Innate immune recognition of *Staphylococcus aureus* cell wall components: Structural and functional requirements

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List of publications

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- C. Rockel, **S. Sigel**, C. Draing, S. Deininger, O. Dehus, A. Ulmer, F. Götz, T. Hartung and S. von Aulock: Lipoteichoic acid from *Staphylococcus aureus* wild type and diagylglycerol transferase deletion mutant possess comparable immunostimulatory activity. *in preparation*

Abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
CD	cluster of differentiation
CpG motifs	repetitive cytosine-guanosine dinucleotide sequences
DNA	desoxyribonucleic acid
EDTA	etylendiamintetraacetat
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FPLC	fast protein liquid chromatography
FCS	fetal calf serum
GC-MS	gas chromatography-mass spectroscopy
G-CSF	granulocyte colony-stimulating factor
HBSS	Hanks balanced salt solution
HDL	high density lipoprotein
HEK	human embryonic kidney cells
HIC	hydrophobic interaction chromatography
IC_{50}	half maximal inhibitory concentration
IFNγ	interferon γ
IgG	immunoglobulin G
kDA	kilodalton
IL	interleukin
LAL	Limulus amoebocyte lysate test
LBP	lipopolysaccharide-binding protein
LDL	low density lipoprotein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MALDI	matrix-assisted laser desorption/ionization
MBL	mannan-binding lectin
MRSA	methicillin-resistant *Staphylococcus aureus*
NF-κB	nuclear factor κB
NOD	nucleotide-binding oligomerization domain
NMR	nuclear magnetic resonance
OD	optical density
PAGE	polyacrylamid gel electrophoresis

$Pam_2Cys\text{-}SK_4$	bispalmitoyloxy-propyl-Cys-Ser-Lys_4-OH
$Pam_3Cys\text{-}SK_4$	bispalmitoyloxy-propyl-N-palmitoyl-Cys-Ser-Lys_4-OH
PAMP	pathogen-associated molecular pattern
PBMC	human peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PGN	peptidoglycan
PRR	pattern recognition receptor
RT	room temperature
SA 113 Δlgt	*Staphylococcus aureus* lipoprotein diacylglycerol transferase deletion mutant
SA 113 wt	*Staphylococcus aureus* wild type
SDS	sodium dodecyl sulfat
SEB	staphylococcal enterotoxin B
SEM	standard error of mean
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumour necrosis factor
Tween20	polyoxyethylensorbitan monolaurate
VLDL	very low density lipoprotein
wt	wild type

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1 Introduction

1.1 Innate and adaptive immunity

When microorganisms overcome the chemical and physical barriers and invade the host organism an immunologic response is recruited to recognize and eliminate infective pathogens. Thereby the innate immune system is the primary line of host defense and relies on soluble components and effector cells that control an infection during the first days. White blood cells, the major components of innate immunity, characterized by their phagocytic capacity, sense, engulf and degrade pathogenic microorganisms through pattern recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMP) on the surface of microbes (54, 61). This results in the production and secretion of inflammatory mediators inducing an acute phase reaction in the liver and thus initiating adaptive immunity. In contrast to the conserved germline encoded recognition molecules of the innate immunity, adaptive immune recognition is mediated by highly diverse antigen receptors on B and T lymphocytes characterized by specificity and memory, and results in a long-lasting protection against secondary infections (33).

1.2 Innate immune recognition of *Staphylococcus aureus*

Staphylococcus aureus, a major human pathogen responsible for a variety of different diseases, often colonizes hosts asymptomatically and lives as a commensal in the human nose. The symptoms caused by *Staphylococcus aureus*, when the skin or mucosal barrier of the body is compromised, differ with the location of the infection. Although restricted most commonly on the skin, *Staphylococcus aureus* bacteria may involve any tissue of the body and progress to invasive systemic infections leading to sepsis and death (136). The increasing frequency of *Staphylococcus aureus*-mediated sepsis during the last decades likely results from the spread of multidrug-resistant bacterial strains like vancomycin intermediate resistant *Staphylococcus aureus* (VISA) (80) or methicillin-resistant *Staphylococcus aureus* (MRSA) which is resistant to *β*-lactam antibiotics (91).

Staphylococcus aureus entering the bloodstream initially provokes an acute inflammatory host response, resulting in activation of the complement system and leukocyte recruitment to the site of infection. Phagocytic cells, such as macrophages and neutrophils play an important role in this first line defense, as they have to efficiently

differentiate between “infectious nonself” and “non-infectious self” (54, 127). Thereby it has been shown that microbial PAMPs can be recognized by either extracellular or intracellular innate immune receptors. Whereas Toll-like receptors, expressed on the surface or within distinct subcellular structures of monocytes, macrophages and dendritic cells are instrumental in sensing extracellular PAMPs or those that are internalized into specialized compartments (1, 139), intracellular Nod-like receptors induce innate immune responses through cytosolic recognition of microbial components (56, 134). Although *Staphylococcus aureus* has traditionally been regarded as an extracellular recognized pathogen (59, 79, 106), there is increasing evidence that internalization of the bacteria and thus presentation of bacterial degradation products to immune receptors recruited to the phagosome essentially contributes to *Staphylococcus aureus* mediated immune response (58, 66, 127).

1.3 Host receptors involved in *Staphylococcus aureus* recognition

To recognize microorganisms entering the host, the innate immune system comprises a variety of highly conserved PRRs. These receptors are expressed on the surface or within the cytosol of the immune cells and are able to detect different microbial structures. The motifs sensed by the PRRs are usually essential for the invading pathogens but are absent in the host organism (96).

1.3.1 Toll-like receptors and co-receptors

To date ten Toll-like receptors (TLR) mediating the recognition of diverse classes of pathogens, have been identified in humans (15, 116). Moreover, TLR11, TLR12, and TLR13 recently have been discovered in mice but so far lack human counterparts (73). All TLRs are type-1 transmembrane receptors characterized by an extracellular leucine-rich repeat domain involved in ligand recognition and an intracellular Toll/IL-1 receptor-like (TIR) domain (98). TLRs are expressed on the surface or within endosomes of antigen-presenting cells like monocytes, macrophages, dendritic cells and neutrophils (55) and, like other pattern recognition receptors sense distinct PAMPs unique to microorganisms. Following ligand binding, TLRs recruit and activate a variety of intracellular adapter molecules and kinases, which finally results in the induction of NF-κB dependent gene expression of proinflammatory mediators (103).

As for other Gram-positive bacteria, innate immune activation by *Staphylococcus aureus* is strongly dependent on TLR2 (53, 131), as demonstrated by the higher mortality and abrogated cytokine induction of TLR2 deficient mice after *Staphylococcus aureus* infection (131). In contrast to the high specificity of other TLRs, TLR2 recognizes various cell wall components of *Staphylococcus aureus*, which is attributed to its ability to form heterodimers with either TLR1 or TLR6. Thereby TLR2 in association with TLR1 is essential for recognizing bacterial lipoproteins (62), whereas binding of lipoteichoic acid requires formation of TLR2/TLR6 receptor complexes (118).

CD14 is a key co-receptor expressed on the surface of lymphocytes, monocytes and macrophages that has previously been described to interact with different *Staphylococcus aureus* cell wall components including peptidoglycan (27, 41) and lipoteichoic acid (16, 118). However, due to reported lipoteichoic acid-contaminations within commercial peptidoglycan preparations (137), the ability of peptidoglycan to interact with CD14 has been called into question. Although the expression of CD14 synergistically increases TLR2-mediated NF-κB activation (150), there was no higher susceptibility to *Staphylococcus aureus* infection observed in CD14 deficient mice (47) indicating that this co-receptor is not crucial for staphylococcal recognition.

1.3.2 Scavenger receptors

Scavenger (SR) receptors represent a broad family of cell surface glycoproteins that, according to their proposed tertiary structure, can be divided into six subgroups (SR-A – SR-F) (72). During bacterial infection, SRs contribute to innate immune response through binding of microorganisms and their products, which subsequently causes their internalization. (112). Among all SRs, the group of SR-A (2) as well as CD36 (53, 127), a member of B class SR, have been described to play crucial roles in immune recognition of *Staphylococcus aureus* through internalization of the whole bacteria as well their cell wall component lipoteichoic acid and thereby mediating an inflammatory response through presentation of staphylococcal compounds to TLRs recruited to the phagosome. Mice deficient in either SR-A or CD36 are considerably more susceptible to *Staphylococcus aureus* infections, resulting from the impaired ability of these mice to clear bacterial burdens (2, 53, 127). Interestingly, expression of CD36 alone in TLR2 deficient cells does not induce lipoteichoic acid-mediated NF-κB activation, indicating that CD36 needs TLR2 as a co-receptor to transduce the signal and initiate cellular response (53).

1.3.3 Nucleotide-binding oligomerization domain protein

In contrast to TLRs, located in either the plasma membrane or in endosomal membranes (107), the intracellular nucleotide-binding oligomerization domain proteins (Nod) 1 and 2 sense bacterial peptidoglycan fragments in the cytosol (65). Whereas Nod1 is activated by peptidoglycan motives containing the amino acid meso-diaminopimelic acid (meso-DAP) produced by mostly Gram-negative bacteria (12), Nod2 recognizes muramyl dipeptides (MDP), a conserved structure of virtually all types of peptidoglycan, and thus senses both Gram-negative and Gram-positive bacteria (37, 57). Like TLRs, Nod1 and Nod2 trigger the activation of NF-κB, resulting in the expression of genes encoding cytokines, chemokines and costimulatory molecules necessary for the defense response (33). In former studies, the evidence that Nod1 and Nod2 contribute to the immune response was restricted to models of gastrointestinal infection, where pathogens interact with cells of the gastrointestinal tract that are largely devoid of TLRs (71, 141). However, recently the general participation of Nod2 in *Staphylococcus aureus* mediated immune response was confirmed by observing a higher susceptibility of Nod2 deficient mice to intraperitoneal *Staphylococcus aureus* challenge (21).

1.4 Serum components involved in *Staphylococcus aureus* recognition

Beside PRRs restricted to antigen-presenting cells, multiple soluble serum components are involved in the innate immune recognition. These serum proteins bind to invading microorganisms and contribute to bacterial clearance through acting as a bridge between bacterial targets and signaling proteins on the surface of the immune cells.

1.4.1 Lipopolysaccharide-binding protein

Lipopolysaccharide-binding protein (LBP), an acute phase protein synthesized in the liver, effectively catalyzes the immune recognition of Gram-negative bacteria through opsonization of intact bacterial cells as wall as aggregates of lipopolysaccharide (LPS) released from the bacterial cell wall during infection (118, 119). Although LBP was found to interact with lipoteichoic acid from the Gram-positive cell wall as well, the underlying mechanism of this interaction was found to be different. Whereas binding of LBP to LPS mediates the transport of LPS to the cell surface of monocytes/macrophages and thereby enhances the sensitivity of cell activation up to

1000-fold *in vitro* (92, 147), formation of lipoteichoic acid-LBP complexes leads to an attenuation of LTA-induced cytokine secretion (102, 118). As LBP deficient mice did not show an increased susceptibility to lipoteichoic acid-induced lung inflammation (70), it seems possible that increased serum levels of LBP protect the host from cytokine-mediated systemic complications during Gram-positive infection.

1.4.2 Serum lipoproteins

Regulation of lipid metabolism during bacterial infection is thought to be part of the host defense, as lipoproteins have been described to considerably modulate immune recognition of bacterial endotoxins like LPS or lipoteichoic acid (40, 93, 135). Among the major serum lipoprotein classes the high-density lipoprotein (HDL) was reported to have the highest affinity for *Staphylococcus aureus* lipoteichoic acid, followed by low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) (77). Interaction of the lipoteichoic acid with serum lipoproteins diminishes its potency to activate human macrophages to release proinflammatory mediators and thus protects the host against lipoteichoic acid-associated toxicity in a similar manner to LBP.

1.4.3 Complement and collectins

The complement system is constituted by a complex cascade of serum proteins and glycoproteins and serves as an important element of innate immune defense against bacterial pathogens (34). It is activated by three biochemical pathways, the classical, the alternative and the lectin pathway (60) that all result in opsonization of microorganisms and thus clearance of the pathogens. Ficolins and mannose-binding lectin (MBL), a member of the collectin family, are able to activate the complement cascade via the lectin pathway (17, 130) through binding of bacterial carbohydrates and thereby initiating lectin-pathway dependent C4 turnover. Among ficolins, L-ficolin was reported in *in vitro* studies (84) to specifically bind to lipoteichoic acid of the cell wall of *Staphylococcus aureus* and thereby participates in innate immune response by triggering the lectin pathway of complement activation. The essential contribution of MBL to *Staphylococcus aureus* induced immune activation has been demonstrated not only by the higher susceptibility of MBL deficient mice to *Staphylococcus aureus* infections (121), resulting from the opsonization capacity of MBL, but also by the direct interaction of MBL with the TLR2/6 receptor complex, leading to an up-regulation of the TLR2-mediated host response (58).

1.5 Bacterial initiators of innate immune response

The cell envelope of Gram-negative bacteria is composed of a thin layer of peptidoglycan enclosed by an outer membrane where the LPS is incorporated. During bacterial infection, LPS is released from the cell wall and thereby its toxic moiety Lipid A is exposed to immune cells and evokes an inflammatory response (139). In contrast, the Gram-positive bacterial cell wall consists of a thick peptidoglycan layer that beside its crosslinking peptides contains teichoic acids, lipoteichoic acids and lipoproteins. Except for teichoic acids (86) these cell wall components have been described to contribute to the pathogenicity of *Staphylococcus aureus* (26, 46, 101).

1.5.1 Lipoteichoic acid

Staphylococcus aureus lipoteichoic acid (LTA), an amphiphilic glycolipid polymer, consists of alternating glycerol-phosphate repeating units substituted with *N*-acetyl-*D*-glucosamine and *D*-alanine (99, 100). The hydrophilic polyglycerol phosphate chain is linked to the plasma membrane via a diacylglycerolipid anchor, but is long enough to protrude the peptidoglycan layer. As for other Gram-positive bacteria, LTA from *Staphylococcus aureus* has been characterized as a major immunostimulatory molecule that is able to induce a broad spectrum of cytokines and chemokines from human whole blood including TNF, IL-1β, IL-6 IL-8 and IL-10 (19, 49) but in contrast to purified LPS, the main stimulus of Gram-negative bacteria, purified *Staphylococcus aureus* LTA selectively fails to induce IL-12 and subsequent release of IFNγ in human whole blood (8).

The immunostimulatory capacity of *Staphylococcus aureus* LTA was recently called into question when lipoprotein contaminations within LTA preparations and not the LTA itself were reported to represent the main immunobiologically active compound of *Staphylococcus aureus* (46). However, LTA preparations of a *Staphylococcus aureus* mutant strain lacking palmitate-labeled lipoproteins (126) were able to induce cytokine release from human blood cells in a potency comparable to LTA from the respective wild type strain (143).

1.5.2 Peptidoglycan

Peptidoglycan (PGN), the most conserved structure of the Gram-positive envelope is constituted of repeating *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine disaccharides (104). The carboxyl group of *N*-acetylmuramic acid is linked to a peptide subunit consisting of four or five alternating L- and D-amino acids which diverge among different Gram-positive bacteria. The tetrapeptide of *Staphylococcus aureus* is composed of L-alanine, D-glutamine, L-lysine and D-alanine and is crosslinked with tetrapeptides of the neighbouring glycan strands via pentaglycin bridges, thereby generating a three-dimensional network that encloses the bacterial cell (104). Although PGN has been demonstrated to be important in innate immune activation and its recognition has been extensively examined, there are still conflicting data on whether PGN-fragments can be recognized by extracellular TLR2 (3, 26, 59) or have to be taken up to induce intracellular signaling via cytosolic Nod1 or Nod2 (37, 57).

1.5.3 Lipoproteins

Lipoproteins are localized in the bacterial cytoplasmic membrane and are common constituents of both Gram-negative and Gram-positive bacteria. Inflammatory responses induced by lipoproteins from Gram-negative bacteria have been described to be mediated via TLR2 (30, 51, 76). In Gram-positive bacteria like *Staphylococcus aureus* some lipoproteins have been identified in the last years (23, 48, 146), and recently the contribution of staphylococcal lipoproteins to immune response has been confirmed as *Staphylococcus aureus* mutants lacking lipoproteins were reported to escape immune recognition and cause lethal infections *in vivo* (10). In comparison to triacetylated lipoproteins of Gram-negative bacteria, Gram-positive bacterial lipoproteins contain conserved diacetylated cysteins at their N-terminus and are postulated to be recognized by a TLR2/TLR6 heterodimer (62). The stimulatory activity of bacterial lipoproteins resides in the acetylated amino terminus and can be mimicked by various synthetic derivatives like $Pam_2Cys\text{-}SK_4$ or $Pam_3Cys\text{-}SK_4$ which have been demonstrated to activate NF-κB in a TLR2-dependent manner (9, 79) Additionally it was reported that LPS preparations, that exhibited partial TLR2 dependence, lost their TLR2 agonistic activity after removing contaminating lipoproteins by re-purification, indicating the importance of TLR2 in the recognition of lipoproteins (52, 113).

2 Aims of the study

Staphylococcus aureus is an important human pathogen that causes diverse diseases ranging from innocuous skin infections to often fatal forms of sepsis. During the initial phase of infection specific molecular patterns of *Staphylococcus aureus* are recognized by innate immune receptors of the host resulting in the induction of an acute inflammatory response. Among these patterns, staphylococcal LTA, a component of the Gram-positive cell wall, represents a major immunostimulatory molecule that triggers immune activation in a TLR2/6 dependent manner. However, despite the prominent role of LTA in inducing different cytokines, purified LTA selectively fails to initiate the release of IFNγ from human blood cells.

- In the first part of this PhD thesis, immune activation by *Staphylococcus aureus* and its derived PGN was investigated with special regard to the induction of IFNγ.

During staphylococcal infection, LTA naturally anchoring to the bacterial cell membrane can be released into the bloodstream. Despite strong evidence that LTA represents a major immunostimulatory principle of Gram-positive bacteria, isolated LTA has been described to be a weak inducer of inflammatory responses *in vivo*. Soluble serum components were found to interact with isolated LTA thereby altering its immunostimulatory capacity.

- The aim of the second part of this PhD thesis was to identify human serum components that interact with soluble LTA and to characterize their influence on LTA-mediated cytokine secretion.

Recent reports suggested that not LTA but lipoproteins are dominant immunostimulatory structures of *Staphylococcus aureus*. The investigators described LTA of a diacylglycerol transferase deletion mutant (Δlgt) *Staphylococcus aureus* lacking palmitate-labeled lipoproteins to be 100-fold less potent in inducing innate immune activation compared to the respective wild type strain. Consequently, the authors suggested that lipoprotein contaminants were responsible for LTA-mediated immune activation. In contrast, our laboratory demonstrated an equipotent cytokine induction between Δlgt- and wt-LTA, resulting in a controversy concerning the immunostimulatory properties of Δlgt-LTA that is still ongoing.

- In the third part of this PhD thesis we investigated the functional requirements for the recognition of Δlgt- and wt-LTA by human blood cells.

