Comparative evaluation of Gram-positive membrane components in activating the innate immune system

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Abbreviations

ANOVA	analysis of variance
BPI	bactericidal/ permeability-increasing protein
CD	cluster of differentiation
CHO	chinese hamster ovary cells
CpG motifs	repetitive cytosine-guanosine dinucleotide sequences
DAP	diaminopimelic acid
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin unit
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC-MS	gas chromatography-mass spectroscopy
GlcNAc	N-acetyl-glucosamine
HEK293	human embryonic kidney cells
HF	hydrofluoric acid
HIC	hydrophobic interaction chromatography
IFNγ	interferon γ
IL	interleukin
LAL	Limulus amoebocyte lysate test
LBP	lipopolysaccharide-binding protein
LP	bacterial lipoproteins
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LTA SA 113	LTA from *Staphylococcus aureus* from strain 113
MALDI	matrix-assisted laser desorption/ionization
MALP-2	*Mycoplasma fermentans* lipopeptide-2
MAP	mitogen-activated protein
MD	Lymphocyte antigen
mRNA	messenger RNA
MRS	bacterial culture medium (DSMZ)
MRSA	methicillin-resistant *Staphylococcus aureus*
MurNAc	N-acetyl-muramic acid

NOD	nucleotide-binding oligomerisation domain
NMR	nuclear magnetic resonance
$Pam_2Cys\text{-}SK_4$	dipalmitoyl S-glycerol Cys-Ser-Lys_4
$Pam_3Cys\text{-}SK_4$	bispalmitoyloxy-propyl-N-palmitoyl-Cys-Ser-Lys_4-OH
PAMP	pathogen-associated molecular pattern
PBMC	human peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGN	peptidoglycan
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
RPMI	cell culture medium
RNA	ribonucleic acid
SA 113 Δdlt	*Staphylococcus aureus* dlt-operon deletion mutant
SA 113 Δlgt	*Staphylococcus aureus* lipoprotein diacylglycerol transferase deletion mutant
SA 113 ΔTA	*Staphylococcus aureus* teichoic acid deletion mutant
SA 113 wt	*Staphylococcus aureus* wild type
SDS	Sodium Dodecyl Sulphate
TA	teichoic acid
TIR	Toll/interleukin-1 receptor
THP-1	Human acute monocytic leukaemia cell line
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	tumor necrosis factor
UV	ultraviolet light
VISA	vancomycin-intermediate *Staphyloccocus aureus*
WTA	wall teichoic acid

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1 INTRODUCTION

1.1 Innate and adaptive immunity

The vertebrate's immune system is a system of specialized cells and organs that protects an organism from infectious threats, i.e. bacteria, nematodes, fungi and viruses, and destroys cancer cells. It is divided into two sections:
The innate immune system comprises soluble components and effector cells that provide an immediate "first-line" of defence to continuously ward off pathogens. A broad variety of organisms share the mechanisms of innate immunity, indicating that this is an ancient form of host defence (101). There are several different types of phagocytic cells, i.e. neutrophils, macrophages, and dendritic cells, which ingest and destroy invading pathogens upon recognition of pathogen-associated molecular patterns (PAMPs) (102 1997) and/or respond by releasing inflammatory cytokines and chemokines, which induce an acute phase reaction in the liver, induce the recruitment of more immune cells and control the inflammatory response. Another cell type, natural killer cells, are especially adept at destroying cells infected with viruses. The complement system consists of normally inactive components of the blood, which are activated to recruit inflammatory cells, coat pathogens to make them more easily phagocytosed, and to insert as destructive pores in the surface of pathogens.

The adaptive or acquired immune system can develop a specific immunity to particular pathogens. This response takes days to develop, and so is not effective at preventing an initial invasion, but it will normally prevent any subsequent infection, and also aids in clearing up longer-lasting infections. One the one hand, B-cells produce specific antibodies designed to target particular pathogens. On the other hand, cytotoxic T-cells recognise infected cells and kill them, and helper T-cells activate and induce the proliferation of immune cells.
The research done in this thesis has focused on the interactions between cells of the human and murine innate immune system with bacteria.

