpp-TLR2. Endocytosis of released antigens from the surface of *S. aureus* during growth or after antibiotic treatment and crosspresentation on MHC class I may have enabled infected DCs to activate CD8^{+} T cells. Additionally, survival of *S. aureus* within DCs and escape from the intracellular compartment by hemolysin-mediated membrane rupture (31) or the release of antigens into the cytoplasm may have lead to the activation of cytotoxic T cells. In a superantigen-mediated lung disease model, CD8^{+} T cell-derived IFN-γ was found responsible for pulmonary pathology (47), but so far, the effect of CD8^{+} T cells on the pathogenesis of systemic *S. aureus* infection is unclear. Interestingly in LCMV infection, MyD88 expression in T cells is required for an intact primary CD8^{+} T cell response (48) and TLR2 engagement is required for an effective anti-tumor CD8^{+} T cell response (49). However, *S. aureus* did not elicit IFN-γ production in naïve purified T cells after infection. Therefore, in our study, a Lpp-stimulation of TLR2 on CD8^{+} T cells does not explain the MyD88-mediated regulation of IFN-γ from these cells.

After *S. aureus* infection of mice, restimulated spleen cells elicited both IFN-γ and IL-10 responses independent of TLR2-signaling, but dependent of MyD88. We had previously found a highly impaired cytokine induction in MyD88-deficient macrophages (9). We and others found this lack of cytokines associated with a higher susceptibility to sepsis of MyD88$^{-/-}$ mice (9-12). The present report extends the findings to DCs, which apparently

also contribute to immune defense through T cell activation. It is however surprising that MyD88 is such a strong regulator of the IFN-γ, IL-17, and IL-10 response in $CD4^+$ and $CD8^+$ T cells. Since a MyD88 effect upon T cell numbers and spleen size was excluded to viable *S. aureus*, the antigen-driven response appears determined by MyD88 costimulation. Infection of MHC class II negative mice will inform on the relative contribution of MHC class II-TCR interaction to IFN-γ from spleen cells ex vivo. For *Toxoplasma gondii* proteins, which act through TLR11-MyD88, it was recently shown that MHC class II has to be expressed together with MyD88 in DC in order to elicit an IFN-γ response in $CD4^+$ T cells (50). In agreement with our in vitro results, the low TLR2-MyD88-dependent release of IL-17 was also observed ex vivo and may be related to the previously described pronounced TLR2-dependent IL-6 production in restimulated spleen cells of Newman-infected mice (9).

We could assign *S. aureus*-induced IFN-γ production to T cells, since we detected significantly less IFN-γ in spleens from *S. aureus*-infected $CD3^{-/-}$ mice and hardly any IFN-γ in those from $Rag2^{-/-}$ mice. Surprisingly, B cells had a suppressive function on T cell derived IFN-γ, IL-10, and IL-17 as we concluded from the excessive cytokine levels produced by B cell-deficient spleen cells from $MuMt^{-/-}$ mice. Since the spleen weight, the number of mononuclear cells and $CD4^+$ T cells in the spleens of MuMt $^{-/-}$ mice are less than 10% of the number observed in the spleens of C57BL/6 mice (51), the high IFN-γ cannot be due to an altered T cell number. It remains to be investigated, whether B cells reduce IFN-γ in C57BL/6 mice because *S. aureus* is better opsonized and elicits less immune activation or whether there is a direct inhibition of a B cell molecule or product upon T cells.

Several studies using the septic arthritis model in *S. aureus* infection, revealed that the IFN-γ dominated immune responses are beneficial for the outcome of mice (18-20). In view of the high IFN-γ in MuMt$^{-/-}$ mice, we expected a better outcome of infection in these mice. B cell deficiency was beneficial for control of *S. aureus*; however, we found no correlation of IFN-γ levels and bacterial load, since CD3$^{-/-}$ and Rag2$^{-/-}$ mice with low or no IFN-γ showed accelerated or improved clearance of *S. aureus* from the organs. The low bacterial load in the mice lacking B and T cells correlated with the absence of infiltrates and lower IL-6 levels in plasma as an indicator for disease severity. These results are in line with our earlier report of a similar liver pathology in mice deficient in MyD88-signaling (9). In these mice, we could relate the better killing of *S. aureus* to the lack of chemokines and phagocyte recruitment, which prevented bacterial hiding in the liver and facilitated access of resident phagocytes to *S. aureus.* Here we show that T and or B cells did not improve the outcome after 6 days of systemic *S. aureus* infection. It was rather worsened, most likely through the inflammation, which is amplified by these cells. Four weeks follow up of the outcome in C57BL/6 and Rag2$^{-/-}$ mice will inform on whether a functional adaptive immune system at a later time point improves bacterial clearing.