3 Peptidoglycan directly and indirectly contributes to *Staphylococcus aureus*-mediated IFNγ release in human whole blood

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3.1 Abstract

Sepsis caused by Gram-positive and Gram-negative bacteria is equally frequent and is associated with similar symptoms of disease. The entire pattern of cytokines induced during Gram-negative infection can be elicited by purified lipopolysaccharide (LPS). In contrast, lipoteichoic acid (LTA), which represents a major immunostimulatory component of Gram-positive bacteria, is unable to induce the release of IFNγ in human whole blood despite potently inducing other relevant cytokines and chemokines. In the present study we aimed to characterize the mechanism of IFNγ release switched on by invading *Staphylococcus aureus* and to identify the IFNγ-inducing component of these bacteria. As digestion of the peptidoglycan (PGN) network by lysozyme or lysostaphin and inhibition of phagocytosis by cytochalasin D abrogated *S. aureus*-mediated IFNγ secretion, we analyzed the contribution of PGN to IFNγ production following engulfment of the bacteria. Using knock-out mice we demonstrated, that the absence of TLR2 or Nod2, both considered essential PGN receptors, did not affect the IFNγ response to *S. aureus* lysates, suggesting that macromolecular PGN acts as a carrier for IFNγ-inducing cell wall components in mediating bacterial uptake, but is not itself responsible for *S. aureus*-induced IFNγ production. In line, intracellular recognition of

lysozyme-digested *S. aureus* lysates could be restored by transfection. In summary we propose that phagocytosis of *S. aureus* depends on polymeric PGN and is essential for IFNγ induction but that PGN itself is not the main inducer of IFNγ release.

3.2 Introduction

During bacterial infection, the immune system becomes activated following recognition of highly conserved microbial structures by innate immune receptors (1, 56, 61). These conserved structures, known as pathogen-associated molecular patterns (PAMPs) include lipopolysaccharide (LPS), lipoteichoic acid (LTA), mannans, flagellin, peptidoglycan (PGN) and DNA sequences containing unmethylated CpG dinucleotides (61, 97). Like LTA of other Gram-positive bacteria, LTA from *S. aureus* has been characterized as a major immunostimulatory molecule that is able to induce a broad spectrum of cytokines and chemokines in human whole blood including TNF, IL-1β, IL-6, IL-8 and IL-10 (49). However, in contrast to purified LPS, the main immune stimulus of Gram-negative bacteria purified *S. aureus* LTA selectively failed to induce IL-12 and subsequent release of IFNγ in humans (8).

S. aureus is a frequent human pathogen that causes a variety of different diseases, including minor skin infections but also severe infections leading to sepsis and death. As for other Gram-positive bacteria, innate immune activation by *S. aureus* is partially mediated by extracellular Toll-like receptor (TLR) 2 (53, 131) that recognizes staphylococcal cell wall components, such as LTA (120). While TLRs recognize bacterial ligands at the cell surface or within endosomes (1), additional receptors induce innate immune responses by cytosolic recognition of microbial components (56, 134). The cytosolic nucleotide-binding oligomerization domain (Nod) protein 2 has been proposed to serve as a potential intracellular sensor for *S. aureus* (33) by recognizing muramyl dipeptides (MDP), a conserved structural component of virtually all types of peptidoglycan (37, 57). PGN is an essential cell wall component of both Gram-positive and Gram-negative bacteria (117) and consists of *β*(1-4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid polymers crosslinked by short peptides (24).

Although PGN has been demonstrated to be important in innate immune activation and its recognition has been extensively examined, there are still conflicting data on whether PGN-fragments can be recognized by extracellular TLR2 (3, 26, 59, 67, 145) or have to be taken up to induce intracellular signaling via cytosolic Nod1 or Nod2 (21, 37, 57, 145). A potential pitfall in characterizing the receptor requirements for anti-bacterial

innate immune responses arises from the use of isolated bacterial components and cellular test systems expressing only single receptors, thereby missing the *in vivo* situation resulting from the interplay of different processes and different activated immune receptors. Only few studies in mice have addressed *S. aureus*-mediated cytokine release by using whole bacteria (66) and human data are rare.

In the current study we aimed to investigate *S. aureus*-mediated IFNγ induction in human whole blood. Beside the underlying mechanisms of IFNγ secretion regarding IL-12 and TNF dependency and producing cell types, we investigated whether IFNγ induction by *S. aureus* depends on the phagocytosis and on an intact PGN network. The role of PGN in the immune activation by *S. aureus* leading to IFNγ production was further defined in mice deficient for the known PGN receptors TLR2 and Nod2 and by overriding the phagocytosis step by transfection of human PBMC.

3.3 Material and Methods

3.3.1 Bacterial strains and growth conditions

Escherichia coli (*E. coli*) were grown in Luria-Bertani medium (MP Biomedicals, Heidelberg, Germany) and *S. aureus* (DSM 20233) were cultivated in tryptic soy broth (BD Biosciences, Heidelberg, Germany), both at 37°C, and were harvested after 16 h. For generating a standardized *S. aureus* lysate, cultivated bacteria were extracted with butanol as described previously (99) and after centrifugation at 13.000 g for 40 min at room temperature the aqueous-phase, the butanol-phase and the pellet containing either water-soluble components (WC), amphiphilic components (AC) or insoluble cell fragments (*S.a.*-ICF), respectively, were lyophilized and frozen at -80°C.

For stimulation of blood cells with whole bacteria, harvested *S. aureus* and *E. coli* were adjusted to 10^8 bacteria/ml and UV-inactivated on ice (UV-Stratalinker, La Jolla, CA) with an irradiation energy of 1 kN/cm^2 (3 mWatt/cm^2 x 300 s) for 5 min in a 6-well cell culture plate (Greiner Bio-One, Frickenhausen, Germany). The inactivation was controlled by growth on blood agar plates (Columbia-blood agar, Heipa Diagnostika, Eppelheim, Germany) after 24 h at 37°C and 5% CO_2.

3.3.2 Stimuli and antibodies

S. aureus (DSM 20233) water-soluble, amphiphilic and insoluble fractions were obtained as described above. LTA from *S. aureus* (DSM 20233) was isolated by butanol extraction and hydrophobic interaction chromatography as previously published (99).
Further substances were LPS from *Escherichia coli* O-113 (National Institute for Biological Standards and Controls, Hertfordshire, UK), LPS from *Salmonella enterica* serovar *abortus equi,* Staphylococcal Enterotoxin B, (Sigma, Deisenhofen, Germany), Poly I:C, peptidoglycan and R-837 (Invivogen, San Diego, USA), $Pam_3Cys\text{-}SK_4$ (EMC microcollections, Tübingen, Germany), CpG 2216 (MWG Biotech AG, Ebersberg, Germany) and muramyl dipeptides (MDP, Bachem, Heidelberg, Germany). Neutralizing antibodies against human IL-12 and human TNF were purchased from R&D Systems (Wiesbaden, Germany).

3.3.3 Cytochalasin D treatment and PGN digestion

To inhibit the process of phagocytosis human whole blood was pretreated with 3 µM cytochalasin D (Sigma) for 30 min in siliconized glass tubes (Vacutainer, Biosciences) before stimulation with various immune stimuli over night. The ability of cytochalasin D to inhibit phagocytosis was controlled by stimulation with R-837, MDP, CpG and Poly I:C as well as with LPS and $Pam_3Cys\text{-}SK_4$, respectively.
For PGN digestion, 1 mg *S.a.*-ICF dissolved in 1 ml PBS was dispersed by sonication. Then lysozyme or lysostaphin (lysostaphin from *Staphylococcus staphylolyticus*, and lysozyme from chicken egg white, Sigma) was added and the sample was incubated for 3 h at 37°C on a shaker. After inactivation of the enzymes at 95°C for 5 min, insoluble material was sedimented by centrifugation at 400 x g for 10 min and washed twice with pyrogen-free water. Supernatants and pellets were lyophilized and stored at -80°C.

3.3.4 Transfection of human PBMC with lysozyme-digested S.a.-ICF

Human PBMC of healthy volunteers were prepared with CPT™ Cell Preparation Tubes (BD Biosciences). After centrifugation at 1600 g for 20 min, PBMC were collected and washed at 300 x g for 5 min with RPMI 1640 and 2.5 IU/ml Liquemin (Hoffmann-La Roche, Mannheim, Germany). The cells were plated at a density of 5×10^5/tube in the absence of any serum. 1 µg lysozyme-digested or 1 µg undigested *S.a.*-ICF in 50 µl HBSS (PAA Laboratories GmbH, Pasching, Austria) was incubated with 5 µl DOTAP

(*N*-(2,3-Dioleoyloxy-1-propyl)trimethylammonium methyl sulphate, Sigma) for 30 min at room temperature to allow complex formation, and added drop-wise to the PBMC. Cells were incubated for 22 h at 37°C and 5% CO_2. Then cell-free supernatants were obtained by centrifugation and stored at -80°C until cytokine measurement by ELISA.

3.3.5 Whole blood incubation and neutralization experiments

Heparinized venous blood was obtained from healthy volunteers. Differential blood cell counts were routinely determined with a Pentra 60 apparatus (ABX Diagnostics, Montpellier, France) to exclude acute infections. Blood was diluted fivefold with RPMI 1640 (Lonza, Verviers, Belgium) and incubated in the presence of the different stimuli in polypropylene vials (Eppendorf, Hamburg, Germany) overnight for 22 h at 37°C and 5% CO_2. For the neutralization experiments, human whole blood stimulation was carried out in the presence of αIL-12 and αTNF antibodies [5 µg/ml]. After the incubation, blood cells were resuspended by gently shaking and then centrifuged at 400 x g for 2 min. The cell-free supernatants were stored at -80°C for cytokine determination.

3.3.6 Isolation of murine spleen cells

Nod2-deficient (B6.129S1-*NOD2*^{tm1Flv}/J, Jackson Laboratory, Maine, USA) and TLR2 deficient (kindly provided by Tularik, South San Francisco, CA, USA) mice as well as the corresponding wild-type mice (129Sv/C57BL/6) were bred in the animal facilities at the University of Konstanz. Mice were killed by terminal pentobarbital anaesthesia (Narcoren; Merial, Halbergmoos, Germany). Splenocytes were harvested by pressing spleen tissue through a sterile nylon cell-strainer (100 µm, BD Biosciences) and resuspending cells in 5 ml RPMI 1640. After lysis of the erythrocytes the lavages were washed once by centrifuging at 350 x g for 10 min. The cells were diluted to 1 x 10^7/ml with RPMI 1640 containing 10% FCS (Lonza) and plated on 96-well cell culture plates for stimulation. After 22 h at 37°C and 5% CO_2, supernatants were frozen at -80°C.

3.3.7 Cytokine measurement

Cytokines released by human blood cells were measured using in-house sandwich ELISA based on commercially available pairs of antibodies and standards. Antibody pairs against human IL-1β and IL-6 were purchased from R&D Systems, against human

IL-10 from BD Biosciences and against human TNF and IFNγ from Endogen (Perbio Science, Bonn, Germany). Recombinant standards for IL-1β, IL-6, TNF and IFNγ were obtained from the NIBSC (Herts, UK) and rIL-10 from BD Biosciences. IFNγ and TNF release by murine spleen cells were measured with the DuoSet-kit from R&D Systems. Assays were carried out in flat-bottom, ultrasorbant 96-well plates (MaxiSorp, Nunc, Wiesbaden, Germany). Binding of biotinylated secondary antibody was quantified using streptavidin-conjugated horseradish peroxidase (Biosource, CA) and TMB (Sigma) was used as substrate. The reaction was stopped with 1 M sculpture acid and the absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm. Cytokine levels are given per milliliter.

3.3.8 Statistics

Statistical analysis was performed using the Graph Pad Prism 3 Program (Graph Pad Software, San Diego, USA). Three or more groups of data were compared by repeated-measure analysis of variance (ANOVA) followed by Dunn's post test. For statistical analysis of two groups of data, the Mann Whitney test was used. In the figures *, ** and *** represent *p* values <0.05, <0.01 and <0.001, respectively.

3.4 Results

3.4.1 Comparison of cytokine induction by E. coli and S. aureus as well as respective purified stimuli, LPS and LTA.

Recent studies demonstrated that *S. aureus* LTA, a prominent innate immune stimulus, is unable to induce IL-12 and subsequent IFNγ release from human whole blood, despite a potent induction of other cytokines (49). To demonstrate that the lack of LTA-mediated cytokine induction is exclusive to IFNγ, we compared IL-1β, IL-6, IL-10, TNF and IFNγ release in human whole blood stimulated with UV-inactivated *S. aureus* or an equipotent amount of *S. aureus* LTA. As shown in figure 1A, comparable amounts of IL-1β, IL-6, IL-10 and TNF were induced by both stimuli, while IFNγ secretion was missing following LTA stimulation but was prominent in case of stimulation with whole *S. aureus*. As observed for *S. aureus*, whole blood stimulation with *E. coli* or *E. coli* derived LPS resulted in a potent and comparable induction of all cytokines tested (IL-1β,

IL-6, IL-10, TNF and IFNγ). These results demonstrate that *S. aureus*-mediated IFNγ induction depends on an immune stimulus that is distinct from LTA.

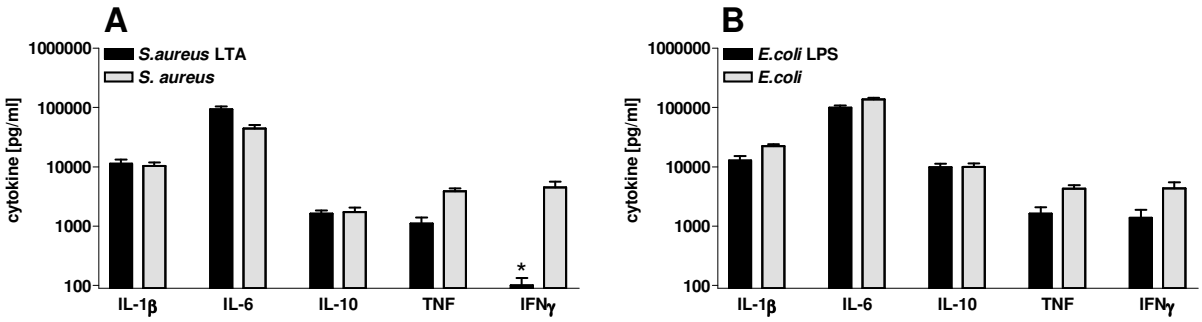

Figure 1: Comparison of *S. aureus*- and *E. coli*-mediated cytokine induction following stimulation with whole bacteria or isolated LTA and LPS. Human whole blood was stimulated with (A) UV-inactivated *S. aureus* [10^7/ml] or *S. aureus* LTA [10 µg/ml] and with (B) UV-inactivated *E. coli* [10^7/ml] or *E. coli* LPS [5 ng/ml]. After incubation for 22 h, released IL-1β, IL-6, IL-10, TNFα and IFNγ was determined from the cell-free supernatants by ELISA. Cytokine release of unstimulated control samples was always below the detection limit of the respective ELISA that ranged from 1 to 15 pg/ml, depending on the cytokine. The data deriving from eight healthy blood donors represent means ± SEM. Significant differences were analyzed by Kruskal-Wallis test followed by Dunn's post test.

3.4.2 S. aureus-mediated IFNγ induction in human whole blood depends on IL-12 and TNF release and is independent of superantigens.

To investigate the mechanism of *S. aureus*-mediated IFNγ production we compared IFNγ release from human whole blood stimulated with LPS, a staphylococcal superantigen (SEB) or *S. aureus* in the presence or absence of neutralizing antibodies against IL-12 and TNF, respectively. For stimulation, we used a standardized *S. aureus* preparation of insoluble cellular fragments (ICF) derived from water/butanol-extracted bacteria that reflected the complete IFNγ-inducing capacity of whole *S. aureus* bacteria (Fig. 2A). As shown in figure 2B, both LPS- and *S.a.*-ICF-induced IFNγ release was significantly inhibited in the presence of anti-monokine antibodies (αIL-12 and αTNF), demonstrating the indispensable role of monocytes/macrophages for *S. aureus*-mediated IFNγ induction. In contrast, stimulation with SEB, a superantigen able to directly activate T cells, led to an IL-12/TNF-independent release of IFNγ. The tendency towards a slightly lower SEB-induced IFNγ release observed in the presence of αIL-12

and αTNF antibodies may be due to the inhibition of the autokrine release of IL-12 and TNF by monocytes upon IFNγ production by lymphocytes (75).

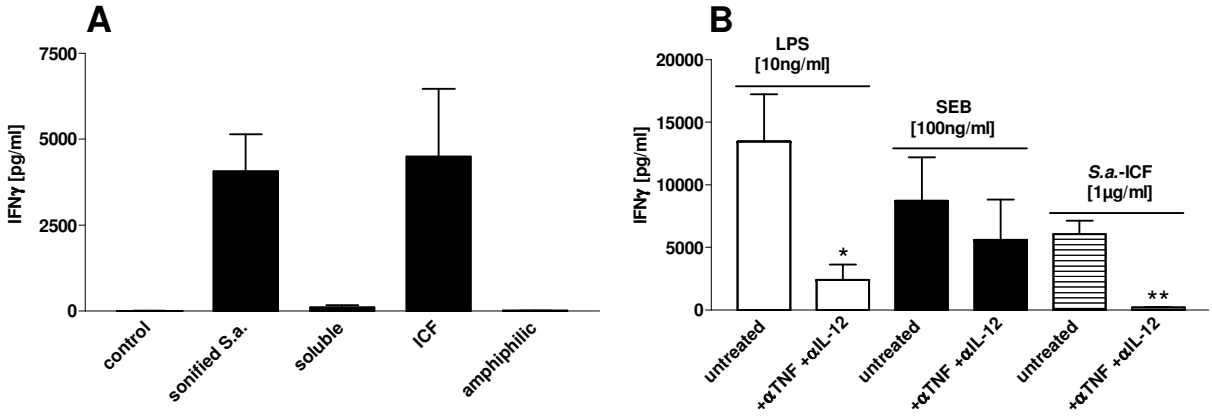

Figure 2: IL-12 and TNF mediate *S. aureus*-induced IFNγ release. Whole *S. aureus* bacteria were disrupted by sonication and subjected to butanol/water extraction resulting in three fractions containing water-soluble components (WC), amphiphilic components (AC) and insoluble cellular fragments (ICF) of *S. aureus*. (A) Human whole blood of eight healthy donors was incubated with sonified *S. aureus* [10 µg/ml] or with *S. aureus* preparations derived from WC, AC or ICF fractions [10 µg/ml each]. After 22 h released IFNγ was determined from the cell-free supernatants by ELISA. (B) Human whole blood, obtained from four healthy volunteers, was stimulated with LPS [10 ng/ml], SEB [10 ng/ml] or *S. a.*-ICF [1 µg/ml] in the absence or presence of neutralizing antibodies against human IL-12 and human TNF. Cell-free supernatants harvested after 22 h were analyzed for IFNγ by ELISA. Data are given as means ± SEM. Significant differences were analyzed by Mann Whitney test.

We further analyzed the type of IFNγ-producing cells in whole blood using intracellular cytokine staining and flow cytometry. As for LPS, following stimulation of whole blood with *S.a.*-ICF, IFNγ-positive NK cells and T cells were observed with comparable kinetics of induction (data not shown). These results indicate that, like for LPS, *S. aureus*-mediated IFNγ production by lymphocytes depends on blood monocyte activation to release IL-12 and TNF.

3.4.3 Inhibition of phagocytosis or digesting of PGN abrogates S. aureus-induced IFNγ release.

Recent reports demonstrated the importance of phagocytic activity for the immune response to *S. aureus* (66, 127). To examine the impact of phagocytosis on *S. aureus*-mediated IFNγ induction, human whole blood was preincubated with or without the phagocytosis inhibitor cytochalasin D and stimulated with *S.a.*-ICF. For control,

stimulation was carried out with Poly I:C, R-837, CpG and MDP, all ligands for intracellular immune receptors (TLR3, 7, 9 and Nod), as well as with LPS and $Pam_3Cys\text{-}SK_4$ representing extracellular immune receptor ligands (TLR4 and TLR2/1).