1.2 Bacterial infection and sepsis

An infection is the destructive colonisation of a host organism by microorganisms. The initial response of the host is a process called inflammation with symptoms like heat, swelling, pain, redness and dysfunction of the organs. An overwhelming inflammation over the whole organism is termed sepsis and includes symptoms like increased heart rate and hyperventilation, fever and drastically modified leukocyte counts. This can result in multiple organ dysfunctions or even death. Sepsis is common and a major cause of death in intensive care units worldwide with mortality rates that range from 35-45% (126). A major problem is the increasing incidence of antibiotic resistance of bacteria, for example methicillin-resistant (MRSA) and vancomycin-intermediate (VISA) strains of *Staphyloccocus aureus*, a major human pathogen that infects both hospitalised and healthy people in community (83, 94). This increasing risk factor for human beings makes it important to understand the interactions between bacteria and the human immune system which can lead to better and new strategies to combat infections and sepsis.

1.3 Composition of bacterial cell walls

Bacteria can be divided into two groups based on the nature of their cell wall. Gram-negative bacteria have a cell wall consisting of a thin peptidoglycan (PGN) layer to which the outer membrane is attached via lipoproteins. This outer membrane contains proteins, phospholipids and lipopolysaccharide (LPS), which is anchored via its hydrophobic part to the phospholipids layer. The thin PGN layer prevents integration of blue or violet dye by method of Gram-staining and the cells remain red or pink. In contrast, Gram-positive have a thick PGN layer of about 40-50 sheets with bound teichoic acids (TA) building a murein sacculus. Lipoteichoic acids (LTA) are non-covalently anchored to their cytoplasmatic membrane (figure 1).

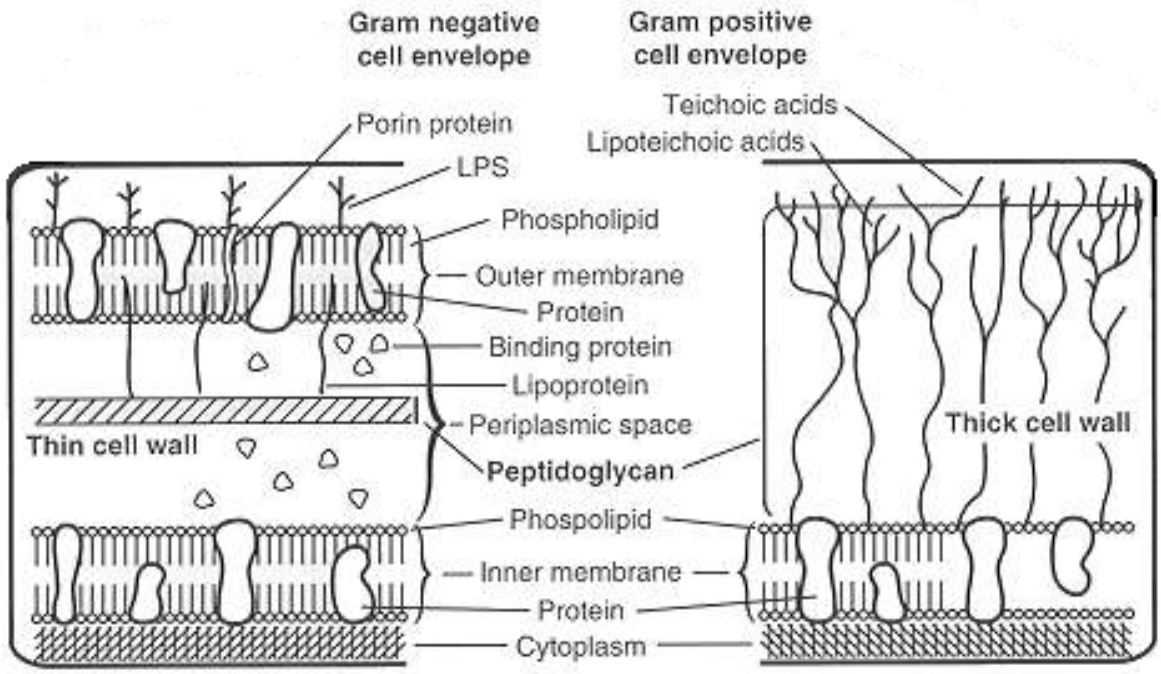

Figure 1

Comparison of the cell wall of Gram-positive and Gram-negative bacteria.