Taken together, although MyD88-mediated immune responses favor the outcome after *S. aureus* infection (9), the host is unable to control *S. aureus* infection by both innate and adaptive immune systems until differentiation and expansion and of T and B cells into effector cells occurs.

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3.4 Part 4 – Role of TLR9 and NOD2 in sensing of *S. aureus*

Introduction

Receptors of the innate immune system have evolved for the recognition of different conserved microbial features, so called PAMPs, to detect the presence of pathogens. *S. aureus* expresses various PAMPs, which interact with TLRs and NOD2, respectively. Several studies investigated the role of individual receptors in sensing of isolated PAMPs.

TLR2 detects several components of the *S. aureus* cell wall preferentially lipoproteins (1-3); TLR4 might interact with leukocidin (4); TLR7 and TLR9 recognize RNA and DNA, respectively (5); IL-1R senses endogenous IL-1 released by activated cells after *S. aureus* infection (6). In addition to TLR receptors, NOD2 senses the peptidoglycan subfragment muramyl dipeptide (MDP) from *S. aureus* (7) in the cytoplasm. However, few studies have addressed the question whether these receptors trigger similar responses when stimulated with viable *S. aureus*. Recognition of whole bacteria can initiate signaling by both extracellular TLRs and intracellular TLRs and NODs. Accordingly, TLR2 and NOD2 have a dual role in activation of cytokine responses and defense against *S. aureus*; they can be either protective (8-10) or detrimental (11).

In the previous three parts, we showed that in response to *S. aureus*, macrophages from MyD88$^{-/-}$ mice fail to produce proinflammatory cytokines including TNF, and animals exhibit an increased susceptibility (1, 2). On the other hand, TLR2$^{-/-}$ macrophages produce normal levels of TNF at later stages in infections and TLR2$^{-/-}$ mice do not display the severe phenotype of MyD88$^{-/-}$ mice (1, 2) suggesting a contribution of intracellular TLRs and MyD88-dependent cytokine receptors. Therefore, we asked the question whether TLR9 and IL-1R contribute to the host responses induced by *S. aureus*.

Furthermore, we and others showed that *S. aureus* gains access to the cytoplasm (12, 13) and thus we postulated a role for NOD2.

Material and Methods

Stimulation of macrophages

Macrophages from C57BL/6, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, IL-1R$^{-/-}$, and NOD2$^{-/-}$ mice were infected with the *S. aureus* strain SA113 as described in Material and Methods in Part 1.

Sepsis infection model

C57BL/6, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, IL-1R$^{-/-}$, and NOD2$^{-/-}$ mice were i.v. infected with 1 x 10^7 CFU of SA113 wt as described in Material and Methods in Part 1.

Statistical analysis

Statistical analyses of cytokines, CFU in organs, cytokines in plasma were analyzed with Mann-Whitney. 2-way ANOVA was used to analyze weight loss of mice in systemic infections. Statistical analysis was done with Prism 5.0a (GraphPad Software, Inc.). A p-value of $p < 0.05$ was considered statistically significant.

Results

IL-1R and TLR9 in S. aureus *infections*

To assess the role of IL-1R and TLR9 in inflammatory responses to viable *S. aureus* SA113 wt in vitro, we analyzed cytokine production of infected IL-1R$^{-/-}$ and TLR9$^{-/-}$ macrophages. Infection with *S. aureus* induced release of TNF, IL-6, IL-10, IL-1β, and MIP-2 in IL-1R$^{-/-}$ and TLR9$^{-/-}$ macrophages (Figure 1A). While cytokine and chemokine levels of IL-1R$^{-/-}$ macrophages were similar to those induced in C57BL/6 macrophages, levels of all cytokines, but not of MIP-2, were decreased in TLR9$^{-/-}$ cells (Figure 1A). These data show that TLR9 but not IL-1R contributes to induction of inflammatory responses after *S. aureus* infection.