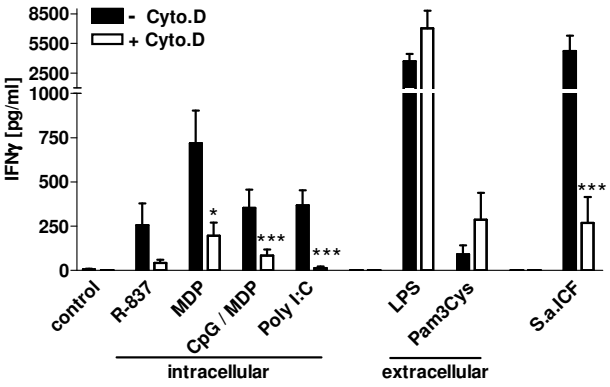

Figure 3: IFNγ induction by *S. aureus* requires phagocytosis. Human whole blood, pretreated with or without cytochalasin D for 30 min, was incubated with R-837 [15 µg/ml], MDP [1 µg/ml], CpG and MDP [2 µM and 500 ng/ml] or Poly I:C [15 µg/ml] for intracellular receptor stimulation and with LPS [10 ng/ml] or $Pam_3Cys\text{-}SK_4$ [10 µg/ml] for extracellular receptor stimulation as well as with *S.a.*-ICF [1 µg/ml]. Following 22 h incubation, the amount of IFNγ in the supernatants was determined by ELISA. Data deriving from 10 healthy blood donors and three independent experiments are presented as means ± SEM. Significant differences between untreated and cytochalasin D-treated samples were determined by Mann Whitney test.

As observed for Poly I:C, CpG and MDP, *S.a.*-ICF mediated IFNγ secretion from whole blood was significantly decreased in the presence of cytochalasin D. Although for R-837 a marked reduction of IFNγ release was found in the presence of cytochalasin D, the difference was not statistically significant owing to the low level of IFNγ induction. In contrast to *S.a.*-ICF, stimulation of whole blood with LPS and $Pam_3Cys\text{-}SK_4$, ligands for extracellular TLR receptors resulted in cytochalasin D-independent IFNγ production. Thus, phagocytosis seems to be pivotal for *S. aureus*-induced IFNγ production in human whole blood, pointing to a role of intracellular immune receptors such as Nod proteins, recognizing *S. aureus* PGN (37, 137). To investigate whether PGN recognition contributes to the IFNγ-inducing capacity of *S. aureus*, whole blood stimulation was carried out with *S.a.*-ICF treated with the PGN-digesting enzymes lysozyme or lysostaphin. As shown in figure 4, following lysozyme treatment, IFNγ induction by *S.a.*-ICF was completely abrogated regardless of whether whole blood was stimulated with soluble (supernatant) or insoluble (pellet) material obtained after the digestion step. Analogues to lysozyme digestion, also lysostaphin treatment resulted in a loss of IFNγ induction capacity (data not shown) suggesting that *S. aureus*-mediated IFNγ release strongly depends on macromolecular PGN.

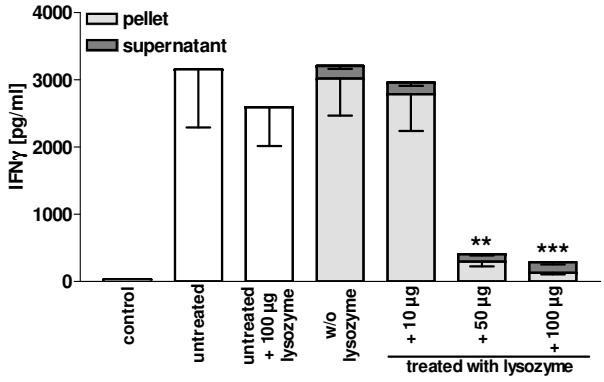

Figure 4: PGN digestion abrogates *S. aureus*-mediated IFNγ release. *S.a.*-ICF was incubated with increasing amounts of lysozyme to digest PGN. Following heat-inactivation respective amounts of generated soluble and insoluble material corresponding to 1 µg/ml undigested *S.a.*-ICF were used to stimulate human whole blood. As control, stimulation was carried out with undigested *S.a.*-ICF [10 µg/ml], either in the presence or absence of 100 µg heat-inactivated lysozyme. After 22 h the release of IFNγ was determined by ELISA. Data deriving from 11 healthy blood donors and three independent experiments are given as means ± SEM. Significant differences between the insoluble material after the digestion and the untreated *S.a.*-ICF were assessed by Kruskal-Wallis test followed by Dunn's post test.

3.4.4 Role of PGN recognition in S. aureus-mediated IFNγ induction.

To analyze whether the phagocytosis and polymeric PGN-dependent IFNγ induction was due to direct PGN recognition, we analyzed *S. aureus*-mediated IFNγ and TNF release from spleen cells of wt mice and mice deficient in TLR2 or Nod2, both demonstrated to be involved in PGN recognition. As observed for the control stimulation with LPS and SEB, *S.a.*-ICF-mediated IFNγ release was comparable among wt, TLR2- and Nod2-deficient mice, respectively (Figure 5A). In contrast, stimulation with PGN led to a slightly decreased IFNγ production among TLR2 and Nod2-deficient mice, which was not observed for *S.a*-ICF. The differences between PGN and *S.a.*-ICF were even more pronounced when TNF release was analyzed, resulting in a marked TLR2-dependency for PGN that was not detectable for *S. aureus*-ICF. Although both stimuli induced less TNF in cells from Nod2-deficient mice, the results above argue for an additional *S. aureus* structure beside PGN able to induce IFNγ release. This would also be in line with the lower IFNγ-inducing capacity of PGN compared to *S.a.*-ICF.

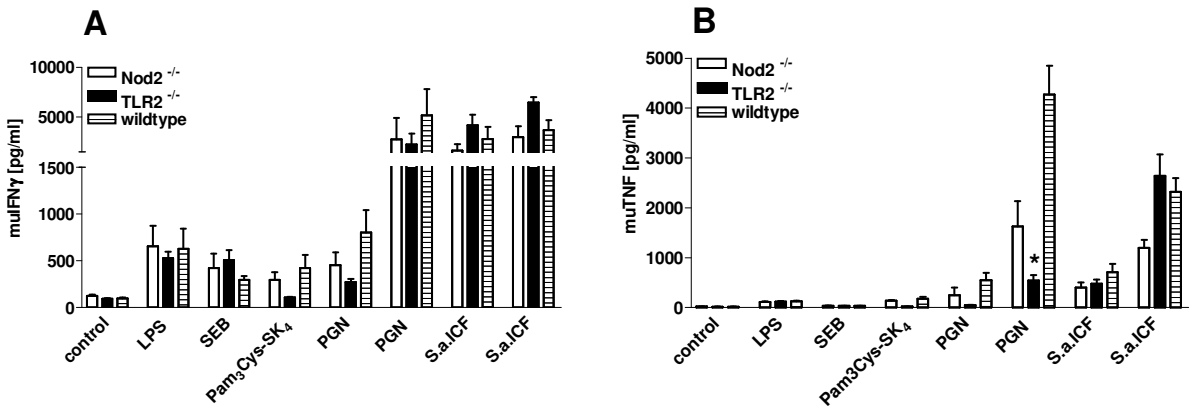

Figure 5: Recognition of *S. aureus* by Nod2- and TLR2-deficient mice. Splenocytes from Nod2-deficient, TLR2-deficient and wt mice were stimulated with LPS [100 ng/ml], SEB [1 µg/ml], $Pam_3Cys\text{-}SK_4$ [20 µg/ml], PGN [1 µg/ml and 10 µg/ml] and *S.a.*-ICF [100 ng/ml and 1 µg/ml]. After 22 h the amount of (A) murine IFNγ and (B) murine TNF released into the supernatants was determined by ELISA. Data from four different animals in each group are given as means ± SEM. Significant differences between $Nod2^{-/-}$ or $TLR2^{-/-}$ and the corresponding wt-mice respectively, were assessed by Kruskal-Wallis test followed by Dunn's post test.

Since digestion of PGN completely abrogated IFNγ release in whole blood (Fig 4), we speculated that polymeric PGN, acting as a carrier, facilitates phagocytosis of the IFNγ-inducing *S. aureus* structure, which is hampered after digestion of PGN. To investigate this hypothesis we overrode the phagocytosis process by transfecting lysozyme-digested *S.a.*-ICF in human PBMC. As shown in figure 6, DOTAP-transfection could partially restore the IFNγ-inducing capacity of the lysozyme-digested *S.a*-CF. In contrast, no effect of DOTAP-transfection was observed for *S. aureus*-ICF that was not digested before stimulation, indicating that PGN digestion renders IFNγ inducing *S. aureus* components unable to be taken up by phagocytosis.

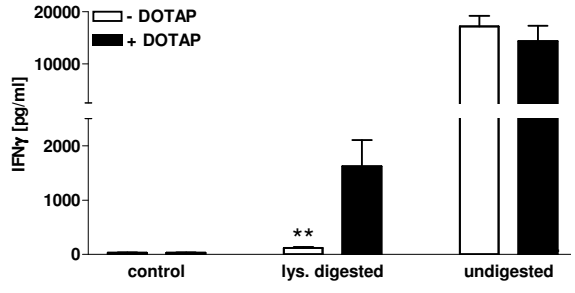

Figure 6: DOTAP transfection of lysozyme-digested *S.a.*-ICF. Human PBMC were stimulated with lysozyme-digested *S. aureus*-ICF [1 µg/ml], either pre-incubated with or without DOTAP reagent. For control, unstimulated PBMC and PBMC stimulated with undigested *S.a.*-ICF [1 µg/ml], either preincubated with or without DOTAP reagent, respectively, were used. IFNγ release was measured after 22 h by ELISA. Data deriving from six healthy blood donors represent means ± SEM. Significant differences between transfected and untransfected samples were assessed by Mann Whitney Test.

3.5 Discussion

Innate immune recognition of both Gram-positive and Gram-negative bacteria results in the induction of a broad spectrum of cytokines and chemokines associated with inflammatory responses. LTA of Gram-positive bacteria is a potent inducer of proinflammatory cytokines in humans, but unlike LPS, the major immunostimulatory molecule of Gram-negative bacteria (94), it fails to initiate IFNγ production (49, 50). In the present study we show that equipotent amounts of *S. aureus* bacteria and LTA are also equipotent in their induction of IL-1β, IL-6, IL-10 and TNF but differ in their ability to induce IFNγ, whereas whole *S. aureus* bacteria are able to induced amounts of IFNγ comparable to *E. coli* or *E. coli* derived LPS. The proinflammatory cytokine IFNγ is mainly produced by NK cells and T lymphocytes in response to IL-12 and TNF secreted by activated monocytes or macrophages, but can also be released via direct T cell activation through bacterial superantigens, such as SEB (89, 128). The results of our study demonstrated that *S. aureus*-mediated IFNγ production in human whole blood was strongly dependent on IL-12 and TNF release. Consistent with previous studies (75, 128), SEB-mediated IFNγ release was IL-12 and TNF independent suggesting that the stimulatory pathway of *S. aureus* leading to IFNγ production differs from that of staphylococcal superantigens. The requirement of IL-12 and TNF for *S. aureus*-mediated IFNγ production points to the role of monocytes and macrophages in the recognition of the stimulus, which elicit inflammatory responses following activation of extracellular but also intracellular receptors. Intracellular recognition has been found to enhance *S. aureus*-mediated immune activation, since inhibition of phagocytosis resulted in markedly decreased cytokine induction (58, 66, 127). In addition, several ligand binding proteins including mannose-binding lectine (58), CD36 (127) or integrin $\alpha_5\beta_1$ (124), have been described to be involved in the uptake or processing of *S. aureus* in the phagosome. We confirmed the pivotal role of intracellular *S. aureus* sensing in whole blood by employing the phagocytosis inhibitor cytochalasin D, which abrogated IFNγ production, as it did for Poly I:C, CpG and MDP, stimuli known to be exclusively recognized by intracellular receptors. These findings indicate a dispensable role of extracellular receptors in the initiation of IFNγ secretion in response to *S. aureus* in human whole blood, which has also been described in mice (66).
A major drawback in the identification of bacterial ligands and the respective innate immune receptors results from the difficulty of isolating contaminant-free bacterial

structures. The cell wall of Gram-positive bacteria mainly consists of a PGN layer that, beside its crosslinking peptides, contains teichoic acids, LTA and lipoproteins. Except for teichoic acids (86) these cell wall components have been described to induce proinflammatory responses (26, 46, 101). The immunostimulatory and inflammatory properties of purified LTA have been extensively investigated (99, 101). However recent studies reported lipoprotein contaminations in purified LTA that were able to activate TLR2 (46), although in human blood, these contaminations were found to make no contribution to LTA-mediated cytokine induction (143). The role of purified PGN in innate immune responses remains unclear, albeit several studies have investigated PGN-dependent immune activation (26, 137). Whereas some investigators found that PGN is sensed primarily by intracellular immune receptors (37, 57), others reported predominant recognition by TLR2 (26). However, improved preparation procedures for PGN resulted in PGN preparations lacking immunostimulatory activity potentially due to the removal of lipoprotein or LTA contaminations ((137) and unpublished results from our group). Furthermore, commercially available PGN preparations were additionally found to be contaminated with bacterial superantigens (78). To avoid discrepancies resulting from the use of isolated bacterial structures, in our experiments we preserved the *S. aureus* cell wall architecture and investigated IFNγ release after treatment with PGN degrading enzymes. Interestingly, lysozyme- or lysostaphin-mediated PGN digestion of *S. aureus* cell wall preparations resulted in a complete loss of IFNγ production in human whole blood. Markedly reduced PGN-mediated NF-κB activation following PGN digestion was also described by others (26, 137), although these studies used either Nod2 or TLR2 transfected cells, lacking the interplay of different immune receptors. Based on the results above, we further investigated whether PGN directly contributes to *S. aureus*-mediated IFNγ induction via activation of either TLR2 or Nod2.

Using splenocytes from TLR2 or Nod2 deficient mice we demonstrated that IFNγ production through *S. aureus* is independent of TLR2 or Nod2, arguing against a pivotal role of PGN in cytokine release. However, commercially available *S. aureus* PGN was able to induce IFNγ, albeit with a lower capacity than the *S. aureus* cell wall preparation. The higher cytokine induction by *S.a.*-ICF argues for an additional component beside PGN, able to trigger IFNγ release. Since DOTAP-transfection of lysozyme-digested *S. aureus* preparations could partially restore IFNγ production, we assume that PGN digestion renders this *S. aureus* compound unable to become internalized by immune cells. However, it is unclear whether this is due to a release of this component out of the

PGN layer resulting in diminished uptake, or due to decreased ability of digested material to trigger scavanger functions. Based on these results it is tempting to speculate, that polymeric PGN, beside its ability to directly mediate IFNγ release, acts as a carrier for PGN associated cell wall components to become internalized and thereby induce IFNγ induction. Further investigations are required to identify the intracellular immune receptors and respective bacterial ligands involved in the cytokine responses of *S. aureus.*

3.6 Acknowledgements

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4 Lipoteichoic acid induced cytokine release is inhibited by apolipoprotein B100

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(submitted)

4.1 Abstract

Little is known about serum mediators involved in immune recognition of lipoteichoic acid (LTA), an important immunostimulatory cell wall component of Gram-positive bacteria. We aimed to identify LTA binding proteins from human plasma and to investigate their role in innate immune activation. Comparative SDS-PAGE profiles of gel chromatography fractions of plasma proteins preincubated either with or without LTA from *Staphylococcus aureus* revealed an interaction between LTA and apolipoproteins (ApoA1, ApoA2, ApoA4 and ApoB100), which was confirmed by solid-phase binding assay. Significant inhibition of LTA-induced cytokine release from human PBMC could be demonstrated for ApoB100 but not for ApoA1 or ApoA2 both for LTA from wild type or from a diacylglycerol tranferase deficient *S. aureus* strain lacking palmitate-labeled lipoproteins. This inhibition mirrored that of preincubation of human LTA with serum before PBMC stimulation. In summary, our data suggest an inhibitory role of plasma-derived ApoB100 in immune activation by LTA from *S. aureus.*

4.2 Introduction

The initial response to pathogens that enter the bloodstream mainly depends on specialized receptors of the innate immune system like nucleotide-binding domain proteins and Toll-like receptors (TLR). In case of Gram-negative bacteria, TLR4 seems to play a pivotal role in the host response through the recognition of lipopolysaccharide (LPS) via a complex cluster of receptors including CD14 and MD-2 (74, 114, 122). In addition to membrane-anchored receptors, a number of soluble serum proteins, like LPS-binding protein (LBP), soluble CD14 or lipoproteins have been described to considerably modulate immune recognition of LPS (93, 135). For example, the binding of LPS to lipoproteins, more specifically to high-density lipoproteins diminishes its potency to activate human macrophages to release proinflammatory mediators like TNF or IL-1β (32, 40).

S. aureus is a frequent human pathogen that causes a variety of different diseases, ranging from minor skin infections to sepsis and death. For *S. aureus*, like for other Gram-positive bacteria, LTA and probably also lipoproteins, both described to be recognized by TLR2/TLR6 or TLR2/TLR1 (62) play an important role in the immune activation (10, 53, 120). Until now, only interactions of LTA with a few soluble serum proteins, such as LBP and CD14, have been reported (49, 102, 118). However, the effects of these interactions on the innate immune response are not yet completely understood, as demonstrated by contradictory reports claiming an accelerating (118) or an inhibitory (102) effect of LBP on LTA-induced cytokine induction. Although in the latter studies LBP was described to partially modulate LTA-mediated immune recognition the observed alterations found in the presence of human serum could not be attributed to LBP alone suggesting the presence of additional compounds able to modulate LTA-mediated immune activation.

In the current study we identified and characterized new LTA-binding proteins from human serum and investigated their influence on LTA-induced cytokine release by human PBMC.

4.3 Material and Methods

4.3.1 Stimuli

LTA from *S. aureus* strain DSM 20233 as well as from SA 113 wild type (SA 113 wt) and the mutant strain SA 113 *lgt::ermB* (SA 113 Δlgt, lacking the lipoprotein diacylglycerol transferase), both strains were kind gifts from A. Peschel, University of Tübingen, Germany, was isolated by butanol extraction and hydrophobic interaction chromatography as described previously (99). Purified human apolipoprotein preparations were obtained from Calbiochem (Darmstadt, Germany).

4.3.2 Tagging of LTA with biotin

500 µg LTA dissolved in 1 ml PBS and 500 µg PFP-biotin (pentafluorophenyl-ester biotin, Pierce, Rockford, USA) dissolved in 50 µl DMSO were mixed and incubated over night at 37°C on a shaker. The mixture was filtered through a pyrogen-free sepharose column (PD-10 desalting column, Amersham Biosciences, Freiburg, Germany), and submitted to ultrafiltration (Microsep 3K, Centricons, MI, USA) to remove the unbound biotin. The yield of the labeled LTA was determined by phosphate content as described previously (22), and the biotin incorporation was determined spectrophotometrically by displacement of HABA dye from avidin (HABA/Avidin reagent, Sigma) according to the manufacturer's specifications.

4.3.3 Identification of LTA-binding proteins

For chromatography, a sephacryl S-200-HR column (HiPrep^{TM}Sephacryl^{TM}-S-200-HR-column, Amersham Pharmacia, Uppsala, Sweden) with a void volume of 35 ml was used. First, the elution behavior of LTA was studied. 40 µg LTA applied to the column already eluted in the void volume, starting at 30 ml, owing to the micelle formation. Next, heparinized blood from healthy donors was spun for 10 min at 600 x g and the plasma was harvested and subjected to albumin and IgG removal (Albumin and IgG removal kit Amersham Biosciences, Freiburg, Germany). To enable the identification of LTA-binding proteins, human plasma was then applied to the column and the macrocomplexes eluting in the void volume were collected and discarded. Afterwards 150 µg plasma proteins derived from column fractions collected after the void volume were incubated either with PBS or with 40 µg wt-LTA in 500 µl PBS for 30 min at room temperature. Then the samples were applied to the column, and the LTA-containing

fractions of the void volume that showed an additional UV absorption peak (fraction 30-32 ml) were pooled and analyzed comparatively by SDS-PAGE and silver staining. The protein bands found only in the LTA-containing samples were excised from the gel and after in-gel tryptic digestion the proteins were identified using MALDI-TOF (matrix assisted laser desorption-time of flight) mass spectrometry at the core facility of the Biomedical Centre at the Ludwig-Maximillians-University Munich, Germany.