[Milton R.J. Salton; Kwang-Shin Kim; http://gsbs.utmb.edu/microbook/ch002.htm]

1.4 Pattern associated molecular patterns

Pathogen associated molecular patterns (PAMPs) are highly conserved small molecular sequences consistently found on different groups of pathogens and are recognised by pattern recognition receptors (PRRs) like toll-like receptors (TLR) or scavenger receptors on immune cells. The engagement of PRRs by PAMPs initiated host defence mechanisms. LPS form Gram-negative bacteria, LTA from Gram-positive bacteria, PGN breakdown products like muropeptides, double-stranded RNA (ds-RNA) from viruses, lipoproteins and bacterial flagellin (107) are typical PAMPs.
Cells of the innate immune system are thus able to recognise and respond quickly and appropriately to infectious threats by focusing on these few highly conserved structures.

1.4.1 Lipopolysaccharide

LPS is the principle component of the outer membrane of Gram-negative bacteria (138). It is an amphiphilic molecule and consists of an outer part made of the O-antigen, a core region and a glycolipid anchor, which embeds the LPS in the outer membrane. The O-antigen is formed by repeating oligosaccharide sequences, which are species-specific for the Gram-negative bacteria strains. The glycolipid anchor, also called Lipid A, is highly conserved and represents a strong stimulus for the innate immune system (167). The release of LPS from degraded bacteria during an infection activates immediate immune responses like release of cytokines and the activation of the complement system, both inducing anti-bacterial defence (5). During bacteraemia, the systemic release of LPS can lead to an excessive TNF release and subsequently septic shock and multi-organ failure (113, 134). LPS is recognised by a complex of the LPS binding protein, the glycosylphosphatidylinositol-anchored membrane protein CD14, together with MD-2 and a homodimer of TLR4 (133, 189). TLR4 was the first mammalian TLR shown to be a PRR (103 1997) and was discovered by the use of mice of the C3H/HeJ strain, which have a point mutation in the TIR domain of TLR4 and are therefore LPS-nonresponsive (133). Furthermore, LPS is well established as the crucial stimulus of immune responses (139).

1.4.2 Lipoteichoic acid

LTA is part of the Gram-positive cell wall and exhibits like LPS an amphiphilic molecule structure. It possesses a lipid part with two fatty acids, which is anchored non-covalently in the cell membrane (115). The hydrophilic part consists of glycerol-phosphate or ribitol-phosphate units and is linked to its lipid anchor via a disaccharide. The backbone units of LTA repeat up to 50 times and its residues are mostly substituted (112).

The role of LTA as an immunostimulatory component has been intensively investigated and discussed (22, 108). LTA binds to TLR2 (88), requires co-receptors like CD14, LPB and MD-2 (58) and engages TLR2 and TLR6 heterodimer (57) and lipid rafts (172). Meanwhile, the importance of LTA as a major immunostimulatory component has been challenged (52) by describing LTA isolated from a diacylglycerol transferase deletion mutant (Δlgt) of *S. aureus* which was 100-fold less

potent than the LTA of the corresponding wild-type. Furthermore it was suggested that not LTA but lipoproteins are the dominant immunobiologically active compound in *Staphylococcus aureus* (51). However, von Aulock et al. have shown that in case of human whole blood, i.e. stimulation of human primary monocytic cells both LTA posses comparable cytokine inducing potency (178). Meanwhile, further studies were conducted and are part of this thesis or are discussed in this thesis to provide further facts and details to this ongoing discussion.

1.4.3 Peptidoglycan

The bacterial cell walls of both, Gram-negative and Gram-positive bacteria contain PGN, a polymer of repeating units of N-acetyl-muramic acid (MurNAc) and N-acetyl-glucosamine (GlcNAc). The MurNac is linked to a peptide chain of amino acids that are cross-linked with the peptide chain of the next MurNac (D-alanine to meso-diaminopimelic acid). The precise sequence of the peptide chain is species-dependent, but it mainly contains L-alanine, D-glutamine, lysine or diaminopimelic acid (DAP). Peptide bridges between amino acids located in different glycan chains lead to the formation of a complex three-dimensional macromolecule, which forms an enormous, covalently bound closed basket around the cytoplasmatic membrane (183). This arrangement of polymeric glycan, cross-linked by peptides, plays a major role in the determination of cell shape and in maintenance of the physical integrity of the bacterium. Although there are numerous subtle variations in the PGN structure among different organisms, the composition and the organization of PGN is highly conserved in nature.