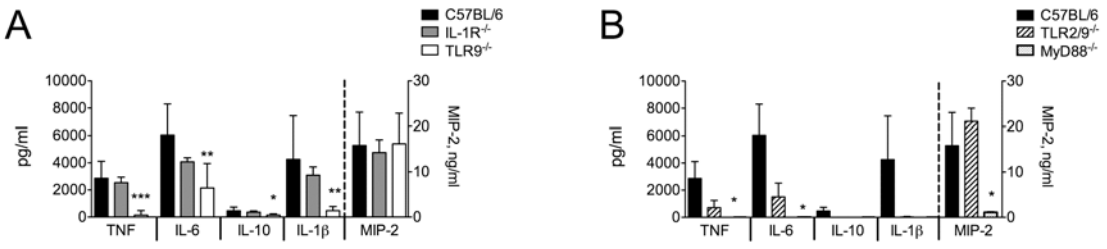

Figure 1. TLR2 and TLR9 enhance cytokine production of macrophages in *S. aureus* infection. Peritoneal macrophages from (A) C57BL/6 (black), IL-1R$^{-/-}$ (gray), TLR9$^{-/-}$ (white), and (B) C57BL/6 (black), TLR2/9$^{-/-}$ (striped), and MyD88$^{-/-}$ (dotted) were infected with SA113 wt at MOI 10. TNF, IL-6, IL-10, IL-1β, and MIP-2 released into the supernatants were analyzed by ELISA after 18h. Significant differences between (A) C57BL/6 vs. TLR9$^{-/-}$ or IL-1R$^{-/-}$ and (B) TLR2/9$^{-/-}$ vs. MyD88$^{-/-}$ cells are indicated as follows: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

We next investigated whether the lack of TLR2/9 abolishes cytokine production to levels observed with MyD88$^{-/-}$ macrophages (described in Part 1). In response to *S. aureus*, both

TLR2/9$^{-/-}$ and MyD88$^{-/-}$ macrophages released significantly less of the measured cytokines compared to C57BL/6 cells (Figure 1B). Whereas MyD88$^{-/-}$ macrophages released no TNF and IL-6, responsiveness in TLR2/9$^{-/-}$ macrophages was impaired but not abolished (Figure 1B). IL-10 and IL-1β were released neither by MyD88$^{-/-}$ nor TLR2/9$^{-/-}$ macrophages. MIP-2 was not dependent on TLR2/9-signaling but on MyD88 (Figure 1B). These data suggest that both TLR2 and TLR9 have to be activated by *S. aureus* to induce an efficient cytokine response in macrophages. However, other MyD88-dependent receptors beyond TLR2 and TLR9 seem to be involved in the induction of TNF, IL-6, and MIP-2.

To elucidate the contribution of TLR9 in vivo, we systemically infected TLR9$^{-/-}$ mice with SA113 wt and compared them to C57BL/6 mice. The absence of TLR9 did not affect weight loss (Figure 2A). Bacterial load in spleen, kidneys, and knees was similar, but reduced in the liver of TLR9$^{-/-}$ mice (Figure 2A). Reduced levels of IL-6 in plasma correlated with less infiltrates in the liver of TLR9$^{-/-}$ mice (Figure 2A). These results suggest that in systemic infection, recognition of staphylococcal DNA by TLR9 is not relevant for killing of *S. aureus*, but required for the attraction of phagocytes and inflammation.