4.3.4 Solid-phase binding assay

To confirm the binding between the identified apolipoproteins and LTA, 0.025 to 2.5 µg purified apolipoproteins were coated to 96-well plates (MaxiSorp, Nunc) in 0.1 M $NaHCO_3$, pH 8.2, over night at 4°C. Then, wells were blocked with 200 µl of 3% BSA in PBS for 2 h at room temperature and washed with PBS containing 0.05% Tween 20. Biotinylated LTA was added to the plate and incubated for 2 h. After washing, bound LTA was quantified using streptavidin-conjugated peroxidase (Biosource, Camarillo, CA) and the substrate 3,3',5,5'-tetramethyl-benzidine (TMB, Sigma). The reaction was stopped with 1 M sulphuric acid and the absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm.

4.3.5 Preparation and incubation of human PBMC

PBMC of healthy volunteers were prepared with CPT™ Cell Preparation Tubes (BD Biosciences, Heidelberg, Germany). After centrifugation at 1600 x g for 20 min, PBMC were collected and washed at 300 x g for 5 min with RPMI 1640 (Lonza, Verviers, Belgium) containing 2.5 IU/ml Liquemin (Hoffmann-La Roche, Mannheim, Germany). After this single washing step, PBMC contain 4-5% residual plasma to ensure the presence of all serum components essential for effective immune activation. To allow binding between LTA and apolipoproteins or serum components, 0.22 µg of wt-LTA or lgt-LTA were preincubated with increasing concentrations of either purified apolipoproteins or autologous serum from the donors in 100 µl RPMI 1640 for 1 h at 37°C in polypropylene cups. Then PBMC were added at a density of 5×10^5cells/tube and the total volume was filled up to 220 µl. Cells were stimulated in the presence of 5% CO_2 at 37°C. Cell-free supernatants were collected 22 h after stimulation and stored at -80°C for ELISA experiments.

4.3.6 Cytokine determination

Cytokine release by human PBMC was measured by in-house sandwich-ELISA using commercially available antibody pairs and standards. Antibody pairs against human IL-1β and IL-6 were purchased from R&D Systems (Wiesbaden, Germany) and against human TNF from Endogen (Perbio Science, Bonn, Germany). Recombinant standards for IL-1β, IL-6 and TNF were obtained from the NIBSC, Herts, UK. Assays were carried out in flat-bottom, ultrasorbant 96-well plates (MaxiSorp, Nunc, Wiesbaden, Germany). Binding of biotinylated secondary antibody was quantified using streptavidin-conjugated horseradish peroxidase (Biosource, CA) and TMB (Sigma) was used as substrate. The reaction was stopped with 1 M sulphuric acid and the absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm. Cytokine levels are given per milliliter.

4.3.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 3 (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison post test was performed. IC_{50} values were obtained by non-linear regression (sigmoidal dose response). Data are given as means ± SEM. Significant differences of $p < 0.05$, < 0.01 and < 0.001 are represented as *, ** and *** in case of lgt-LTA and $^{\#}$, $^{\#\#}$, $^{\#\#\#}$ in case of wt-LTA respectively.

4.4 Results and Discussion

4.4.1 Inhibition of LTA-induced cytokine release by human plasma

Previous studies have shown that serum components can bind to bacterial products and thus specifically influence their recognition by innate immune receptors. To investigate the role of serum components in the immune recognition of LTA the cytokine release from human PBMC was determined after stimulation with both LTA from an *S. aureus* wild type strain (wt-LTA) and from a lipoprotein diacylglycerol transferase deletion mutant (lgt-LTA), both preincubated with increasing concentrations of autologous serum.

The LTA from the mutant *S. aureus* strain was additionally used in this study since the role of LTA as an immunostimulatory molecule was recently called into question when LTA purified from a diacylglycerol transferase mutant was unable to stimulate murine

cell lines, suggesting that not LTA but lipoprotein impurities in the LTA preparations represent the main immunobiologically active compound of *S. aureus* (46). The mutant strain lacks palmitate-labeled lipoproteins (126) and thus any controversially discussed (46, 143) possible lipoprotein contaminations can be excluded.

Compared to wt-LTA without addition of serum, preincubation of wt-LTA with serum led to a significant inhibition of IL-1β and TNF release, although IL-6 was not affected (Fig. 1). This suppressive effect of the serum preincubation was even more pronounced in case of lgt-LTA, which led to a significant inhibition of IL-1β, IL-6 and TNF secretion already at 0.125% human serum.

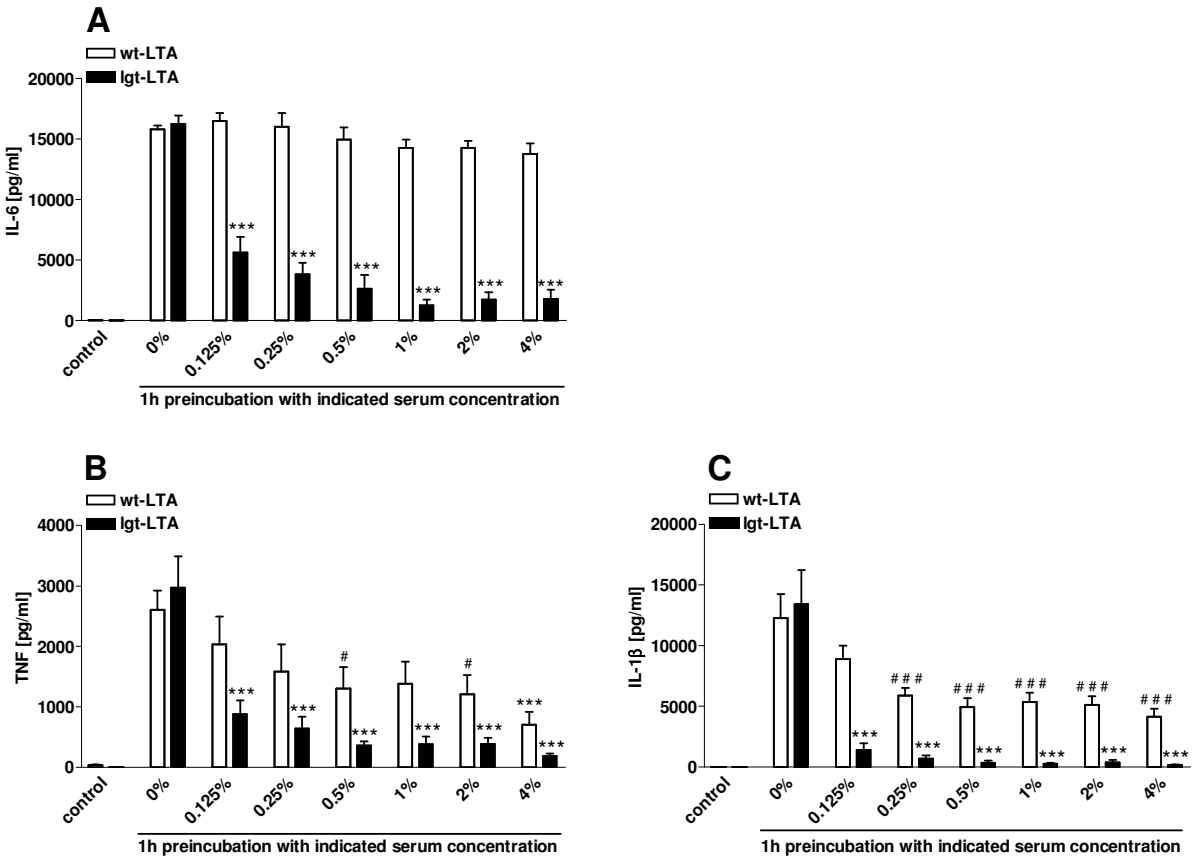

Figure 1: Attenuation of LTA-induced cytokine release in the presence of human plasma. 10 µg/ml of wt-LTA or lgt-LTA and increasing concentrations of human serum were preincubated for 1 h before incubation with human PBMC (containing 5% autologous serum) from 8 healthy donors. Cell-free supernatants were harvested after 24 h and analyzed for TNF, IL-1β and IL-6 release by ELISA. Data are presented as means ± SEM. Significant differences compared to the respective sample without serum preincubation were assessed by Bonferroni's multiple comparison test.

Since the PBMC used for the experiment still contained 4-5% plasma after preparation, the observed inhibition of cytokine release is likely due to direct binding of serum components to LTA during the preincubation time and not to a decreased responsiveness of the PBMC resulting from the addition of 0.125% serum. In line with these results a recent report demonstrated a suppressive effect of human serum on LTA-mediated cytokine secretion that in part could be explained by the presence of soluble LBP in the serum (102). However, the near complete inhibition of the LTA-induced cytokine release from human PBMC in the presence of autologous serum in our experiments suggested that further serum compounds could be involved in attenuating immune recognition of LTA.

4.4.2 Isolation of LTA-binding proteins from human plasma

To identify the serum proteins able to bind LTA and thus possibly to suppress LTA-induced immune activation, human plasma from which albumin, IgG and the macrocomplexes had been removed was applied to gel chromatography in the presence or absence of wt-LTA from *S. aureus*, and the resulting fractions were analyzed by SDS-PAGE. As shown in figure 2A the analysis of early chromatography fractions containing the LTA, as determined by a previous run with only LTA, revealed additional plasma protein bands compared to the same fractions when plasma was subjected to gel chromatography without added LTA. Using MALDI-TOF mass spectrometry analysis of peptide fingerprints of these protein bands, the apolipoproteins A1, A2, A4 and B100 were identified. The identification of these apolipoproteins concurs with observations that in human plasma about 95% of added LTA is bound to lipoproteins (77), mostly to high-density lipoproteins followed by low-density lipoproteins and very low-density lipoproteins, a pattern that was comparable to that of LPS (69, 77). For the latter, a direct interaction with proteins derived from plasma lipoprotein complexes (85) that was further able to neutralize LPS-mediated immune-activation (31, 115, 148) has been reported. To investigate whether the identified lipoproteins can also directly bind to LTA, a solid-phase binding assay was performed using purified preparations of the identified apolipoproteins. Except for ApoA4, which was not commercially available, the purified apolipoproteins A1, A2 and B100 were immobilized to ELISA wells, and their ability to bind biotinylated wt-LTA was quantified using peroxidase conjugated streptavidin (Fig. 2B). With increasing concentrations of all three apolipoproteins coated to ELISA plates we observed increased binding of wt-LTA which

was more pronounced for ApoB100 than for ApoA1 and A2. The higher amount of LTA detected in the wells that were coated with ApoB100 might be due to a higher binding affinity of ApoB100 to LTA or due to more LTA binding sites owing to the higher molecular weight of ApoB100.

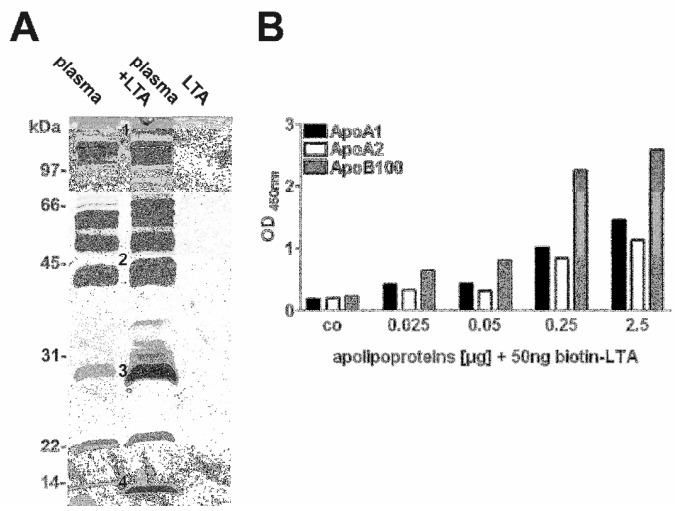

Figure 2: Identification of LTA-binding proteins from human plasma. A, Following preincubation of LTA with human plasma, samples were subjected to an octyl-sepharose column. As control either LTA or human plasma were separately passed through the column. The fractions containing the LTA were analyzed for their protein content in comparison to equivalent fractions derived from plasma proteins not preincubated with LTA, by SDS-PAGE. Proteins of interest were excised for tryptic digestion and mass spectrometry analysis. The positions of the identified apolipoproteins are indicated by small numbers (1: ApoB100, 2: ApoA4, 3: ApoA1, 4: ApoA2). B, Increasing concentrations of apolipoproteins were coated to 96-well ELISA-plates and incubated with 50ng/well biotin-LTA. Binding of biotinylated LTA was quantified using streptavidin-peroxidase

4.4.3 Role of LTA-binding proteins in immune recognition

Having shown that ApoA1, A2 and B100 can bind to LTA, we were interested in whether the identified apolipoproteins are able to modulate LTA-induced cytokine release. When LTA was preincubated with increasing concentrations of ApoB100 before stimulation with PBMC, a significant decrease of cytokine release was observed. In case of IL-6 and IL-1β, the reduction was more pronounced for lgt-LTA compared to wt-LTA, while the decrease in TNF induction by ApoB100 was similar for both wt-LTA and lgt-LTA (Fig. 3). In contrast to ApoB100 we observed no inhibitory effect of ApoA1 or A2 (data not shown) at concentrations up to 5 µg/ml. Interestingly, consistent with the observations for human serum (Fig.1) ApoB100 was unable to inhibit the IL-6 response to wt-LTA but significantly inhibited lgt-LTA mediated IL-6 induction. The comparable ability of human serum and ApoB100 to suppress cytokines, especially reflected by the same differences observed for IL-6, argues for a dominant role of ApoB100 in serum-mediated suppression of LTA-induced immune activation. The maximal

concentration of ApoB100 used in our experiments to inhibit the LTA-mediated cytokine release was 200fold lower than the concentration of ApoB100 in human serum (about 1 mg/ml). Although most of the AboB100 under physiological conditions is likely associated with lipids in lipoproteins, such as very low-density lipoproteins and low-density lipoproteins, the high inhibitory potency of free ApoB100 argues for an immunosuppressive role *in vivo*. We could not yet clarify the reason for the different sensitivities of wt-LTA and lgt-LTA towards ApoB100- or serum-mediated inhibition of cytokine release. At present, neither a low-grade bacterial lipoprotein contamination of the wt-LTA nor structural differences between the LTAs of the two strains can be excluded (143).

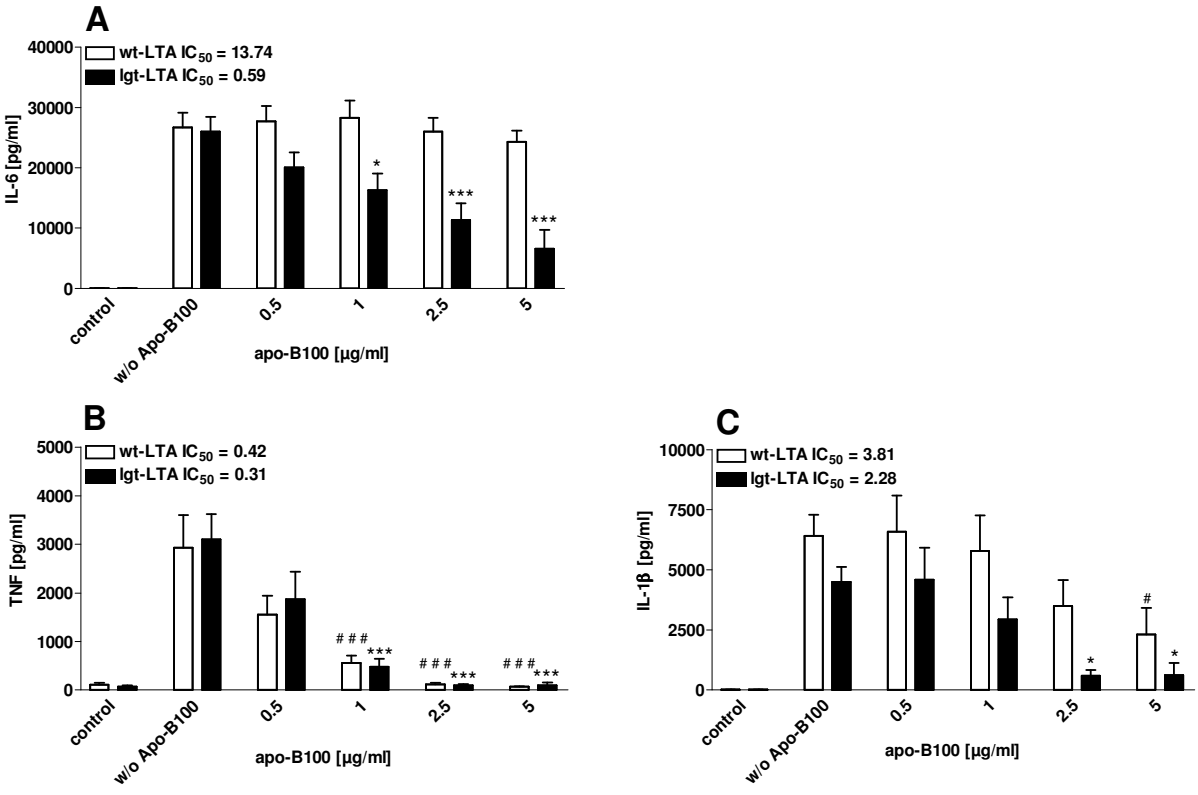

Figure 3: Effect of human ApoB100 on LPS/LTA-induced IL-1β release. Human PBMC from 9 healthy donors, containing 5% autologous serum, were stimulated with 1 µg/ml of wt-LTA or lgt-LTA and increasing concentrations of ApoB100. After 22 h, IL-1β, IL-6 and TNF were measured in the cell-free supernatant by ELISA. Data are means ± SEM. IC_{50} values were determined by non-linear regression. Significant differences compared to the respective sample without ApoB100 preincubation were assessed by Bonferroni's multiple comparison test.

4.5 Concluding remarks

The present study identifies apolipoprotein B100 as an important serum protein able to inhibit the cytokine induction by LTA from *S. aureus.* ApoB100 represents a major compound of the lipid metabolism known to play an important role in host defense by modulating effector functions of the innate immune system (6, 40). The near complete suppression of LTA-induced cytokine secretion by ApoB100 demonstrates the importance of lipid mechanism components in the modulation of the innate immune response. ApoB100 appears to act as a sink for free lipoteichoic acids in the blood, increasing the activation threshold of the inflammatory response. Furthermore, recent studies report that inflammatory cytokines upregulate ApoB100 production in hepatocytes (4, 5), indicating that ApoB100 may not only play a role in preventing but also in turning off an inflammatory reaction. Further studies elucidating the binding characteristics of ApoB100 to LTA and investigating its possible role in binding and immune modulation of other amphiphilic molecules are warranted.

4.6 Acknowledgements

We thank Andreas Peschel for providing the *S. aureus* mutant strain SA 113 *lgt::ermB* and are grateful to Thomas Hartung, Corinna Hermann, Christof Hauck and Christian Hesslinger for helpful discussions. The technical assistance of Leonardo Cobianchi and Margarete Kreuer-Ullmann is greatly appreciated. This work was supported by the German Research Council (International Research Training Group 1331).