Pattern recognition of PGN in humans is intensively discussed. First studies indicated signalling via TLR2 (149, 192) using TLR2 transfected HEK293 and CHO cells. Furthermore this was confirmed using TLR2 knock-out mice (164). However, recent studies have shown that PGN has to be purified intensively which abolishes the immunostimulatory properties (171). They further concluded that it is more likely that PGN detection occurs intracellularly via NOD1/2. However, the relationship between PGN and immune recognition in human being is not yet solved and one part of this thesis contributes more information and facts to this discussion.

1.4.4 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 (38, 61, 86). For Gram-positive bacteria, some lipoproteins have been identified, e.g. for *Staphylococcus aureus* (28, 55, 187). In contrast to triacylated lipoproteins of Gram-negative bacteria, Gram-positive bacterial lipoproteins contain conserved diacylated cysteins at their N-terminus and are postulated to be recognised by a TLR2/6 heterodimer (70). Recently, a S. aureus mutant lacking lipoproteins based on a deletion of the diacylglycerol transferase was reported to evade the human immune system and cause lethal infections *in vivo* (11). As it seems to be difficult to isolate lipoprotein structures from Gram-positive bacteria, the immunostimulatory properties of bacterial lipoproteins can be mimicked by synthetic variants like $Pam_2Cys\text{-}SK_4$ or $Pam_3Cys\text{-}SK_4$ which have been demonstrated to activate TLR2 (9, 93).

2 Aims of the study

For Gram-positive bacteria the role of the different major cell wall components i.e. lipoteichoic acid (LTA), peptidoglycan (PGN) and cell wall incorporated lipoproteins (LP), to activate the immune system is highly controversial. In this study, we aim to investigate the role of each of these structures. First a meta-analysis and therefore a systematic review addressing the fulfilment of the four well-known criteria of Koch and Dale by LTA, PGN and LP shall be done. The criteria are:

- Evidence for presence of LTA, PGN or LP in cytokine inducing bacteria
- Synthesis inhibition of LTA, PGN or LP impairs cytokine induction
- Exposure to LTA, PGN or LP induces cytokine induction
- Blocking of LTA, PGN or LP by antibodies or antagonists prevents or reduces cytokine induction

The approach is to compile current best evidence for a causal role of LTA, PGN and LP as inducers of inflammatory cytokine release from human monocytes / macrophages and therefore be able to draw conclusions of the role of these structures in human innate immune recognition of Gram-positive bacteria.
Second we aim to study the role of the different cell wall components in activating human and murine innate immune cells by using *S. aureus* (SA) 113 wt and respective S, aureus mutants. The bacterial mutants include: SA 113 *lgt*::*ermB* (SA 113 Δlgt) lacking the lipoprotein diacylglycerol transferase resulting in a lack of lipoproteins in the bacterial cell wall, SA 113 ΔdltA (SA 113 Δdlt) lacking the dlt operon and thus leading to a reduced alanine content of the LTA and SA 113 ΔtagO (SA 113 ΔTA) lacking wall teichoic acids (WTA). As read-out system, cytokine release from human whole blood and primary murine cells is used. To gain more insights about the precise role of each cell wall component, either whole bacteria or the cell wall components LTA and PGN will be prepared from the different *S. aureus* strains and be used as a stimulus for the immune cells. LTA shall be prepared according to an established protocol by Morath et al. (108), in case of PGN, the purification procedure shall be established according to Girardin et al. (43).
As especially the role of lipoprotein contaminations of LTA in recognition of Gram-positive bacteria by the human immune system is discussed, we aimed to compare

purified LTA from SA 113 wt and SA 113 Δlgt, the mutant lacking lipoproteins in the cell wall, in more detail in the third part of this thesis. Historically, LTA was first described as an immune stimulus signalling via Toll-like receptor 2 (TLR2), but this was challenged recently by Hashimoto et al. (52) isolating an inactive LTA from the S. aureus mutant Δlgt resulting in a loss of lipoproteins. The LTAs were used as a stimulus for human whole blood, monocytic cell lines, mouse models and a microarray analysis to identify and deduce differences in immune recognition and their origins. Furthermore, these studies should provide us with more information about the role of lipoproteins and LTA in innate immune recognition.