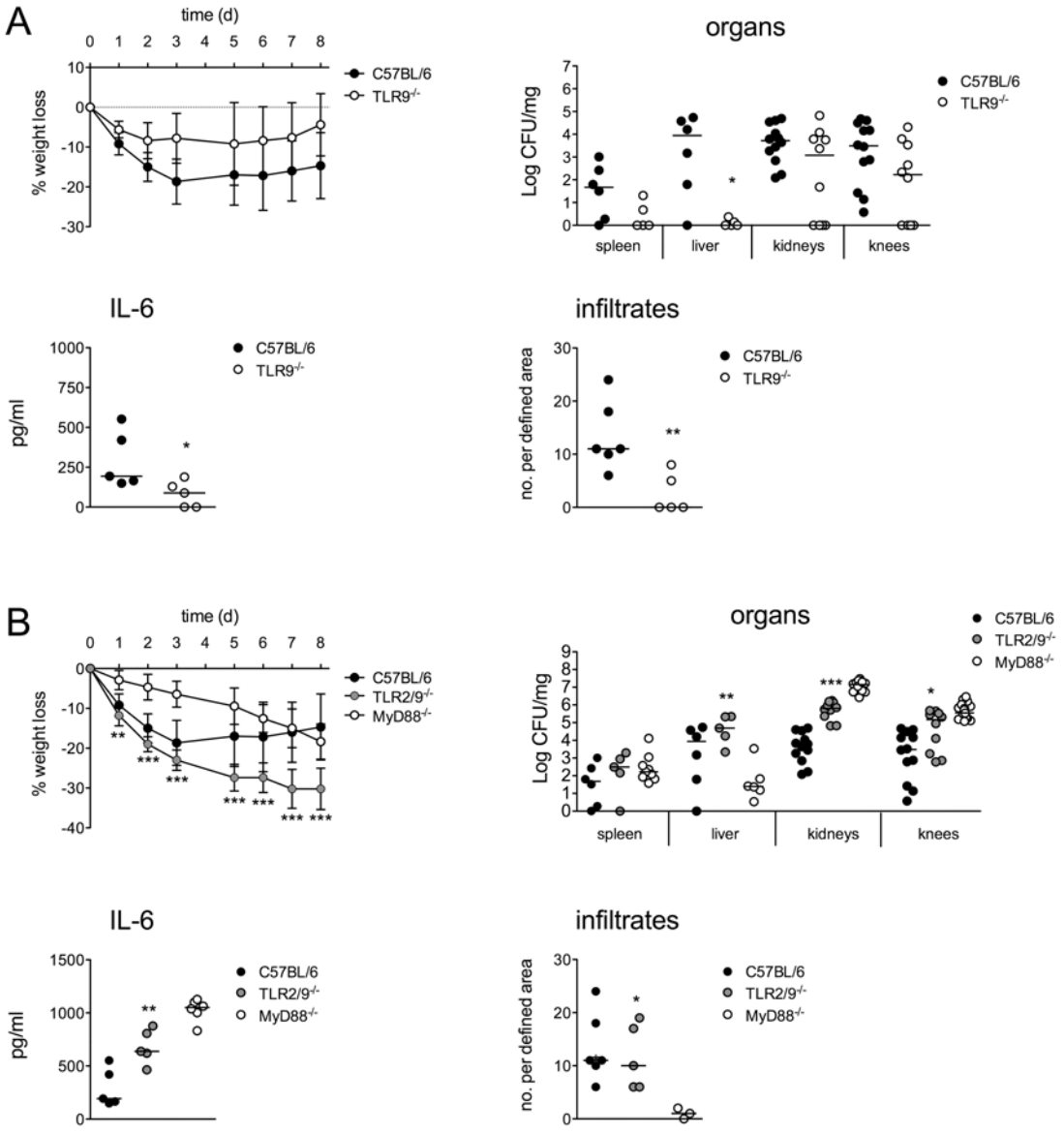

Figure 2. Concurrent signaling by TLR2 and TLR9 is required for virulence of *S. aureus*. (A) C57BL/6 (black) and TLR9$^{-/-}$ mice (white) and (B) C57BL/6 (black), TLR2/9$^{-/-}$ (gray), and MyD88$^{-/-}$ mice (white) were infected with 1 x 10^7 CFU of SA113 wt. Weight loss of mice was measured during infection and bacterial load in spleen, liver, both kidneys, and both knees, IL-6 amounts in plasma, and number of infiltrates in the liver was determined on day 8. Significant differences between (A) C57BL/6 and TLR9$^{-/-}$ mice and (B) TLR2/9$^{-/-}$ vs. MyD88$^{-/-}$ mice are indicated as follows: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$.

Infected TLR2/9$^{-/-}$ mice had a more severe weight loss than C57BL/6 and MyD88$^{-/-}$ mice (Figure 2B). TLR2/9$^{-/-}$ mice also had higher bacterial load in liver, kidneys, and knees, more IL-6 in plasma but similar numbers of infiltrates compared to C57BL/6 mice (Figure 2B).

Compared to MyD88$^{-/-}$ mice, TLR2/9$^{-/-}$ mice had more bacteria in the liver, but less in kidneys and knees; IL-6 in plasma was lower, whereas infiltrates were more numerous in TLR2/9$^{-/-}$ mice (Figure 2B). Interestingly, the phenotype of TLR2/9$^{-/-}$ mice strikingly resembled the TLR2$^{-/-}$ phenotype (see Part 1, Figure 3). Taken together, our data suggest that TLR2 is more important than TLR9, and TLR2/9$^{-/-}$ mice are not as susceptible to *S. aureus* as MyD88$^{-/-}$ mice.