5 Immune activation by lipoteichoic acid depends on presence of immunoglobulin G: Role of IgG-mediated opsonization

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(submitted)

5.1 Abstract

Lipoteichoic acid (LTA), a cell surface molecule of Gram-positive bacteria, represents a major immunostimulatory component able to elicit a strong inflammatory host response. Despite the compounding evidence of a pivotal role of LTA in immune activation, recent reports suggested stimulatory lipoprotein contaminations within LTA preparations to be the source of immunostimulatory activity of *Staphylococcus aureus* LTA. In the current study we investigated the requirements for immune recognition of LTA, from a diacylglycerol transferase mutant *S. aureus* strain (Δlgt), which lack palmitate-labeled lipoproteins, to address these conflicting data. We demonstrate that cytokine induction by Δlgt-LTA in human whole blood critically depends both on immobilization of LTA and phagocytic activity of the blood cells. Furthermore, Δlgt-LTA-induced immune activation was strongly augmented by LTA-specific IgG antibodies found to be present in human sera at varying levels, suggesting a novel mechanism for ΔLTA-mediated immune activation in human blood cells that involves opsonization-dependent uptake of LTA, enabling recognition within intracellular compartments. Under experimental conditions, conducive to optimal immune recognition of LTA, we found no differences in the immunostimulatory capacity of Δlgt-LTA and the respective wild type LTA, arguing for very limited contribution of possible lipoprotein contaminations even in wild type LTA.

5.2 Introduction

Staphylococcus aureus (*S. aureus*), a common cause of severe infections acquired in hospitals, often colonize human skin asymptomatically and can be isolated from the nasal mucosa of up to 40% of healthy individuals (144). Having overcome skin or mucosal barriers *S. aureus* establish an infection that, although mostly restricted to the skin, may involve tissue or systemic blood bacteraemia associated with sepsis and sometimes death (136). Upon contact with immune cells, *S. aureus* initially provoke an innate immune response resulting in secretion of cytokines and chemokines as well as in up-regulation of phagocytosis and co-stimulatory molecules able to trigger adaptive immune responses to control bacteraemia (63, 133). This response is set in motion by the recognition of highly conserved bacterial molecules, called pathogen-associated molecular patterns (PAMPs), via dedicated receptors, such as Toll-like receptors (TLRs) or cytosolic receptors containing a nucleotide-binding oligomerization domain (NOD) (1, 56, 61).

As for other Gram-positive bacteria, the innate immune recognition of *S. aureus* strongly depends on Toll-like receptor (TLR) 2 (53, 131). Human TLR2 is expressed on the cell surface and within endosomes of antigen presenting cells like monocytes, macrophages, dendritic cells and neutrophils (55). It recognizes various staphylococcal cell wall components including lipoteichoic acid (LTA) (120), lipoproteins (11) and peptidoglycan (25) albeit recognition of the latter is controversially discussed (26, 137). This broad range of different bacterial structures described as TLR2 ligands can be explained by heterodimer formation between TLR2 and other TLRs, such as TLR1 or TLR6 (62). Beside TLR2, the mannose-binding lectin (MBL) and CD36, both able to directly interact with LTA, have been shown to make an important contribution to the host response upon *S. aureus* infection (53, 58). Whereas CD36 was found to augment TLR2/6-dependent recognition of *S. aureus* and its LTA via enhancing their intracellular uptake (127), MBL was described to directly interact with TLR2 within the phagosome possibly improving LTA delivery to the TLR2/6 heterodimer (58).

Despite strong evidence for a major role of LTA in the immune activation by Gram-positive bacteria (19, 99, 101), recent reports suggested that not LTA but lipoproteins are dominant immunobiologically active structures of *S. aureus* (44, 46). The latter authors demonstrated a 100-fold decreased immunostimulatory capacity of LTA preparations derived from an Δlgt deletion mutant *S. aureus* lacking palmitate-labeled lipoproteins compared to LTA from the respective wild type strain.

From this finding and from other observations they concluded contaminating lipoproteins in the LTA preparations to be responsible for LTA-mediated immune activation (44-46). Contrarily to the latter investigators (44), we found that Δlgt-LTA and wt-LTA were equipotent in inducing cytokine release from human whole blood, although we could confirm the blunted ability of Δlgt-LTA to stimulate murine macrophages and human TLR2-transfected cell lines ((143) and unpublished data). The major discrepancy in LTA activation capacity observed in the two groups both using human whole blood were puzzling and led us to comparably investigate the specific requirements for Δlgt- and wt-LTA-mediated cytokine induction in human blood cells in order to find an explanation.

In this report, we describe that cytokine induction by Δlgt-LTA in human whole blood is critically dependent on surface immobilization of LTA, the presence of serum components as well as the phagocytic activity of blood cells. We further provide evidence that Δlgt-LTA-mediated immune activation is strongly augmented by LTA-specific IgG antibodies found to be present in human sera at varying levels. The results presented here not only confirm the equipotent immune activation between Δlgt- and wt-LTA in human whole blood observed previously (143), but also provide a conclusive explanation for the controversial findings obtained in previous experiments comparing the immunostimulatory capacity of Δlgt-LTA and wt-LTA.

5.3 Material and Methods

5.3.1 Stimuli

LTA from *S. aureus* wild type (SA 113 wt, wt-LTA) and mutant strain SA 113 *lgt::ermB* (SA 113 Δlgt, lacking the lipoprotein diacylglycerol transferase, Δlgt-LTA), both strains were kind gifts from A. Peschel, University of Tübingen, Germany, was isolated by butanol extraction and hydrophobic interaction chromatography as described previously (99). For UV inactivation and subsequent whole blood stimulation, *S. aureus* (SA 113 wt) were cultivated in tryptic soy broth (BD Biosciences, Heidelberg, Germany) for 16 h at 37°C. Harvested bacteria were adjusted to 10^8 bacteria/ml and 1 ml per well was irradiated on ice (UV-Stratalinker, La Jolla, CA) with an energy density of 1 kN/cm^2 (3 mWatt/cm^2 x 300 s) for 5 min in a 6-well cell culture plate (Greiner Bio-One, Frickenhausen, Germany). The inactivation was controlled by growth on blood agar plates (Columbia-blood agar, Heipa Diagnostika, Eppelheim, Germany) after 24 h at 37°C and 5% CO_2.

Other substances were LPS from *Salmonella enterica* serovar *abortus equi* and cytochalsin D from *Zygosporium masoni* (Sigma, Deisenhofen, Germany), Poly I:C (Invivogen, San Diego, USA), $Pam_2Cys\text{-}SK_4$ (EMC microcollections, Tübingen, Germany), CpG 2216 (MWG Biotech AG, Ebersberg, Germany), muramyl dipeptides (MDP, Bachem, Heidelberg, Germany), Endobulin (Baxter, Wien, Österreich), Avastin (Roche, Grenzach-Wyhlen, Deutschland) and pagibaximab (BSYX-A1110, Biosynexus, Gaithersburg, MD, USA).

5.3.2 Human whole blood incubation

Heparinized venous blood was obtained from healthy volunteers. Differential blood cell counts were routinely determined with a Pentra 60 apparatus (ABX Diagnostics, Montpellier, France) to exclude acute infections. Blood was diluted fivefold with RPMI 1640 (Lonza, Verviers, Belgium) and 500 µl were incubated in the presence of the different stimuli in polypropylene tubes (Eppendorf, Hamburg, Germany) overnight for 22 h at 37°C and 5% CO_2. For LTA immobilization, 50 µl of wt-LTA, Δlgt-LTA in RPMI at different concentrations was preincubated in tubes for up to 1 h at room temperature before the addition of whole blood and RPMI 1640. In case of LTA immobilization, all other control stimuli, such as LPS or UV-inactivated *S. aureus* were also preincubated before used for stimulation experiments. To assess the role of phagocytosis in LTA-mediated cytokine induction, diluted human whole blood was first pretreated with 3 µM cytochalasin D for 30 min in siliconized glass tubes (Vacutainer, Biosciences) and then applied to the preincubated LTA. The ability of cytochalasin D to inhibit phagocytosis was controlled by stimulation with MDP, CpG and Poly I:C as well as with LPS and $Pam_2Cys\text{-}SK_4$, respectively. After 22 h, blood cells were suspended by gentle shaking and centrifuged at 400 x g for 2 min. The cell-free supernatants were stored at -80°C for cytokine determination.

5.3.3 Preparation and stimulation of human peripheral blood mononuclear cells

PBMC of healthy volunteers were prepared with CPT™ Cell Preparation Tubes (BD Biosciences, Heidelberg, Germany). After centrifugation at 1600 x g for 20 min, PBMC were collected and washed four times at 300 x g for 10 min with RPMI 1640 containing 2.5 IU/ml Liquemin (Hoffmann-La Roche, Mannheim, Germany) to remove serum residues. PBMC stimulation with LTA and other stimuli were performed according to the conditions described for whole blood in a total volume of 220 µl. PBMC

were added at a density of 5 x 10^5cells/tube and stimulated either without or with human autologous serum at different concentrations or in the presence of different human antibodies or IgG-depleted serum. Cell-free supernatants were collected 22 h after stimulation and stored at -80°C for ELISA experiments.

5.3.4 Identification of LTA-binding proteins

For the identification of LTA-interaction components from human serum, the wells of a 6-well cell culture plate (Greiner Bio-One) were incubated with either 10 µg Δlgt-LTA/well or with only PBS, over night at 4°C and afterwards washed twice with PBS. Then 1 ml/well of 5% human serum in PBS was added for 1 h at room temperature, again followed by two washing steps with PBS. Surface-bound proteins from six wells were pooled in a total volume of 100 µl PBS containing 1% SDS and analyzed by SDS-PAGE and silver staining. The two protein bands at 25 kDa and 50 kDa, found only in the wells preincubated with Δlgt-LTA and not in the PBS-controls, were excised from the gel and, after in-gel tryptic digestion, were identified using MALDI-TOF (matrix assisted laser desorption-time of flight) mass spectrometry at the core facility of the Biomedical Centre at the Ludwig-Maximillians-University Munich, Germany.

5.3.5 Depletion of immunoglobulin G from human serum and Western blot

Immunoglobulin G was removed from human serum using HiTrap™ protein A columns (1 ml, GE Healthcare, Munich, Germany). According to the manufacturer's protocol, 1 ml of 50% serum in PBS was applied to the column and the flow-through was collected. In order to decrease remaining IgG residues, three columns were connected in series. The removal of IgG was confirmed by Western blot analysis. IgG-depleted serum-samples were applied to 12% SDS-PAGE and blotted to nitrocellulose membranes (Pall Corporation, Dreieich, Germany). Immunoglobulin G was detected by immunoblotting with horse radish peroxidase-conjugated polyclonal rabbit anti-human-IgG antibodies (DakoCytomation, Glostrup, Denmark) and enhanced chemiluminescence detection using an LAS-3000 imaging system (Fuji).

5.3.6 Cytokine measurement

Cytokines released by human whole blood were measured by in-house sandwich ELISA based on commercially available pairs of antibodies and standards. Antibody pairs against human IL-1β and IL-6 were purchased from R&D Systems and against human TNF and IFN-γ from Endogen (Perbio Science, Bonn, Germany). Recombinant standards for IL-1β, IL-6, TNF and IFN-γ were obtained from the NIBSC, Herts, UK. Assays were carried out in flat-bottom, ultrasorbent 96-well plates (MaxiSorp, Nunc, Wiesbaden, Germany). Binding of biotinylated secondary antibody was quantified using streptavidin-conjugated horseradish peroxidase (Biosource, CA) and TMB (Sigma) was used as substrate. The reaction was stopped with 1 M sulphuric acid and the absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm. Cytokine levels are given per ml.

5.3.7 Determination of anti-LTA antibody serum titer

The amount of LTA-binding IgG was determined in human serum obtained from 57 healthy volunteers. Flat-bottom 96-well plates (MaxiSorp; Nunc) were coated overnight with 10 µg ∆lgt-LTA in PBS at 4°C. Then, wells were blocked with 200 µl of 3% BSA in PBS for 2 h at room temperature and washed with PBS before addition of 4% serum in PBS and further incubation for 1 h at room temperature. After washing, horse radish peroxidase-conjugated polyclonal rabbit anti-human-IgG antibody was added to each well for another 30 min. Enzymatic activity was detected with 3,3',5,5',-tetramethybenzidine substrate (TMB, Sigma) and stopped with 1 M sulphuric acid. The absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm.

5.3.8 Statistics

Statistical analysis was performed using the Graph Pad Prism 3 Program (Graph Pad Software, San Diego, USA). Three or more groups of data were compared by repeated-measure analysis of variance (ANOVA) followed by Dunn's post test. For statistical analysis of two groups of data, the Mann Whitney test was used. In the figures *, ** and *** represent p values <0.05, <0.01 and <0.001, respectively.

5.4 Results

5.4.1 Immobilized but not soluble LTA of mutant ∆lgt S. aureus induces cytokine release in human whole blood

There are conflicting reports concerning the immunostimulatory capacity of lipoteichoic acid isolated from the lipoprotein diacylglycerol transferase deletion mutant (∆lgt) *S. aureus* that lacks lipoproteins (44, 46, 143). A probable cause of these discrepancies may be the immobilization-dependent immune activation of LTA (20, 106).

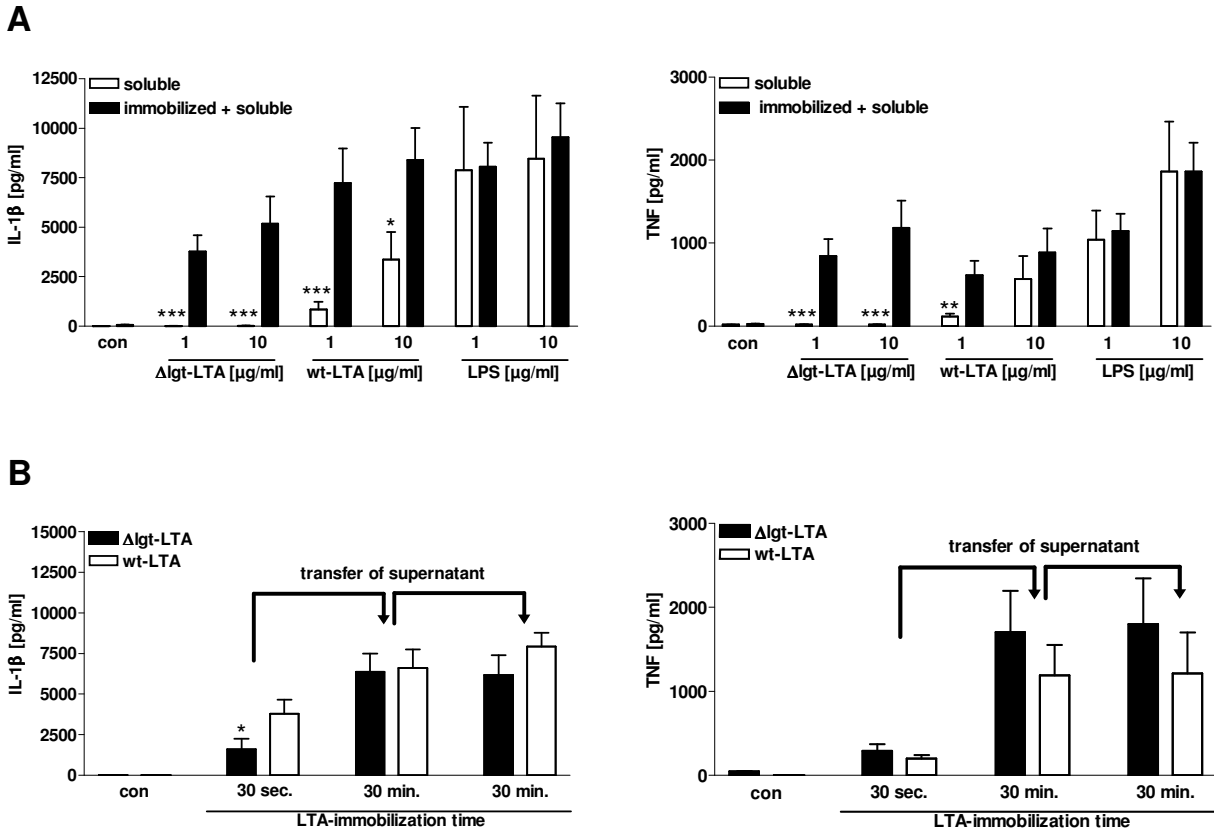

Figure 1: Stimulation with immobilized but not soluble ∆lgt-LTA induces cytokine release in human whole blood. Whole blood of healthy human donors was stimulated with *S. aureus* LTA from wild type (wt-LTA) or ∆lgt mutant strain (∆lgt-LTA) under immobilizing and non-immobilizing conditions. As controls, stimulations were carried out with LPS, or blood was left without stimulus (con). After 22 h, TNF and IL-1β release was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM. *A*, Whole blood of different donors (n=11) was stimulated with 1 µg/ml and 10 µg/ml ∆lgt-LTA, wt-LTA or LPS in polypropylene tubes. The stimuli were directly applied to the blood (soluble) or were coated for 1 hour before blood was added allowing immobilization of stimuli (immobilized). Significant differences between samples with soluble and immobilized stimuli were analyzed by Mann Whitney test. *B*, 10 µg/ml wt-LTA or ∆lgt-LTA were immobilized by preincubation in polypropylene tubes for 30 sec. Supernatants with unbound LTA were completely removed and transferred to another tube for 30 min, removed again and incubated in the last tubes for 30 min without subsequent removal of the supernatant. Human whole blood (n=8) was added directly after immobilization and samples were incubated for 22 h before cytokine determination by ELISA. Significant differences between all three conditions of the same stimulus were analyzed by Kruskal-Wallis test followed by Dunn's post testing.

To address this, we analyzed cytokine induction in human whole blood stimulated with LTA from the mutant strain (Δlgt-LTA) and from the respective wild type *S. aureus* strain SA113 (wt-LTA), either under immobilizing or non-immobilizing conditions. For immobilization, LTA was preincubated in the reaction tubes prior to the addition of medium and blood, whereas for non-immobilizing conditions LTA was added to the tubes after medium and blood.

Immobilization of LTA in polypropylene tubes led to a potent induction of IL-1β and TNF that was comparable between Δlgt-LTA, wt-LTA and LPS (Fig. 1A). In contrast, without immobilization of Δlgt-LTA and wt-LTA the release of IL-1β and TNF from whole blood was fully abrogated or significantly reduced, whereas LPS-mediated cytokine induction was unaffected. Notably, in contrast to wt-LTA, which induced low cytokine levels also without prior immobilization, Δlgt-LTA added freshly to human whole blood was unable to induce cytokines under these conditions.

To confirm that LTA does adhere to the polypropylene tubes, we analyzed the cytokine-inducing capacity of only the LTA bound during the immobilization procedure. For this, LTA was incubated in polypropylene tubes for 30 seconds and the residual, unbound LTA in the supernatant was transferred to another tube, and then incubated for 30 min, followed by another transfer and 30 min incubation but without subsequent removal of the supernatant. Even after incubation in the vial for only 30 sec, the bound Δlgt-LTA and wt-LTA were able to induce IL-1β and TNF, albeit in low amounts, which was strongly enhanced after 30 min preincubation. Interestingly, the cytokine-inducing capacity of bound LTA was not diminished in tubes incubated with supernatants from previous incubations (Fig. 1B). No decrease in cytokine induction by the LTA containing supernatant was observed even when it was transferred up to six times (unpublished observations), indicating that only a small proportion of LTA binds to the cup thereby enabled to induce cytokine release.

5.4.2 Δlgt-LTA-induced cytokine release by human PBMC is abrogated by cytochalasin D treatment and in the absence of human serum.