3 A systematic review of membrane components responsible for inducing human monocyte / macrophage cytokine release by Gram-positive bacteria

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3.1 ABSTRACT

50 years after the elucidation of lipopolysaccharides as the principal structure of Gram-negative bacteria activating the human immune system, its Gram-positive counterpart is still under debate. Following evidence-based medicine approaches, a systematic review of existing evidence was undertaken. For the three major constituents currently discussed, i.e. peptidoglycan, lipoteichoic acids and bacterial lipoproteins, the questions to be answered and a search strategy for relevant literature starting in MedLine was developed and reviewed. The evaluation was based on the Koch-Dale criteria for a mediator of an effect. 380 articles for peptidoglycan, 391 for bacterial lipoproteins and 285 for lipoteichoic acid were retrieved of which 12 (peptidoglycan), 8 (lipoproteins) and 24 (LTA) fulfilled the pre-defined inclusion criteria. The compiled data suggest that for peptidoglycan two Koch-Dale criteria are fulfilled, four for lipoteichoic acids and two for immunostimulatory bacterial lipoproteins. In conclusion, based on the best currently available evidence, LTA is the only substance which fulfils all of the four Koch/Dale criteria with the limitation that only one paper was found to support the criterion 4. LTA has been isolated from a large number of bacteria, results in cytokine release patterns inducible also with synthetic LTA. Reduction in bacterial cytokine induction with an inhibitor for LTA was shown. These findings support the importance of LTA for immune recognition in humans. However, this meta-analysis can not exclude that other stimulatory compounds complement or substitute for LTA in being the counterpart to LPS in some Gram-positive bacteria.

3.2 INTRODUCTION

The scientific literature on Gram-negative lipopolysaccharides includes about 50.000 Medline-listed articles. Gram-positive bacteria are clinically by no mean less important, but neither has a structural counterpart for the induction of inflammatory reactions such as cytokine release by macrophages been unambiguously identified nor made available to give rise to similar scientific literature.

The amount of scientific publications concerning a special topic has made it very difficult to follow a given field. One possible method to manage this overwhelming amount of literature is meta-analysis, which follows well-defined rules and leads to a possibility to condense our current state of knowledge from the existing literature. Systematic reviews with meta-analysis of compiled evidence are the principal approach of Evidence-based Medicine (EBM). Systematic reviews have to be distinguished from the more common narrative reviews in the life sciences: A systematic review answers a very narrow set of precisely defined questions; the strategy to identify relevant evidence (typically by a decision tree) and how to qualify, include / exclude and to compile and interpret it are predefined and reviewed. The result is expressed if possible with a measure of uncertainty. Neugebauer et al. (121) have shown first that such EBM approaches can be translated from clinical studies to animal and in vitro findings.

The advantages and shortcomings of meta-analysis have been widely discussed (15, 16, 46, 122, 123). It is probable that the application of a tree of yes/no decisions raises the production of new faults and the preference to use methodological criteria for the selection of publications will lead to the simple result that the most actual methods deliver the most accurate data. Construction of a decision tree starts from a subjective point of view and must not be necessarily correct. In spite of such limitations, systematic reviews and meta-analysis appear to date to represent the most appropriate methods to extract the current state of art from the literature.

Here, the approach of a systematic review was taken to compile current best evidence for a causal role of peptidoglycan, lipoteichoic acids and lipoproteins as inducers of human monocyte / macrophage inflammatory cytokine release.

3.3 AIM

Different structures of the Gram-positive cell wall have been proposed as inducers of cytokine release in human monocyte / macrophages. In a systematic review adopting principles of evidence-based medicine, the fulfilment of Koch-Dale (K/D) postulates for the three most prominent candidate inducers of cytokine release lipoteichoic acids (LTA), lipoproteins (LP) and peptidoglycan (PGN) should be evaluated. To exclude a species discrepancy between the recognition of bacterial cell wall components in human or animals, only articles on primary human monocytes / macrophages and derive cell lines were used.