Role of MyD88-independent receptors recognizing Peptidoglycan motifs

To assess the contribution of NOD2 to the release of inflammatory mediators, we infected NOD2$^{-/-}$ macrophages with *S. aureus* SA113 wt. NOD2$^{-/-}$ macrophages showed diminished ability to release TNF, IL-6, IL-10, and IL-1β but not MIP-2 (Figure 3A) indicating that NOD2 is a crucial receptor to establish a strong inflammatory immune response after *S. aureus* infection.

After infection of NOD2$^{-/-}$ mice with *S. aureus* SA113 wt, weight loss of NOD2$^{-/-}$ mice was comparable to C57BL/6 mice during infection (Figure 3B). Bacterial load was lower in liver but not in spleen, kidneys, and knees of NOD2$^{-/-}$ than of C57BL/6 mice (Figure 3B). In the absence of NOD2-signaling, IL-6 in plasma was lower and infiltrates were less numerous in the liver (Figure 3B). Similar to the results of TLR9$^{-/-}$ mice, NOD2 does

not contribute to weight loss and bacterial killing in most organs, but is necessary for recruitment of inflammatory cells and cytokines.

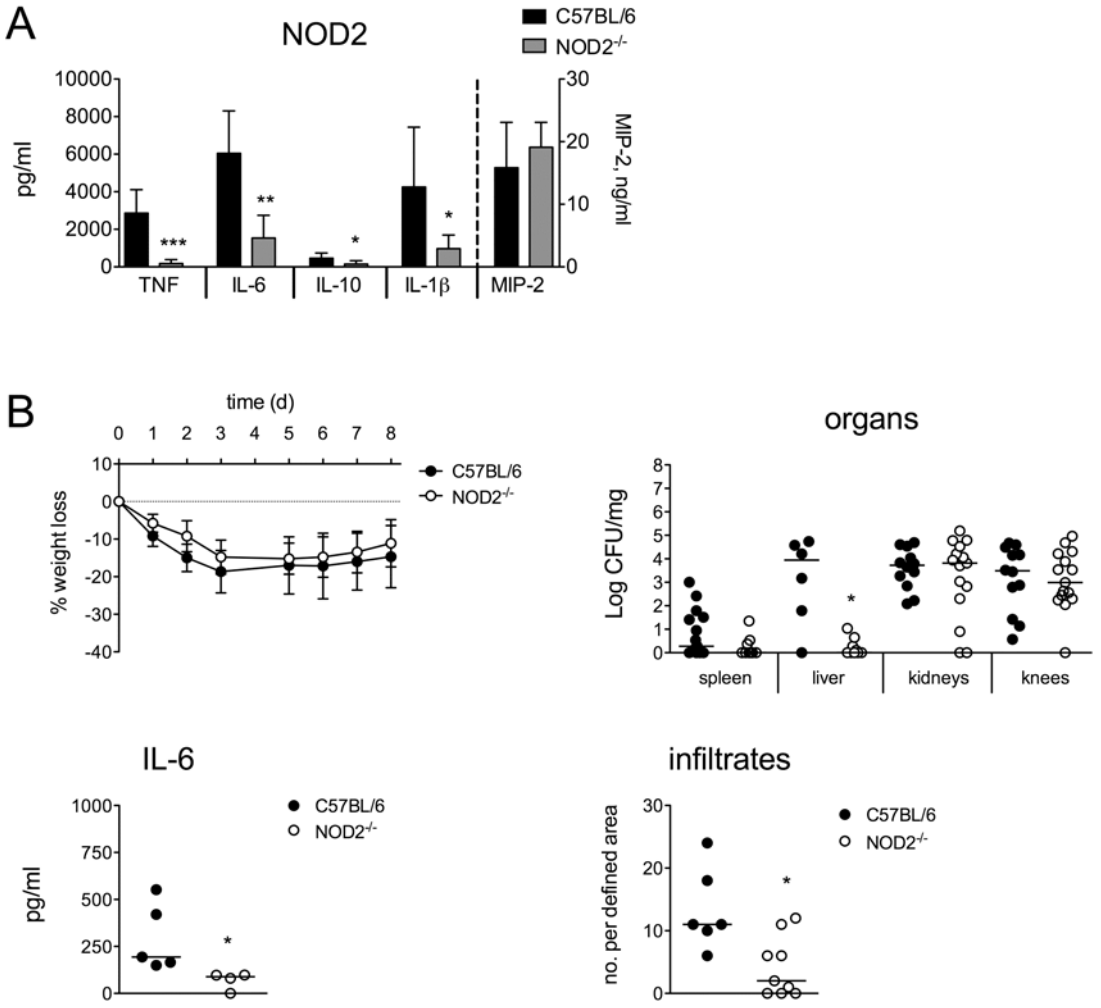

Figure 3. Signaling by NOD2 is required for virulence of *S. aureus*. (A) Peritoneal macrophages from C57BL/6 (black) and NOD2$^{-/-}$ (gray) were infected with SA113 wt at MOI of 10. TNF, IL-6, IL-10, IL-1β, and MIP-2 released into the supernatants were analyzed by ELISA after 18h. Significant differences between groups are indicated as follows: *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$. (B) C57BL/6 (black) and NOD2$^{-/-}$ mice (white) were infected with 1 x 10^7 CFU of SA113 wt. Weight loss of mice was measured during infection and bacterial load in spleen, liver, both kidneys, and both knees, IL-6 amounts in plasma, and number of infiltrates in

the liver was determined on day 8. Significant differences between groups are indicated as follows: *, $p < 0.05$.

Discussion

Distinct PRRs can cooperate to detect different ligands with converging signaling pathways. TLRs and IL-1R contain an intracellular TIR-domain to which MyD88 is recruited (except TLR3) for signal transduction via NF-κB. Activation of more than one MyD88-dependent receptor might have an impact on the expression of many immune and inflammatory genes. In *S. aureus* infection models, the role of TLR2 and MyD88 has been established (1, 2, 6, 14-16), but the function of other TIR-domain receptors contributing to the phenotype of MyD88$^{-/-}$ mice are not clearly understood. Additionally, the use of different strains of *S. aureus* complicates the interpretation of the results.