Recognition of LTA was described to be amplified by CD36-mediated phagocytosis and by the presence of serum MBL via a mechanism also dependent on the intracellular uptake of LTA (58, 127). These findings prompted us to investigate the influence of phagocytosis and human serum components on LTA-induced cytokine release in human blood cells. Using cytochalasin D to inhibit phagocytic activity we analyzed the

cytokine inducing capacity of Δlgt-LTA and wt-LTA in human whole blood. For control purposes, stimulations were carried out with different ligands known to activate immune receptors located either intracellular or extracellular. Surprisingly, in the presence of cytochalasin D the induction of IL-1β and TNF by Δlgt-LTA was completely abrogated and in case of wt-LTA strongly attenuated, which was also observed for the intracellular receptor ligands CpG and plus MDP as well as for Poly I:C (Fig 2A). In contrast, recognition of the extracellular TLR ligands LPS and $Pam_2Cys\text{-}SK_4$ was not affected by cytochalasin D treatment suggesting a pivotal role of phagocytosis for LTA-mediated cytokine induction.

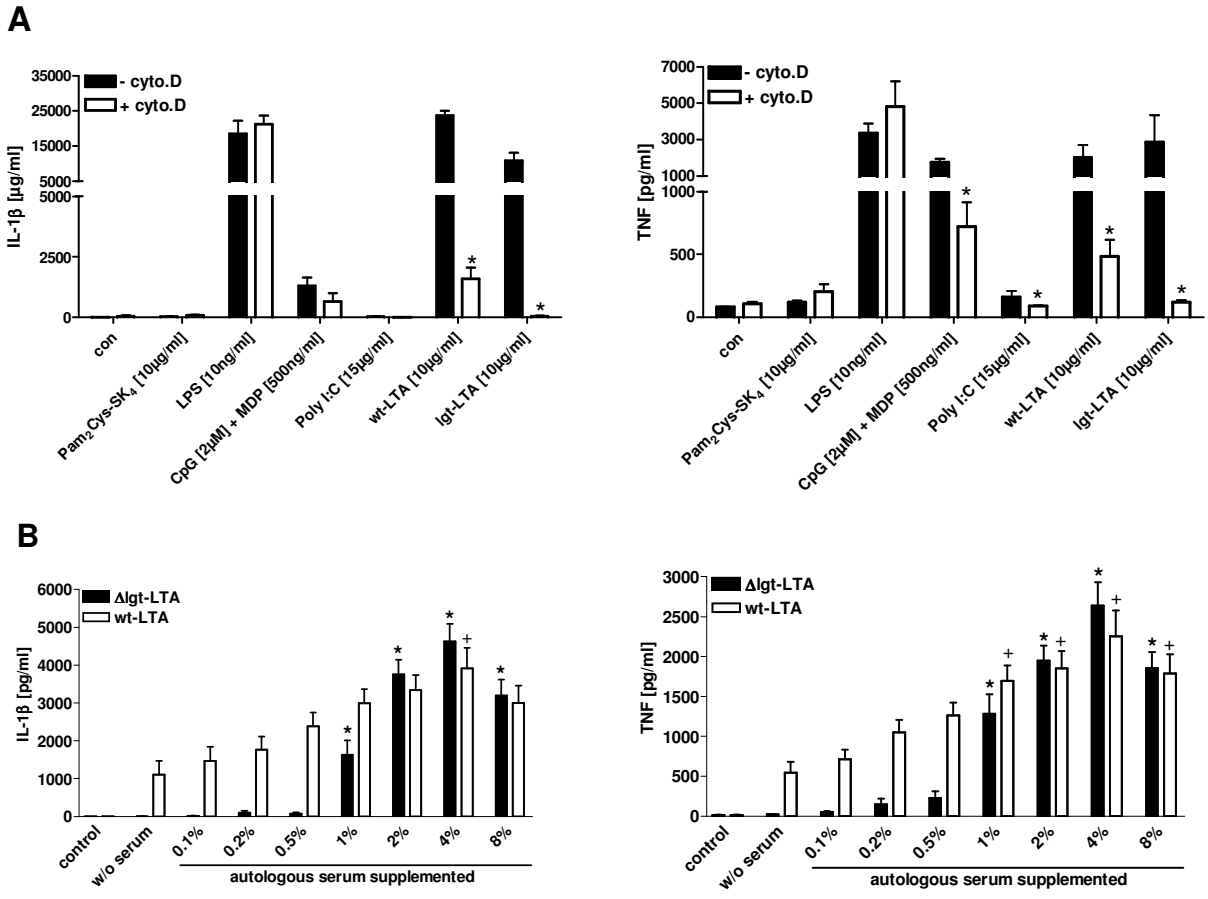

Figure 2: Δlgt-LTA-mediated cytokine release is abrogated by cytochalasin D treatment and in the absence of human serum. Whole blood or PBMC of healthy human donors were stimulated with Δlgt- or wt-LTA immobilized for 1 h to polypropylene tubes. Stimulations were carried out in the presence or absence of the phagocytosis inhibitor cytochalasin D or in the presence of increasing concentrations of autologous serum. After 22 h, TNF and IL-1β release was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM. *A*, Stimulation of cytochalasin D-treated blood samples (n=4) with 10 µg/ml Δlgt-LTA or wt-LTA. For control stimulations of extracellular and intracellular TLR receptors, $Pam_2Cys\text{-}SK_4$ or LPS and CpG, MDP or Poly I:C were used, respectively. Significant differences between samples pretreated with or left without cytochalasin D were analyzed by Mann Whitney test. *B*, Stimulation of human PBMC (n=10) with 10 µg/ml Δlgt-LTA or wt-LTA in the presence of increasing concentrations of autologous sera. Significant differences between the serum-free samples and the samples containing serum were assessed by Kruskal-Wallis test followed by Dunn's post testing.

To investigate the role of serum components we determined lgt-LTA and wt-LTA-mediated cytokine release from serum-free PBMC supplemented with increasing concentrations of autologous human serum. For immobilized but also non-immobilized (not shown) ∆lgt-LTA we detected no cytokine release from PBMC in the absence of human serum, which could be regained by the addition of autologous human serum (Fig. 2B). Already at concentrations of 1% serum significant cytokine induction by ∆lgt-LTA was observed. For wt-LTA a comparable enhancement of cytokine release in the presence of human serum was found, but in contrast to ∆lgt-LTA, wt-LTA was already able to induce cytokines under serum-free conditions, albeit at low amounts (Fig. 2B). The cytokine induction in PBMC by ∆lgt-LTA and wt-LTA peaked at a concentration of 4% serum and the amounts of released IL-1β and TNF were comparable between both LTA underlining their equal immunostimulatory capacity.

5.4.3 Immobilized ∆lgt-LTA interacts with apolipoprotein A1 and cationic immune globulins from human serum

To identify serum components that possibly interact with immobilized LTA resulting in immune activation, cell culture plate wells pretreated with or without ∆lgt-LTA were incubated with 4% serum from different human donors and bound components were analyzed by SDS-PAGE. As shown exemplarily in Fig. 3, the SDS-PAGE profiles of serum components derived from wells containing immobilized ∆lgt-LTA showed two abundant protein bands (25 kDa and 50 kDa) that were absent in serum samples obtained from control wells without bound LTA. The two protein bands were observed in the SDS-PAGE profiles of six tested donors albeit the band intensity among these donors was slightly different (data not shown).

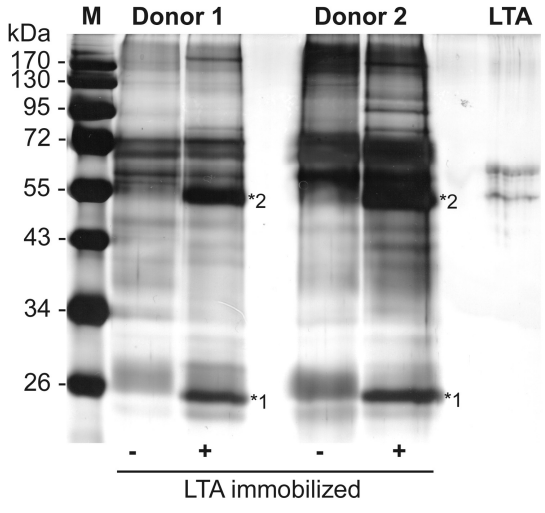

Figure 3: Identification of serum proteins interacting with immobilized ∆lgt-LTA. Cell culture plate wells (6-well) with or without immobilized ∆lgt-LTA were washed twice and then incubated with 4% human serum of two different donors. After removal of residual soluble serum components, the bound proteins were detached by addition of 1% SDS and analyzed by SDS-PAGE and silver staining. As control, SDS-PAGE analysis was performed with 10 µg ∆lgt-LTA. A standard protein marker (M) was used. The two protein bands (25 kDa and 50 kDa) marked with *1 and *2 were excised from the gel, subjected to tryptic digestion and then analyzed by peptide mass fingerprinting (for results see Table 1).

Using tandem mass spectrometry analysis of peptides derived from the 25 kDa protein band we identified two different human proteins, i.e. apolipoprotein A1 and immunoglobulin kappa light chain, whereas the analysis of the 50 kDa protein band revealed only the heavy chain of immunoglobulin (Table 1). In addition to the complete immunoglobulin light chain sequences with a predicted molecular mass of 23 kDa, a Mascot database search revealed highest identification scores also for only the variable domains of light chains derived from anti-DNA and anti-cardiolipin antibodies. Interestingly, DNA and cardiolipin share putative epitopes consisting of phosphodiester groups separated by three adjacent carbon atoms together with the polyglycerolphoshate backbone of LTA (64) arguing for a specific interaction between the detected antibodies and LTA.

Table 1 Identification of serum proteins interacting with immobilized ΔIgt-LTA

Sample	Accession no.	Protein	MS/MS Score	Peptides matched	Molecular Mass [kDa]
1 (25kDa)	gi|90108664	Apolipoprotein A1	702	49	28.0
	gi|1407576	Immunoglobulin light chain, variable region (anti-DNA antibody)	369	13	11.9
	gi|157838230	Immunoglobulin kappa light chain, constant and variable region	313	10	23.0
	gi|18092618	Immunoglobulin light chain, variable region (anti-cardiolipin antibody)	236	7	11.9
2 (50kDa)	gi|34365168	immunoglobulin heavy chain	419	41	51.1

5.4.4 LTA-specific IgG augments Δlgt-LTA-mediated cytokine induction in PBMC

To analyze the influence of apolipoprotein A1 and LTA-specific immunoglobulins we performed supplementation experiments using serum-free PBMC. The addition of up to 10 µg/ml apolipoprotein A1 was unable to restore cytokine release in serum-free PBMC stimulated with immobilized Δlgt-LTA (data not shown). In contrast, stimulation of PBMC supplemented with human clinical IgG preparations resulted in significant cytokine release and human chimeric LTA-specific antibodies induced even stronger cytokine release, which was not observed with humanized control IgG1 antibodies (Fig. 4A).

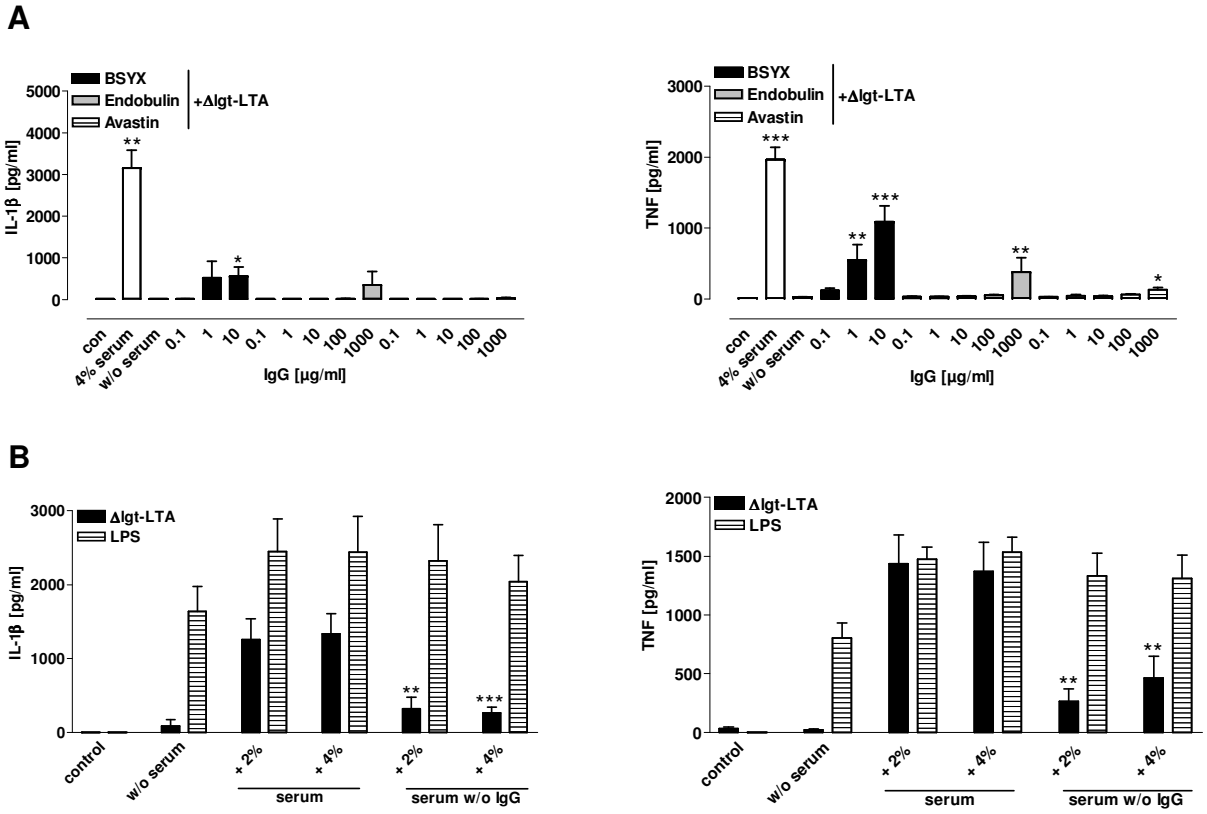

Figure 4: Δlgt-LTA-mediated cytokine release from human PBMC depends on the presence of specific immunoglobulin G. Human serum-free PBMC were stimulated with immobilized Δlgt-LTA in the presence of LTA-specific or -unspecific human IgG antibodies or in the presence of human autologous serum or IgG-depleted human serum. After 22 h, TNF and IL-1β release were measured in the cell-free supernatants by ELISA. Data are given as means ± SEM. *A*, Δlgt-LTA-mediated stimulation of serum-free PBMC (n=8) supplemented with increasing concentrations of human chimeric anti-LTA IgG1 (BSYX-A110) or humanized control IgG1 antibodies (Avastin) or with clinical preparations of total human IgG (Endobulin). As control, PBMC were left without stimulus or were stimulated in the presence of 4% human autologous serum. Significant differences in comparison to serum-free conditions were analyzed Kruskal-Wallis test followed by Dunn's post testing. *B*, Δlgt-LTA- and LPS-mediated stimulation of PBMC (n=10) in presence of 2% or 4% normal serum or IgG-depleted serum. Control stimulations were carried out under serum-free conditions. Significant differences between samples with normal serum and IgG-depleted serum were analyzed by Mann Whitney test.

However, even in the presence of LTA-specific antibodies we observed only a partial restoration of ΔIgt-LTA-mediated cytokines in serum-free PBMC compared to PBMC with 4% serum. In our stimulation experiments, we used high concentrations of clinical IgG and LTA-specific antibodies, which contained more LTA-specific IgG antibodies than are present in 4% human serum, thus suggesting the requirement of additional serum components for the recognition of immobilized ΔIgt-LTA by human blood cells. We therefore addressed the role of immunoglobulin by analyzing ΔIgt-LTA-mediated cytokine release from PBMC supplemented with either normal serum or IgG-depleted serum. As shown in Fig 4B, compared to PBMC containing normal serum, cytokine induction by ΔIgt-LTA was significantly reduced in the presence of IgG-depleted serum. This reduction was found to be specific for LTA, since LPS-mediated cytokine release was not affected by IgG-depletion. These findings indicate that specific IgG in the serum of human donors augments LTA-induced cytokine release from human blood cells. However, beside these antibodies, further serum components are needed for full immune activation.

5.4.5 The amount of LTA-specific serum IgG correlates with Δlgt-LTA- but not S. aureus-mediated immune activation in human whole blood

Immune recognition of whole *S. aureus* strongly depends on internalization of bacteria, since decreased phagocytosis in CD36-deficient mice or cytochalasin D treatment of murine immune cells leads to attenuated or abrogated immune activation by *S. aureus* (58, 127). In the present study we found that LTA-mediated cytokine induction is sensitive towards cytochalasin D treatment and requires LTA-specific antibodies, suggesting an opsonization-dependent LTA uptake and recognition process that could also contribute to the recognition of whole *S. aureus* presenting LTA at their surface. To investigate this, we correlated LTA-specific IgG titers in the sera of different donors with the respective cytokine response of their whole blood after stimulation with ΔIgt-LTA, whole *S. aureus* or LPS. Among 57 human donors we observed pronounced differences in LTA-specific IgG titers, as revealed by ELISA experiments using immobilized ΔIgt-LTA (Fig. 5A). Consistent with the results above, compared to blood from donors with high titers of specific IgG, blood from donors with low titers showed a significantly decreased induction of IL-1β and TNF after stimulation with ΔIgt-LTA, a difference that was less pronounced for IL-6 release (Fig. 5B).

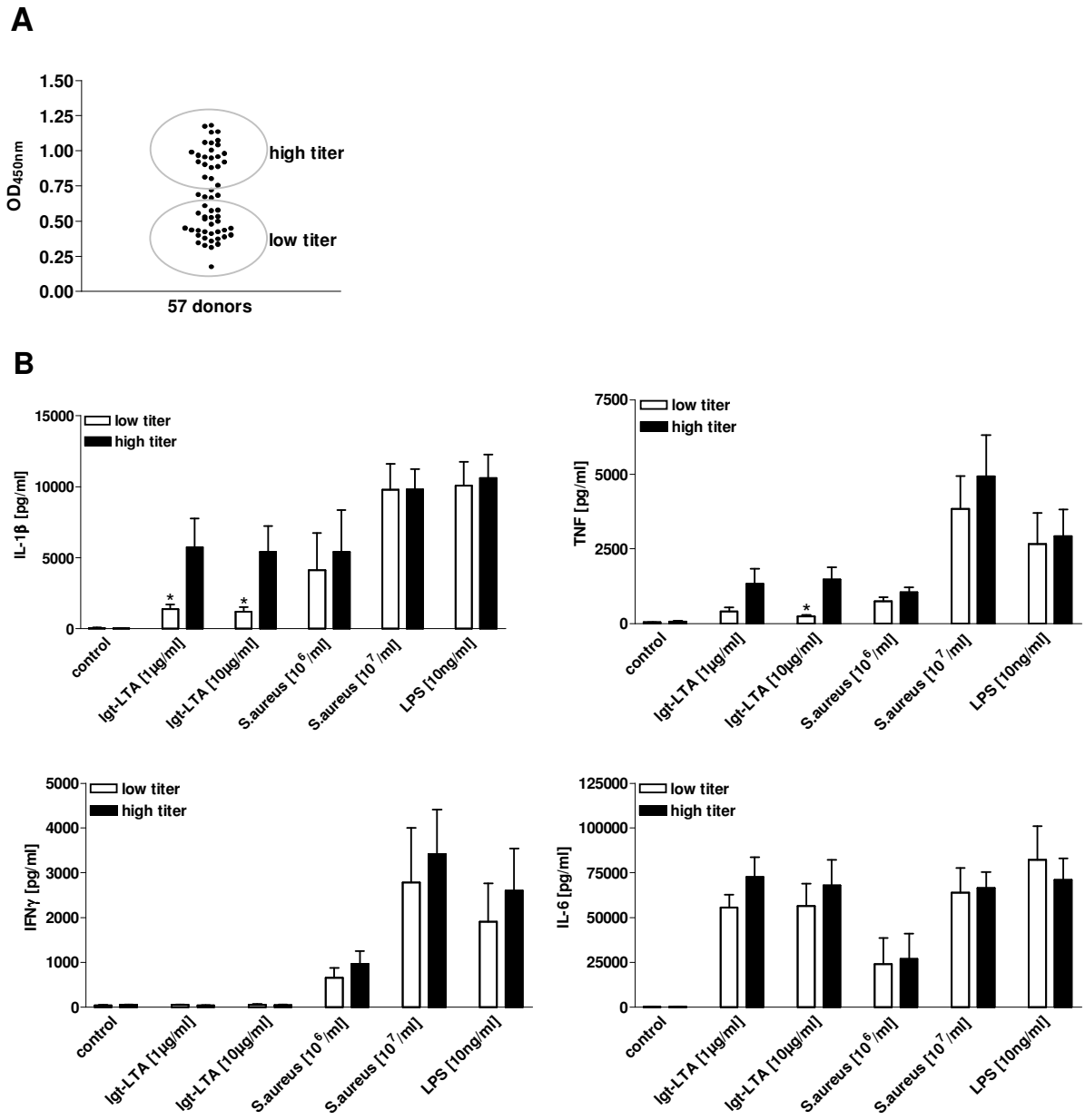

Figure 5: LTA-specific serum IgG titers determine immune activation by Δlgt-LTA but not by *S. aureus* or LPS in human whole blood. *A*, Δlgt-LTA immobilized in 96-well plates was used to determine the titer of LTA-specific IgG antibodies in serum samples obtained from 57 healthy blood donors. Blood from the donors showing the highest (n=20) and the lowest LTA-specific IgG titers (n=20) was used for further experiments. *B*, Whole blood from donors with either high or low titers of LTA-specific antibodies was stimulated with immobilized Δlgt-LTA (1 µg/ml and 10 µg/ml), UV-inactivated *S. aureus* (10^6 and 10^7 /ml) or LPS (10 ng/ml). After 22 h, the amount of released IL-1β, TNF, IFN-γ and IL-6 was determined by ELISA. Data representing six different donors in each group are given as means ± SEM. Significant differences in cytokine release between these two donors groups were assessed by Mann Whitney test.