Koch-Dale criteria adapted to the relevant question are:

1. Evidence for presence of LTA, PGN or LP in cytokine inducing bacteria
2. Synthesis inhibition of LTA, PGN or LP impairs cytokine induction
3. Exposure to LTA, PGN or LP induces cytokine induction
4. Blocking of LTA, PGN or LP by antibodies or antagonists prevents or reduces cytokine induction

3.4 K/D CRITERION 1

3.4.1 Database for K/D criterion 1

First, it was important to verify if the three analyzed surface molecules were found or even purified from cytokine inducing Gram-positive bacteria. PGN is well known to be ubiquitous in Gram-positive bacteria (177), whereas some Gram-positive bacteria lack LTA i.e. several Micrococci (*M. luteus, M. flavus, M. sodonensis*), *Bifidobacterium bifidum* and Mycobacteria (127, 163). Bacterial lipoproteins are also essential for Gram-positive bacteria (6, 119), but we aimed to find information about isolation of these structures (Table 3). For K/D criterion 1 we therefore focused on finding publications that were able to isolate LTA from Gram-positive bacteria. Using the keywords “isolation lipoteichoic acid gram positive bacteria” we revealed 179 publications from the library of the U.S. National Library of Medicine and the National Institutes of Health (NCBI). All publications were collected in full length; their abstracts, key words, headlines, and authors were stored as a data file. For reports on bacteria lacking LTA, a secondary research was undertaken to identify for these

specific bacteria reports on cytokine induction (or its lack of) as well as other specific cytokine inducing cell wall components reported for these bacteria.

3.4.2 Selection of the literature for K/D criterion 1

To identify the publications that isolated LTA, the pool of publications was preselected according to decision tree 1 (fig. 1) and was constructed with the following demands:

1. **Original article on isolation of LTA**

   Only original articles that were able to isolate LTA confirmed by structural analysis were included, articles only reporting on the use of commercial LTA material were excluded and reviews were not taken into account as well as publications that did not reveal detailed information about the isolation procedure of LTA.

2. **Methods appropriate**

   Appropriate methods were hot or cold phenol extraction with subsequent gel chromatography, chloroform-methanol, or butanol extraction, hydrophobic interaction chromatography (HIC) and ion-exchange chromatography for purification.

The two demands led to 58 articles out of 179 publications and are listed in table 1.

name	bacteria	reference	isolation method
Serological studies on the teichoic acids of Lactobacillus plantarum	Lactobacillus plantarum	(78)	Phenol, Gel chromatography, Gel chromatography, Gel chromatography
Comparative studies on the isolation of membrane lipoteichoic acid from Lactobacillus fermenti	Lactobacillus fermenti	(184)	Chloroform-Methanol
Pneumococcal Forssman antigen. A choline-containing lipoteichoic acid	Pneumococci	(10)	Chloroform-Octanol
Lipoteichoic acid localization in mesosomal vesicles of Staphylococcus aureus	Staphylococcus aureus (ATCC 6538P)	(63)	Chloroform-Methanol

Characterization of group N streptococcus lipoteichoic acid	**Streptococcus lactis (ATCC 9936)**	**(185)**	**Phenol, Gel chromatography**
Lipoteichoic acid and lipoteichoic acid carrier in Staphylococcus aureus H	**Staphylococcus aureus H**	**(27)**	**Phenol, Gel chromatography**
Extraction and purification of lipoteichoic acids from gram-positive bacteria	**Staphylococcus aureus H, Micrococcus 2102, Bacillus subtilis 168, Bacillus subtilis W-23**	**(17)**	**Phenol, Gel chromatography**
Cellular localization of lipoteichoic acid in Streptococcus faecalis	**Bacillus licheniformis 6346 MH-1**	**(71)**	**Phenol, Gel chromatography**
Trihexosyldiacylglycerol and acyltrihexosyldiacylglycerol as lipid anchors of the lipoteichoic acid of Lactobacillus casei DSM 20021	**Lactobacillus casei DSM 20021**	**(116)**	**Phenol, Gel chromatography**
Purification of lipoteichoic acids by using phosphatidyl choline vesicles	**Streptococcus mutans BHT, AHT**	**(157)**	**gel filtration, HIC**
Alanine ester-containing native lipoteichoic acids do not act as lipoteichoic acid carrier. Isolation, structural and functional characterization	**Staphylococcus aureus (DSM 20233), Lactobacillus helveticus (DSM 20075), Streptococcus lactis (NCDO 712)**	**(36)**	**HIC**
Isolation of a Bacillus globigii cell wall component involved in binding to human lymphocytes	**Bacillus globigii**	**(153)**	**Phenol, Gel chromatography**
Immunisation of rhesus monkeys with Streptococcus mutans, Lactobacillus acidophilus and lipoteichoic acid for protection against dental caries	**Streptococcus mutans**	**(14)**	**Phenol, Gel chromatography**
Association of elevated levels of cellular lipoteichoic acids of group B streptococci with human neonatal disease	**Group B Streptococci (GBS), different strains**	**(120)**	**Phenol, Gel chromatography**