In the present part, we have analyzed the contribution of IL-1R, TLR9, and NOD2 in sensing of staphylococcal components and in inducing a host response after infection of macrophages and mice. We demonstrate that cytokine induction in macrophages is mediated by TLR9 and NOD2 but not by IL-1R. The phenotype of the MyD88$^{-/-}$ mice after *S. aureus* infection is partially dependent on the concurrent signaling of TLR2 and TLR9, but other receptors signal in addition through MyD88 to initiate cytokine responses in vitro. Surprisingly, in systemic infections, the importance of TLR9, TLR2/9, and NOD2 in defense against *S. aureus* is less evident.

The inflammatory cytokines like TNF, IL-6, IL-1β, the anti-inflammatory cytokine IL-10, and chemokine MIP-2 are critical mediators in systemic infections (17). They are induced after infection of macrophages with viable *S. aureus* in a MyD88-dependent fashion (1). We found however no influence of IL-1R-signaling upon the production of

these mediators in macrophages. In line with our observation, heat-killed *S. aureus* induced TNF in macrophages without altering the cytokine production in the presence of an IL-1R agonist (10). In subcutaneous infection, IL-1R$^{-/-}$ mice showed similar levels of IL-1β, MIP-2, and KC after 24h (6). In contrast, IL-1R protected the host from uncontrolled growth of *S. aureus* in organs preventing the development of severe sepsis and arthritis after systemic infections (18).

We demonstrate that TLR9 was required for optimal induction of all cytokines except MIP-2. This finding contrasts with a recent study showing that heat-killed *S. aureus* stimulates macrophage-cytokines independent of TLR9 (10). This discrepancy might be due to the fact that heat-killed *S. aureus* present altered or other PAMPs to PRRs than viable bacteria. In addition, viable *S. aureus* need first to be engulfed and degraded to activate PRRs in the phagosome, whereas heat-killed bacteria also activate surface-expressed PRRs. However, in line with our data, the TLR9-effect upon the inflammatory response was also evident by the reduced plasma IL-6 level and low number of infiltrates in TLR9$^{-/-}$ mice after systemic infection. The data suggest that DNA activation of TLR9 provides strong signals for acute phase response and leukocyte recruitment to mount a robust immune response against *S. aureus*. Surprisingly, the reduced inflammatory response in TLR9$^{-/-}$ mice did not result in impaired killing of *S. aureus* in systemic infection (this study), which is in agreement with data from a corneal infection model (16).