As previously observed for wt-LTA (49), Δlgt-LTA was unable to induce IFN-γ in human whole blood. When whole blood stimulation was carried out with UV-inactivated *S. aureus* or LPS, no differences in the cytokine induction between donors with low and

high titers of LTA-specific antibodies were observed. The latter results suggest that despite the necessity of LTA-specific IgG antibodies to recognize LTA, these are dispensable for the immune recognition of *S. aureus* in human whole blood arguing for a mechanism of *S. aureus* recognition different from that of LTA.

5.5 Discussion

The immunostimulatory capacity of LTA derived from an *S. aureus* deletion mutant lacking diacylated lipoproteins (∆lgt-LTA) is controversially discussed. This is of seminal interest for the field of innate immunity, as it questions the ability of LTA *per se* to induce cytokine release. Although fully synthetic LTA was found to mirror the immunostimulatory activity of butanol-extracted LTA from wt *S. aureus* (19, 101), Hashimoto *et al*. propose that the activity of the latter stems entirely from purported, highly immunostimulatory lipoprotein contaminations not present in ∆lgt-LTA, which was inactive in their cellular systems and whole blood (44, 46). However, using the same bacterial strains in a previous report, we have reported equipotent immunostimulatory activity of wt-LTA and ∆lgt-LTA in human whole blood (143). Notably, the present study investigating the requirements for ∆lgt-LTA-mediated immune activation reveal an explanation for this discrepant activity of ∆lgt-LTA observed in human whole blood.

Our results clearly confirm that cytokine induction by ∆lgt-LTA is equipotent to LTA from wt *S. aureus*, but also show that ∆lgt-LTA requires more stringent conditions to be recognized by human immune cells than wt-LTA.

We demonstrate that ∆lgt-LTA-mediated cytokine release from whole blood or PBMC can only be observed after immobilization of LTA and requires phagocytic activity of stimulated cells as well as the presence of human serum components, including LTA-specific antibodies. As ∆lgt-LTA does not contain lipoprotein contaminants, the activity and these requirements must stem from the LTA itself. As described previously, wt-LTA induced cytokine release was also potentiated after immobilization (20, 106), but in contrast to ∆lgt-LTA, a residual activity was observed under non-immobilizing conditions. Compared to ∆lgt-LTA, wt-LTA was also found to exhibit residual activity in cytochalasin D treated blood cells and showed an incomplete dependency on serum supplementation. Since we (manuscript in preparation) and others (46) found no structural differences between wt-LTA and ∆lgt-LTA, it appears that the residual activity results from the lipoproteins in these LTA preparations, which were shown by Hashimoto and co-workers (44, 46) to elute in the same hydrophobic interaction

chromatography fractions than LTA. Although the full immunostimulatory activity of wt-LTA and ∆lgt-LTA did not differ, all further experiments were performed with ∆lgt-LTA to exclude contributions by possible lipopeptides. In all our experiments where ∆lgt-LTA was added directly to human whole blood or serum-containing PBMC without preincubation (non-immobilizing conditions) we observed a complete loss of cytokine induction suggesting a role of human serum components able to prevent LTA immobilization on the reaction tube. Indeed, serum lipoproteins can bind soluble LTA with very fast kinetics (77) and, in addition to apolipoproteins and LBP, have been found to inhibit LTA-mediated immune activation under non-immobilizing conditions (40, 102). In line with this we identified specific serum apolipoproteins able to completely block the cytokine induction of ∆lgt-LTA when applied together with LTA during immobilization process (manuscript submitted). This mechanism likely also inhibited ∆lgt-LTA-mediated immune activation in the experiments of Hashimoto et al. (44), who stimulated human whole blood with soluble but not immobilized LTA, thereby rendering ∆lgt-LTA unable to induce cytokines. Our observations may further explain why, soluble LTA is less pyrogenic than LPS *in vivo* (18). In contrast to LTA isolated in the laboratory, LTA in *S. aureus* anchors in the bacterial cell membrane via its two fatty acids and projects through the peptidoglycan. We speculate that, comparable to the immobilization process observed here, this arrangement shields the fatty acids from interactions with serum apolipoproteins, thereby allowing optimal recognition by human immune cells.

Our observation that ∆lgt-LTA-mediated cytokine release was completely abrogated in blood cells under phagocytosis inhibition with cytochalasin D demonstrates an absolute requirement of phagosomal recognition of LTA. This is in line with recent reports finding that the scavenger receptor CD36, a specific sensor of LTA and other diacylglycerides (53), augments *S. aureus*-induced immune activation in a TLR 2/6 dependent manner by enhancing phagocytosis of LTA or whole bacteria and by its subsequent cooperation with the TLR signaling pathway (127). Also, the mannose-binding lectin was found to amplify the host response to *S. aureus* in a comparable, but CD36-independent manner (58). The authors described a mechanism in which MBL binds to LTA, leading to specific enhancement of TLR 2/6 signaling by supporting intracellular receptor complex formation. Interestingly, augmented recognition of LTA through MBL could only be observed for LTA efficiently delivered to the phagosome, whereas no effect was observed for soluble LTA. The requirement of phagocytic activity for its recognition likely

explains the failure of ∆lgt-LTA to induce activation of human TLR-2 transfected HEK293 cells or other cells with poor phagocytic activity.

A important observation of this study was the enhanced LTA-mediated cytokine release found in the presence of LTA-specific IgG antibodies. Using IgG-depleted serum we found significantly reduced cytokine induction by LTA in human PBMC. Despite this pronounced effect, we assume that, beside antibodies, other serum components are required for full activation, since LTA-mediated cytokine release from PBMC was completely lacking under serum-free conditions. This is also in line with the inability of total human IgG fraction or LTA-specific antibodies alone to fully restore LTA-mediated immune activation under serum-free conditions. Antibodies recognizing the polyglycerol phosphate backbone of LTA have been shown to be present in normal human serum with titers that can become elevated during streptococcal infection (35, 88). Our data also demonstrated striking differences in LTA-specific IgG antibody levels among 57 healthy human donors. Intriguingly, the titer of LTA-specific antibodies significantly correlated with the LTA-induced cytokine levels, which were found to be up to fivefold increased in whole blood of donors with high LTA antibody titers. This further underlines the pivotal role of IgG antibodies for LTA-mediated cytokine induction in human whole blood. With regard to the internalization-dependent process of LTA recognition we speculate that, in the presence of human serum, LTA is opsonized by specific antibodies leading to Fc receptor-mediated uptake of LTA by phagocytic immune cells. Interestingly, this would also explain the inability of LTA to induce IL-12 and subsequent IFN-γ release, since ligation of phagocytic receptors on immune cells during stimulation with TLR ligands has been found to selectively downregulate IL-12 transcription (39, 90, 129).

Recently, an indispensable role of *S. aureus*-specific IgG antibodies has also been shown for the activation of human plasmacytoid dendritic cells (pDC) by whole *S. aureus* (110). The authors described an FcγRIIA-dependent uptake process of antibody-opsonized bacteria to be responsible for pDC activation after stimulation with *S. aureus*. However, we observed no differences in cytokine release in blood from donors with high or low LTA antibody titers stimulated with *S. aureus*. It seems likely that, in contrast to purified LTA of *S. aureus*, the whole bacteria can be taken up by alternative mechanisms independent of antibody-mediated opsonization.

The high concurrence of the requirements for immune activation by LTA and whole *S. aureus*, such as phagosomal uptake and presence of CD36, MBL as well as TLR 2/6,

is consistent with the idea that LTA represents the major component triggering activation upon stimulation of immune cells with whole bacteria. Although lipoproteins play an important role in the host defense against *S. aureus* in mice (10) and the possible lipoproteins contaminants of LTA do stimulate cytokine release in human whole blood, our experiments find no differences in the immunostimulatory capacity of ∆lgt-LTA and wt-LTA under optimal conditions, arguing that the contribution of lipopeptide contaminations in LTA preparations to the immunostimulatory activity is minimal. Notably, in line with a previous report the stimulatory capacity of LTA is strongly underestimated, since only the low proportion of LTA that becomes immobilized (1-4%) (20) reflects the situation of *S. aureus*-bound LTA and is able trigger immune activation.

Based on our data we suggest a mechanism for LTA-mediated immune activation in human blood cells that involves opsonization of immobilized LTA by specific antibodies, thereby enabling phagocytic uptake of LTA and subsequent recognition within intracellular compartments. Although the antibody-mediated uptake of LTA might play only a minor role for the recognition of LTA anchoring in the cell membrane of *S. aureus*, the present study uncovers important requirements for LTA-induced immune activation in general that should be taken into consideration when working with purified LTA. Furthermore, it offers a satisfying explanation of discrepancies found between two laboratories working with lipoproteins-free ∆lgt-LTA and human whole blood.

5.6 Acknowledgements

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6 Summarizing Discussion

Staphylococcus aureus is a major human pathogen that causes superficial skin- or tissue-infections but also life-threatening systemic diseases, leading to sepsis and death. The incidence of *Staphylococcus aureus* infections is on the rise, and the main limitations of the treatment of these infections result from the emergence of multidrug-resistant strains known as meticillin-resistant *Staphylococcus aureus* (MRSA) (83). Furthermore, the isolation of MRSA strains that have also become resistant to vancomycin, led to more treatment failures and higher mortality rates during the last years (80, 81). *Staphylococcus aureus* invading the bloodstream initially activate the innate immune system through interaction of their exposed cell wall components with highly conserved immune receptors. This results in the release of a wide range of inflammatory chemokines and cytokines important for host defense. However an inflammatory response with inflated production of proinflammatory cytokines induces side-effects and can lead to sepsis and death (140). Monocytes and macrophages are essential for host defense by recognizing, engulfing and killing bacteria, but they also play a critical role in the pathophysiology of bacterial sepsis, as they represent the main source of proinflammatory cytokines responsible for septic shock (33, 87).

As the symptoms of systemic Gram-negative sepsis are clinically indistinguishable from those caused by Gram-positive bacteria (7, 125), we compared the patterns of innate immune activation of Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* in a human *ex vivo* model. In line with other studies (150) we observed a potent and comparable induction of IL-1β, IL-6, IL-10, TNF and IFNγ upon stimulation of human whole blood with equipotent amounts of *Escherichia coli* and *Staphylococcus aureus*, suggesting that the described clinical parallels between Gram-negative and Gram-positive bacterial infections result from a similar progression of the inflammatory responses. However, unlike the prominent role of LPS in the pathogenesis of Gram-negative sepsis (105) which renders its toxic moiety lipid A a therapeutically target (123), the contribution of immunostimulatory molecules of Gram-positive bacteria to overall immune response is still under debate. Although the proinflammatory properties of different staphylococcal cell wall components are discussed controversially (26, 46, 137, 143), the important role of TLR2 in *Staphylococcus aureus* infections has been widely reported in various staphylococcal infection models (38, 53, 131, 144). In contrast to the high ligand specificity of TLR4, which beside few virus-derived proteins only recognizes bacterial LPS (95), TLR2 is

described to form functional complexes with further TLRs to increase its range of detection (109) and accommodate diverse ligands like staphylococcal PGN (25), LTA (118) and lipoproteins (45).
Here the immunostimulatory capacity of different constituents of the staphylococcal cell envelope that are implicated in staphylococcal septic shock (125) was investigated and the underlying mechanisms of their recognition by the innate immune system were characterized.

6.1 Role of peptidoglycan in innate immune recognition

The exoskeleton of Gram-positive bacteria mainly consists of PGN, a polymer of β-1,4-linked *N*-acetylglucosamine-*N*-acetylmuramic acid glycan strands crosslinked by short peptides (142) that form a netlike structure surrounding the cytoplasmic membrane. During bacterial growth or after antibiotic treatment, PGN breakdown products are released into the bloodstream and can be detected by innate immune receptors. Among these muropeptides, composed of *N*-acetylmuramic acid and oligopeptides, muramyl dipeptides (MDP) represent the minimal PGN motif common to Gram-negative and Gram-positive bacteria (28). However, albeit extensively studied, the role of bacterial PGN in innate immune activation remains controversial since there are conflicting data on whether PGN-fragments can be recognized by extracellular TLR2 (3, 26, 59, 67, 145) or have to be taken up to induce intracellular signaling via cytosolic Nod1 or Nod2 (21, 37, 57, 145). A major challenge concerning the investigation of host-pathogen interactions and subsequently identification of specific innate immune receptors arises from the difficulty of isolating contaminant-free bacterial structures. Most studies performed to identify the immunostimulatory capacity of PGN were carried out with commercially available PGN preparations, however these preparations were found to contain LTA (137) as well as bacterial superantigens (78). As hydrolysis of LTA with hydrofluoric acid and thus removal of possible LTA contaminations resulted in PGN preparations lacking immunostimulatory capacity, it was hypothesized that immune activation is not attributable to PGN itself but rather to other inflammatory cell wall components contaminating the commercial PGN preparations (137).
The role of *Staphylococcus aureus*-derived PGN in innate immune recognition with special regard to PGN-mediated IFNγ induction was investigated in the first part of this PhD thesis. To avoid erroneous conclusions resulting from possible contaminations of

isolated PGN, the complex bacterial cell wall architecture was preserved and PGN-induced cytokine release in human whole blood was determined after treatment of *Staphylococcus aureus* cell wall preparations with lysozyme, a PGN degrading enzyme that specifically hydrolyzes glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid. The digestion of the bacterial PGN rendered the staphylococcal cell wall preparation unable to induce cytokine secretion, pointing to polymeric PGN rather than disaccharide-peptide PGN fragments being recognized by the immune cells. These results are consistent with those described by Dziarski et al. (26), who also showed markedly reduced PGN-mediated NF-κB activation following PGN digestion. However, the latter studies were performed with transfected HEK cells, while in the present study human whole blood was used to conserve the interplay of different immune receptors that might be critical for the induction of immune responses.

Inhibition of phagocytosis also resulted in a significantly decreased cytokine secretion in human whole blood after stimulation with *Staphylococcus aureus* cell wall preparations, suggesting a dispensable role of extracellular immune receptors in PGN recognition. Taken together, it appeared that polymeric PGN probably due to its size, initiates internalization and subsequently triggers proinflammatory signaling through intracellular immune receptors. Considering the frequently reported contribution of cytosolic Nod2 to PGN-mediated inflammatory response (21, 37, 57, 111), but also the alternative of TLR2 representing an intracellular PGN sensor recruited to the phagosome (109, 138) splenocytes from Nod2 or TLR2 deficient mice were stimulated with *Staphylococcus aureus* cell wall preparation. Interestingly, staphylococcal cell wall-mediated cytokine secretion turned out to be independent from Nod2 and TLR2, suggesting that not PGN itself but rather PGN associated cell wall components represent crucial bacterial PAMPs that mediate inflammatory responses upon stimulation of human blood cells. This would also be in line with the results obtained by Travassos and co-workers (137), who demonstrated PGN-mediated immune activation to result from LTA and lipoprotein contaminations present in PGN preparations. Furthermore, PGN-derived MDP, a major component of complete Freud's adjuvant (13, 14), as well as polymeric PGN have been reported to synergistically enhance immune responses towards the staphylococcal cell wall components (132, 149). Therefore, it seems likely that bacterial PGN amplifies host responses to staphylococcal PAMPs incorporated in the PGN exoskeleton. This function could be either mediated by improved presentation of these PAMPs to the

respective immune receptors or by shielding potential immunostimulatory cell wall constituents from interactions with inhibitory serum components.

6.2 Role of lipoteichoic acid in innate immune recognition

In Gram-positive bacteria, LTA is anchored to the plasma membrane via a diacylglycerol moiety but bears a polyglycerolphosphate appendage that protrudes through the PGN layer and is exposed to the surface (29). LTA is known to function as a prominent bacterial PAMP capable of inducing an inflammatory response that is qualitatively similar to the pattern of immunostimulation and proinflammatory mediator production elicited by LPS (68). During microbial invasion, LPS and LTA can be released into the bloodstream when the bacteria are killed either by the cells of the immune system or following antibiotic treatment (36). However, in contrast to the large quantities of spontaneously accumulated LPS detected in the serum of patients suffering from Gram-negative sepsis (108), the amount of LTA released during Gram-positive infections as well as its effect on the clinical outcome remains controversial. Whereas some studies demonstrated soluble LTA released after antibiotic treatment to enhance innate immune activation characterized by increased chemokine release as well as phagocytic activity (82), other investigators observed attenuated cytokine induction by soluble LTA due to interaction with inhibitory serum components (77, 102). As LTA was found to attach to the surface of hydrophobic but not hydrophilic incubation vessels, resulting in a strongly enhanced LTA-mediated cytokine release (20), the discrepancies described above might be explained by the use of different experimental setups. Unlike soluble LTA, the immobilized molecule bound to hydrophobic surfaces (20) rather reflects the physiological situation, where LTA is anchored to the staphylococcal cell membrane via its two fatty acids potentially shielding it from previously observed interactions with inhibitory serum proteins (40, 102). To address this, the requirements for LTA-mediated cytokine induction in human whole blood as well as potential serum interaction partners influencing the immune activation were analyzed in the second and the third part of this PhD thesis. Thereby the discrimination between soluble and immobilized LTA considered possibly different consequences on the immune response.

In recent years the immunostimulatory capacity of *Staphylococcus aureus* derived LTA has been discussed controversially (46, 143) since bacterial lipoproteins were hypothesized to contaminate LTA preparations (45). Due to potentially similar

chromatography properties of both molecules, minor amounts of lipoprotein contaminations below the detection limit of analytical chemical methods are conceivable to elute in the same hydrophobic interaction chromatography fractions as LTA. However, these putative lipoproteins are not yet identified and their contribution to innate immune activation remains to be investigated. Recently, Stoll et al. (126) constructed a *Staphylococcus aureus lgt::ermB* mutant that completely lacks palmitate-labeled lipoproteins. Compared to the wild type LTA, we observed equipotent cytokine release in human whole blood induced by LTA from this ∆lgt-mutant and furthermore we were not able to detect any differences between both molecules regarding their structure and chain length or composition of the fatty acids as revealed by NMR-analysis and GC-MS analysis. Therefore, LTA extracted from this *lgt* mutant strain was additionally used in all parts of this PhD thesis addressing the immunostimulatory potency of *Staphylococcus aureus* LTA to exclude any possible lipoprotein contaminations.