TLR2 and TLR9 activate cytokine responses through the central adapter MyD88, which is essential for production of proinflammatory cytokines (1, 2). Reduction in cytokine amounts in TLR2/9$^{-/-}$ macrophages give evidence that recognition of Lpp and DNA from

S. aureus trigger MyD88-dependent signaling pathways that partially explain the phenotype of MyD88$^{-/-}$ mice. However, TLR2/9$^{-/-}$ macrophages did not fail to produce cytokines and mice were not as susceptible as MyD88$^{-/-}$ mice suggesting that beside TLR2 and TLR9 other unknown MyD88-dependent receptors are involved in vitro and in vivo. One candidate is TLR7, which was not yet investigated in *S. aureus* infections. However, in vitro experiments showed the importance of bacterial RNA recognition (5, 19). Additionally, studies with multiple knock-out mice were not yet done.

NOD2 as an intracellular receptor for detection of MDP from several bacteria is also involved in the recognition of staphylococcal PGN or *S. aureus* (7-10). In agreement with reduced IL-6 levels in BMM (9), we found that macrophages infected with viable *S. aureus* were highly impaired in the production of proinflammatory and anti-inflammatory cytokines suggesting that intracellular NOD2 recognizes *S. aureus* fragments in the cytoplasm. To date we know that staphylococcal hemolysins and ATP facilitate intracellular delivery of MDP to cytosolic NOD2 (20) but the exact pathway of MDP delivery remains elusive. Furthermore, reduced amounts of IL-1β in *S. aureus*-infected NOD2$^{-/-}$ macrophages suggested that processing of pro-IL-1β to active IL-1β was impeded. Indeed, a recent study showed that MDP-induced caspase-1 activation via NLrp3 resulted in processing of IL-1β (20). In previous studies using subcutaneous and peritoneal *S. aureus* infection, NOD2$^{-/-}$ mice were more susceptible, which was associated with higher bacterial load (8, 9) and lower inflammatory responses (9). Since both models are dependent on the attraction of cells, an impaired inflammatory response might influence survival of *S. aureus* in the absence of NOD2. In systemic infection, we found NOD2 required for the IL-6 response and recruitment of inflammatory cells,

although it did not affect the outcome. The lower bacterial load in the liver of NOD2-/- mice might be explained by reduced leukocyte infiltrates that allowed immune cells better accessibility to kill *S. aureus* (1).

In conclusion, *S. aureus* present a wide variety of PAMPs that phagocytes recognize by PRRs. No single receptor, as shown for TLR2, TLR9, and NOD2, is the sole mediator of a protective immune response, whereas signaling by the MyD88-adaptor is essential. Future studies will help to understand synergism and crosstalk of different PRRs and provide a new basis for identification of new therapeutic targets.

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4 Perspectives

We undertook our studies on *S. aureus* Lpp to understand their pathogenetic role in staphylococcal infection and to uncover their potential use as therapeutic target.

More and more variants of *S. aureus* have developed resistance against available antibiotics (78). MRSA is the most common cause of nosocomial infections; community-associated MRSA strains can spread rapidly among healthy individuals and have now been transmitted in hospitals (79, 80). The exposure of community-associated MRSA to nosocomial strains may lead to untreatable *S. aureus* infections due to horizontal gene transfer and antibiotic pressure, which will encourage the emergence of multiple resistances (78, 80). Therefore, the major task is to understand the interaction of *S. aureus* and the host to develop new preventive and therapeutic strategies including antibiotics and vaccines. Furthermore, the identification of new therapeutical targets is indispensible.

Colonization is a complex process that requires nutrient acquisition, adherence to the host tissue, and protection against host defenses. Since colonization is a risk factor for systemic infection (2, 3), reducing the fitness of *S. aureus* by therapeutic agents might prevent colonization and more importantly invasion. As shown in part 1, Lpp maturation enhances the fitness of *S. aureus* by enhancing iron acquisition. Blocking the Lgt enzyme leads to a stronger release of non-mature Lpp and an impaired iron uptake of *S. aureus* (58). Indeed, we were able to show that the intrabacterial iron content has dramatic consequences for *S. aureus*; survival and growth of iron-restricted *S. aureus* were significantly reduced causing a less severe infection. Our data are supported by another

study showing that *S. aureus* deficient in the Lpp-containing ABC-transporter (Isd) display an attenuated phenotype during infection (81). Therefore, most likely the acquisition of iron is also required in the nasopharynx for successful colonization and infection. After successful colonization or adhesion, *S. aureus* can invade to gain access to the large reservoir of substrates within the host. We demonstrated that maturation of Lpp enhances the invasive capacity of *S. aureus*. This suggests that blocking the maturation of Lpp might also prevent the crossing of physical barriers. Further experiments will elucidate the need of Lpp maturation in *S. aureus* for invasion and uptake of other nutrients such as amino acids and metals in competition with commensals and the host.