6.2.1 Innate immune recognition of soluble LTA

Isolated LTA, released from the bacterial cell wall during infection, initially comes into contact with components of human serum that possibly influence its interaction with innate immune receptors. To mimic this mechanism of immune recognition in a human *ex vivo* model, we stimulated human blood cells with soluble LTA preincubated with autologous serum and determined the release of proinflammatory cytokines. In accordance with previous reports (40, 102), we observed a suppressive effect of serum preincubation on LTA-induced cytokine release, arguing for an interaction of soluble LTA with inhibitory serum components able to prevent the LTA-mediated immune response. In the literature, this immunosuppressive serum effect is partially attributed to LBP (102, 118), however in the present study apolipoprotein B100 was also found to bind and subsequently neutralize cytokine induction of soluble LTA. In contrast to LTA embedded in the bacterial cell wall, the lipophilic diacylglycerol anchor of soluble LTA is not shielded and presumably renders the fatty acids a target for serum lipoproteins. This also concurs with the observation that all human lipoprotein classes are capable of binding soluble LTA with high-density lipoproteins displaying the highest affinity for LTA followed by low-density lipoproteins and very low-density (77). Furthermore, the physiological relevance of serum lipoproteins during bacterial infection was confirmed by incubation of LPS with lipoproteins prior to *in vivo* administration, which strongly

attenuated LPS-mediated cytokine secretion (42, 43). Taken together, our results indicate that among serum lipoproteins apolipoprotein B100 in particular represents an important serum component that attenuates LTA-mediated activation of monocytes and thus prevents the host from an adverse overproduction of proinflammatory cytokines.

6.2.2 Innate immune recognition of immobilized LTA

In the last part of this PhD thesis the immunostimulatory capacity of LTA attached to a hydrophobic surface was investigated. Unlike the soluble form of LTA probably released in a particular amount during Gram-positive infection but neutralized by serum components with very fast kinetics (77), LTA in its natural environment anchors to the plasma membrane via its two fatty acids. In agreement with previous studies (20, 106), we observed strongly potentiated cytokine induction with LTA immobilized to a surface prior to stimulation of human blood cells. However, the use of Δlgt-LTA for the first time demonstrated that LTA-induced cytokine release is not only enhanced by binding to a surface but necessarily requires immobilization. Therefore we suggest that the immobilization process reflects the physiological situation in the staphylococcal cell wall and shields the fatty acids from association with inhibitory serum proteins. Considering the fact that only a very small proportion of LTA (~1-4%) becomes immobilized during incubation (20), it seems likely that the immunostimulatory capacity of LTA anchored to the staphylococcal cell wall is drastically underestimated by stimulation of immune cells with isolated LTA attached to a surface.

Due to already described interactions between soluble LTA and serum components considerably influencing the innate immune response towards soluble LTA, we further examined the role of serum in cytokine secretion induced by immobilized LTA. Interestingly, stimulation of human blood cells, in the absence of any supplemented serum, completely abrogated LTA-induced cytokine release even under immobilizing conditions. Employing interaction studies followed by SDS-PAGE profiles and MALDI-TOF mass spectrometry analysis we were able to identify Ag-specific IgG antibodies interacting with immobilized LTA. Using IgG-depleted serum we further observed significantly reduced cytokine induction from human blood cells, pointing to a pivotal role of IgG in mediating LTA-induced cytokine release. As antibodies recognizing the polyglycerol phosphate backbone of LTA have been demonstrated to become elevated during Gram-positive bacterial infection (88), we further investigated possible correlations between LTA-specific IgG antibody serum titers and cytokine response

following LTA stimulation. Thereby LTA-induced cytokine levels turned out to be up to fivefold increased in whole blood of donors showing high LTA antibody titers. With regard to the recently observed essential contribution of phagocytosis to *Staphylococcus aureus* mediated immune response (58, 66, 127) it is tempting to speculate that in the presence of human serum LTA is opsonized by specific antibodies leading to Fc receptor-mediated uptake of LTA by phagocytic immune cells and subsequent recognition within intracellular compartments. Intriguingly this would also explain the inability of LTA to induce IL-12 and subsequent IFN-γ observed in our experimental system (50), since ligation of phagocytic receptors on immune cells during stimulation with TLR ligands has been found to selectively downregulate IL-12 transcription (39, 90, 129). In summary, our data suggest a mechanism for LTA-mediated immune activation in human whole blood that involves opsonization of immobilized LTA by specific antibodies, thereby enabling phagocytic uptake of LTA and subsequent recognition within the intracellular compartment.

6.3 Contributions to the understanding of immune recognition of *S. aureus*

There is growing evidence that *S. aureus*-mediated innate immune activation depends on a highly complex interplay between different receptors and receptor-associated adapter molecules, either located at the surface of the host cells or within intracellular compartments. This thesis, analyzing structural and functional requirements for the recognition of specific staphylococcal cell wall components by human whole blood, reveals novel findings that might contribute to the understanding of how *S. aureus* is sensed during infection. Since for the first time the influence of IgG antibodies in the recognition of LTA has been described, it would be interesting to analyse whether these antibodies are truly LTA-specific or rather show cross-reactivity with DNA, potentially sharing putative epitopes. Assuming its specificity for LTA, it might be of high interest to investigate the source of these antibodies. It seems to be possible that LTA-specific antibodies are produced during infection with Gram-positive bacteria, which was likely experienced at some stage by all blood donors tested (> 22 years old) in this thesis. However, unlike the latter donors, LTA-specific antibodies might be lacking or present only in very low amounts in neonates or very young children, which could be of importance for their control of *S. aureus* infection. Although here *S. aureus*-mediated immune activation in whole blood was found to be comparable between donors with high and low LTA antibody titers, we cannot exclude that individuals lacking LTA

antibodies for any reason, would display a decreased response to *S. aureus* infection. This potentially also explains, why the commercial anti-LTA antibody used in this thesis was beneficial in preventing Gram-positive infection in neonates, as found in a phase II study.

The data of this thesis also strongly support a novel model in which *S. aureus* recognition is mainly mediated within intracellular compartments. As observed for whole *S. aureus* bacteria, recognition of purified LTA was strongly dependent on intracellular uptake. Furthermore, immune activation by *S. aureus* PGN was also found to be sensitive to phagocytosis inhibition albeit, it cannot be excluded that this finding is due to LTA contaminations in PGN preparations. With regard to the intracellular recognition of LTA, it would be interesting to investigate the mechanism and the receptors involved in this uptake and signaling process. Since antibodies were found to play a prominent role in LTA recognition we assume that Fc receptor-mediated activation of phagocytosis initiates the uptake into intracellular compartments. In this respect, it needs to be clarified how LTA in its immobilized form, here described as a prerequisite for immune activation, can efficiently be internalized by the immune cells.

Taken together, the findings of this thesis support a novel mechanism for LTA-mediated immune activation in human whole blood that involves opsonization-dependent uptake of LTA, enabling intracellular recognition.

7 Summary

Staphylococcus aureus (*S. aureus*), a frequent human pathogen, often colonizes humans asymptomatically but can also establish severe infections of tissue or spread to the blood, leading to sepsis and sometimes death. Upon infection, the bacteria are recognized by specific immune receptors of the host able to bind to different molecular patterns of *S. aureus*, thereby inducing anti-bacterial responses, such as inflammation. Innate immune activation by *S. aureus* strongly depends on Toll-like receptor (TLR) 2, which, beside other staphylococcal cell wall components, recognizes lipoteichoic acid (LTA), a major immunostimulatory molecule of all Gram-positive bacteria. Despite the prominent role of LTA in *S. aureus*-mediated immune activation, purified LTA selectively fails to initiate the release of IL-12 and subsequent IFNγ in human whole blood. In contrast, stimulation of blood cells with whole *S. aureus* results in a strong IFNγ release, suggesting further staphylococcal components to be responsible for its production. In the first part of this thesis, the IFNγ inducing principle of *S. aureus* cell walls was characterized.

- Stimulation of human whole blood with staphylococcal cell wall preparations revealed that *S. aureus* triggers IFNγ production in NK cells and T cells in a TNF and IL-12 dependent manner. IFNγ release was found to be sensitive to phagocytosis inhibition and to treatment with enzymes digesting peptidoglycan (PGN), pointing to an important role of ingested PGN in *S. aureus*-induced IFNγ production. However, knock-out mice deficient in TLR2 or Nod2, both considered essential PGN receptors, did not show significantly altered IFNγ release upon stimulation with *S. aureus* cell wall preparations. The experiments indicate that not *S. aureus* PGN itself represents a major IFNγ-inducing component but that it amplifies the recognition of PGN-associated immunostimulatory molecules by mediating their intracellular uptake or presentation to the respective immune receptors.

Despite the pivotal role of LTA in *S. aureus*-mediated immune activation, soluble LTA has been described to be a weak inducer of inflammatory responses *in vivo*. Furthermore, soluble LTA released from the staphylococcal membrane was found to interact with human serum components, which might influence its recognition by innate immune receptors. In the second part of this PhD thesis LTA-binding proteins were

identified from human serum and their influence on LTA-mediated cytokine secretion was investigated.

- SDS-PAGE profiles of human serum proteins, fractionated by gel chromatography in the presence or absence of *S. aureus* derived LTA, revealed interactions between LTA and different apolipoproteins. Among these apolipoproteins, significant inhibition of LTA-induced cytokine release was demonstrated for Apo B100 and could be mirrored by incubation of soluble LTA with human serum prior to stimulation of human blood cells.

Despite strong evidence that LTA represents a major immunostimulatory principle of Gram-positive bacteria, recent reports suggested that not LTA but lipoproteins are dominant immunobiologically active structures of *S. aureus* and that the activity of purified LTA lies in contaminating lipoproteins. This was concluded from a 100-fold decreased immunostimulatory capacity of LTA preparations from mutant *S. aureus* lacking palmitate-labeled lipoproteins (Δlgt) compared to LTA from the respective wild type strain (wt). In contrast, using the same bacterial strains and comparable experimental systems, i.e. human whole blood, we were able to demonstrate equipotent cytokine induction between Δlgt-LTA and wt-LTA. In order to find an explanation for these discrepancies in Δlgt-LTA-mediated immune activation, the specific requirements for Δlgt-LTA recognition were investigated in the last part of this thesis.

- Cytokine induction by Δlgt-LTA in human whole blood was critically dependent on LTA immobilization, phagocytic activity of blood cells and the presence of human serum, whereas wt-LTA showed only an incomplete dependency. Furthermore, Δlgt-LTA-induced immune activation was strongly augmented by LTA-specific IgG antibodies found to be present in human sera at varying levels. These results suggest a novel mechanism for ΔLTA-mediated immune activation in human blood cells that involves opsonization-dependent uptake of LTA enabling intracellular recognition and further explain why a previous study was unable to detect Δlgt-LTA-induced immune activation.

In summary, the results of this thesis contribute to the understanding of the innate immune response against *Staphylococcus aureus* with regard to interactions of conserved bacterial patterns with components of the human immune system. The presented data may open new approaches to anti-inflammatory or anti-infectious treatment of *Staphylococcus aureus* associated diseases.

8 Zusammenfassung

Staphylococcus aureus (*S. aureus*) ist eines der wichtigsten humanpathogenen Bakterien. Neben lokalen Entzündungen kann der Erreger schwere systemische Erkrankungen auslösen, bei denen sich die Bakterien über den Blutstrom verbreiten und die nicht selten einen tödlichen Verlauf nehmen. Im Verlauf einer Infektion werden charakteristische Merkmale der Bakterien von den Zellen des angeborenen Immunsystems über spezifische Rezeptoren erkannt. Dadurch wird eine Entzündungsreaktion ausgelöst, die letztlich das Absterben der Bakterien herbeiführt. In der Immunerkennung von *S. aureus* stellt Lipoteichonsäure (LTA) eine wesentliche immunstimulatorische Komponente dar, die neben weiteren Bestandteilen der Gram-positiven Zellwand von Toll-like Rezeptor (TLR) 2 erkannt wird. Das immunstimulatorische Potenzial von *S. aureus* ist größtenteils auf LTA zurückzuführen, die in der Lage ist, die Immunzellen zur Freisetzung zahlreicher proinflammatorischer Zytokine zu stimulieren. Allerdings konnte gezeigt werden, dass lösliche LTA im Gegensatz zu ganzen *S. aureus* Bakterien keine IFNγ induzierende Aktivität aufweist. Dies deutet darauf hin, dass *S. aureus* neben LTA weitere immunstimulatorische Komponenten besitzt, die im Zuge einer Infektion für die Freisetzung von IFNγ verantwortlich sind. Im ersten Teil dieser Arbeit wurde das IFNγ induzierende Prinzip von *S. aureus* charakterisiert.

- Nach Stimulation von humanem Vollblut mit einem bakteriellen Zellwandlysat konnte gezeigt werde, dass IFNγ von NK Zellen sowie T Zellen freigesetzt und durch die Monokine TNF und IL-12 initiiert wird. Durch Inhibition der Phagozytoseaktivität, sowie durch den enzymatischen Verdau des PGN-Netzwerks konnte die IFNγ Ausschüttung deutlich vermindert werden, was darauf hindeutet, dass durch die Aufnahme von PGN intrazelluläre Immunrezeptoren angesprochen werden. Da sowohl TLR2 als auch Nod2 als mögliche Rezeptoren für PGN diskutiert werden, wurde im folgenden die Rezeptorabgängigkeit des *S. aureus* Zellwandlysats mit Hilfe von $TLR2^{-/-}$ und $Nod2^{-/-}$ Mäusen untersucht. Allerdings konnte nach der Simulation mit *S.* aureus-Zellwandlysat weder bei $TLR2^{-/-}$ noch $Nod2^{-/-}$ Mäusen eine Veränderung hinsichtlich der IFNγ Freisetzung beobachtet werden. Diese Ergebnisse deuten darauf hin, dass die Sekretion von IFNγ nicht durch PGN selbst induziert wird, sondern die Phagozytose und anschließende intrazelluläre

Immunerkennung von weiteren Zellwand-assoziierten Bakterienbestandteilen durch ein intaktes PGN-Netzwerk ermöglicht werden.

Obwohl LTA bereits als entscheidende immunstimulatorische Komponente der bakteriellen Zellwand identifiziert wurde, konnten mit löslicher LTA im Tiermodel bisher nur geringfügige Entzündungsreaktionen hervorgerufen werden. Da während einer Infektion mit Gram-positiven Bakterien LTA aus der Zellwand freigesetzt werden kann, besteht die Möglichkeit, dass Komponenten des humanen Serums mit löslicher LTA interagieren und dadurch ihre Immunerkennung beeinflussen. In zweiten Teil dieser Arbeit wurden LTA Bindeproteine aus humanem Serum identifiziert und deren Einfluss auf LTA-induzierte Zytokinfreisetzung untersucht.

- Vergleichende Analysen humaner Serumproben, die entweder mit oder ohne *S. aureus* LTA inkubiert wurden, konnten zeigen, dass lösliche LTA von verschiedenen Serum-Apolipoproteinen gebunden wird, wobei jedoch nur ein Apolipoprotein in der Lage war LTA-induzierte Zytokinfreisetzung in humanem Vollblut signifikant zu verringern. Dieser von Apo B100 hervorgerufene inhibitorische Effekt konnte ebenfalls durch autologes Serum nachgestellt werden, wenn dieses vor Stimulation der Blutzellen mit LTA zusammen inkubiert wurde.

Obwohl LTA generell als immunstimulatorisches Prinzip Gram-positiver Bakterien angesehen wird, wurden die immunstimulatorischen Eigenschaften der LTA kürzlich in Frage gestellt. Nachdem LTA einer *S. aureus* Mutante (Δlgt), die aufgrund der Deletion des Gens für das Enzym „Lipoprotein Diacylglycerol Transferase“ keine Lipoproteine bildet, in einer Studie nur noch eine um 100fach verringerte Zytokinfreisetzung aufwies, wurde das immunstimulatorische Potenzial der Wildtyp-LTA Lipoprotein-Kontaminationen zugeschrieben. Im Gegensatz dazu konnte in unserem Labor gezeigt werden, dass Δlgt-LTA und wt-LTA unter denselben experimentellen Bedingungen eine vergleichbare Zytokin-Antwort in humanem Vollblut induzieren. Um eine Erklärung für diese Widersprüche zu finden, wurden im letzten Teil dieser Arbeit die spezifischen Bedingungen für eine erfolgreiche Immunerkennung von Δlgt-LTA untersucht.

- Es konnte gezeigt werden, dass die Immunerkennung der Δlgt-LTA sowohl von der Immobilisierung der LTA an bestimmten Oberflächen, als auch von der Phagozytoseaktivität der Blutzellen abhängt. Darüber hinaus wurde Δlgt-LTA-induzierte Zytokinfreisetzung durch LTA-spezifische IgG Antikörper in

humanem Serum verstärkt, wobei die Menge an freigesetzten Zytokinen mit dem Antikörpertiter des jeweiligen Blutspenders korrelierte. Diese Ergebnisse deuten auf einen neuen Mechanismus der LTA-induzierten Immunaktivierung in humanem Vollblut hin. Ferner erklären sie, warum Δlgt-LTA in der vorangegangenen Studie nicht in der Lage war, eine Immunantwort auszulösen.

Zusammenfassend lässt sich sagen, dass diese Arbeit zu einem besseren Verständnis der angeborenen Immunantwort gegen *S. aureus* beiträgt. Die hier präsentierten Daten verbessern nicht nur unser Wissen über die vielfältigen Wechselwirkungen zwischen charakteristischen Bakterienbestandteilen und Komponenten des menschlichen Immunsystems, sondern eröffnen auch neue Möglichkeiten zur anti-infektiösen oder anti-entzündlichen Behandlung von *S. aureus* assoziierten Krankheiten.

9 Declaration of authors' contributions

- **S. Sigel,** V. Herrmann, C. Draing, S. Bunk, T. Hartung and S. von Aulock: Peptidoglycan directly and indirectly contributes to *Staphylococcus aureus*-mediated IFNγ release in human whole blood. *Submitted*

The experiments were performed by Stefanie Sigel and Valerie Hermann under the supervision of Christian Draing and Sonja von Aulock. The study was designed by Thomas Hartung, Sonja von Aulock, Stefanie Sigel and Sebastian Bunk. The manuscript was written by Stefanie Sigel.

- **S. Sigel,** S. Bunk, J. Hoffmann, S. Deininger, T. Meergans and S. von Aulock: Lipoteichoic acid induced cytokine release is inhibited by apolipoprotein B100. *Submitted*

The experiments were performed by Stefanie Sigel and Julia Hoffmann under the supervision of Sonja von Aulock, Sebastian Bunk and Thomas Meergans. The study was designed by Sonja von Aulock. The manuscript was written by Stefanie Sigel.

- **S. Sigel,** S. Bunk, D. Metzdorf, T. Hartung and S. von Aulock: Immune activation by lipoteichoic acid depends on presence of immunoglobulin G: Role of IgG-mediated opsonization. *Submitted*

The experiments were performed by Stefanie Sigel, Sebastian Bunk and Daniela Metzdorf under the supervision of Sebastian Bunk and Sonja von Aulock. The study was designed by Sebastian Bunk, Stefanie Sigel, Sonja von Aulock and Thomas Hartung. The manuscript was written by Stefanie Sigel.

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