Besides nutrient acquisition, Lpp allow the detection of *S. aureus* by TLR2, which induces leukocyte recruitment to the local site of infection. A recent study showed that a neutrophil-rich environment favors staphylococcal survival in wound infections (82) supporting that a too strong immune response is harmful for the host. This raises the question whether preventing Lpp-TLR2-signaling on the host side would be a potential approach in therapy. However, deficiencies in TLR2 and IL-17 facilitate the colonization of *S. aureus*, which might be due to impaired induction of neutrophil chemoattractants and β-defensins in epithelial cells (60, 83). From this perspective, blocking TLR2 might be the wrong approach. In contrast, inactivation of the Lgt enzyme would reduce, but not abolish TLR2-signaling due to residual stimulation by LTA and PGN and allow maintaining a well-balanced recruitment of cells to protect the mucosa.

In contrast to local infection, TLR2 blockage might be a therapeutic option in systemic infection. *S. aureus* activates several receptors including TLR2, TLR9, and NOD2 for signaling through NF-κB. The activation of NF-κB has a central role in the generation and expansion of the inflammatory response and the development of organ dysfunction (84). In addition to the exogenous stimuli, endogenous DAMPs such as alarmins (85) are released during sepsis (86). As a consequence of the overstimulation of immune cells, beneficial immune responses convert into excessive, damaging inflammation (86). Septic shock induced by bacterial lipopeptides was found to be dependent on TLR2 (87) while toxic effects of CpG-DNA are mediated by TLR9 (35). Therefore, targeting TLRs is promising in the treatment of sepsis. This assumption is sustained by two TLR4-antagonists for treatment of severe Gram-negative sepsis (TAK-242 and Eritoran, clinical Phase III) (27), which bind to TLR4 but do not induce signaling. Similarly, the synthesis of TLR2 antagonists preventing overstimulation of the host might be beneficial for patients with *S. aureus* sepsis.

The type of primary T cell response and the proportions of IFN-γ producing T_H1 cells and IL-17-producing T_H17 cells respectively may also determine the outcome of *S. aureus* infection. We observed an Lpp-TLR2-dependent recruitment of myeloid cells into the liver. However, in this organ a moderate immune response is required for maximal *S. aureus* killing, since large numbers of abscesses and infiltrates hamper bacterial killing. We further demonstrated a TLR2-MyD88-dependent IL-17 release from infected spleen cells during infection. Taking into account that IL-17 attracts granulocytes (55, 83) and that enhanced neutrophil recruitment improves staphylococcal survival in infected

wounds (82), it remains to be investigated whether a modulation of T_H17 activity is beneficial for the host.

Another approach in *S. aureus* treatment is the induction of memory responses. Whether Lpp-TLR2-signaling after *S. aureus* infection promotes differentiation toward memory immune cells is an open question. Recent data show that injection of staphylococcal Lpp is protective against challenge with *S. aureus* in a colonization and invasion model (Gordon Conference, 2009 and ISSSI, 2006). It is noteworthy that on the one hand, Lpp are able to stimulate TLR2 in the phagosome and on the other hand, Lpp-derived peptides can be presented on MHC molecules. Both signals result in a more efficient presentation of antigens to $CD4^+$ T cells. Whether DC-dependent instruction of T cells is helpful for B cells and which type of antibodies is protective is focus of future studies. Furthermore, the exact mechanism of antibody-mediated protection has to be resolved, since Lpp are anchored in the membrane and antibodies are not able to cross the bacterial cell wall.

The protective effect of Lpp as described above was mediated by injection of selected Lpp. Whether other Lpp confer better protection remains to be elucidated. To understand the role of individual Lpp, it is important to determine what Lpp are expressed during infections. A group investigated the mRNA expression of Lpp under normal growth conditions in vitro (88). The next step is to change the growth conditions to predict possible functions of Lpp and to characterize their expression in infection. 2D-gel electrophoresis would be an ideal tool to analyze individual Lpp of *S. aureus*. The Lpp expressed under in vivo conditions will be the most promising Lpp to combine in a vaccine.

Our studies demonstrated the contribution of Lpp to the pathogenesis of staphylococcal diseases and will greatly assist future strategies for prophylactic or therapeutic intervention in *S. aureus* infection.

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7 Curriculum vitae

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