Improved Preparation of Lipoteichoic Acids	**Bacillus licheniformis DSM 13, Lactobacillus casei DSM 20021, Staphylococcus aureus DSM 20233, Streptococcus faecalis ATCC 9790, Streptococcus faccalis subsp. zymogenes Kiel27138, Streptococcus luctis Kiel42172, Streptococcus lactis NCDO 712, Streptococcus pyogenes I1 D698, Micrococcus varians ATCC 29750**	**(35)**	**Phenol, Gel chromatography, HIC**
Lipoteichoic acid from Listeria monocytogenes	**Listeria monocytogenes**	**(59)**	**Phenol, Gel chromatography**
Structure of the lipoteichoic acids from Bifidobacterium bifidum spp. pennsylvanicum	**Bifidobacterium bifidum spp. pennsylvanicum**	**(125)**	**Phenol, Gel chromatography**
Activation of the alternative complement pathway by pneumococcal lipoteichoic acid	**Streptococcus pneumoniae R36A**	**(64)**	**Chloroform-Methanol**
Chemical properties and immunobiological activities of streptococcal lipoteichoic acids	**Streptococcus pyogenes Sv, Streptococcus mutans 6715, and Streptococcus sanguis ATCC 10556**	**(48)**	**Phenol, Gel chromatography**
Comparative studies of lipoteichoic acids from several Bacillus strains	**Bacillus subtilis, Bacillus licheniformis, Bacillus pumilus, Bacillus coagulans, Bacillus megaterium**	**(67)**	**Phenol, Gel chromatography**
Lipoteichoic acids in Lactobacillus strains that colonize the mouse gastric epithelium	**Lactobacillus fermentum, Lactobacillus leichmanii, Lactobacillus reuteri, Lactobacillus acidophilus, Lactobacillus murinus**	**(152)**	**Phenol, Gel chromatography**
Structural studies on lipoteichoic acids from four	**Listeria monocytogenes**	**(173)**	**Phenol, Gel chromatography**

Listeria strains			
Structural studies of a teichoic acid from Streptococcus agalactiae type III	**Streptococcus agalactiae type III**	**(30)**	**Phenol, Gel chromatography**
Lipoteichoic acid from Bacillus subtilis subsp. niger WM: isolation and effects on cell wall autolysis and turnover	**Bacillus subtilis subsp. niger WM**	**(105)**	**Phenol, Gel chromatography**
Mediation of Staphylococcus saprophyticus adherence to uroepithelial cells by lipoteichoic acid	**Staphylococcus saprophyticus**	**(168)**	**Phenol, Gel chromatography**
'Lipoteichoic acid' of Bifidobacterium bifidum subspecies pennsylvanicum DSM 20239. A lipoglycan with monoglycerophosphate side chains	**Bifidobacterium bifidum subsp. pennsylvanicum DSM 20239**	**(33)**	**Phenol, Gel chromatography**
Stimulation of human monocyte chemiluminescence by staphylococcal lipoteichoic acid	**Staphylococcus saprophyticus strains S1 and S35**	**(124)**	**Phenol, Gel chromatography**
Structure and glycosylation of lipoteichoic acids in Bacillus strains	**Bacillus cereus (4 strains), Bacillus subtilis (5 strains), Bacillus licheniformis (1 strain), Bacillus polymyxa (2 strains), and Bacillus circulans (3 strains)**	**(68)**	**Phenol, Gel chromatography**
Distribution analyses of chain substituents of lipoteichoic acids by chemical degradation	**Lactococcus lactis Kiel 48337**	**(148)**	**Phenol, Gel chromatography**

Occurrence and structure of lipoteichoic acids in the genus Staphylococcus	**Stahpylococcus aureus, S. capitis DSM 20326, S. carnosus DSM 20501, S. epiderrnidis DSM 20044, S. hemolyticus DSM 20263, S. saccharolyticus DSM 20359, S. sciuri DSM 20352, S. warneri DSM 20316, S. cohnii DSM 20260, S. homnis DSM 20328, S. saprophyticus DSM 20229, S. simulans DSM 20322**	**(142)**	**Phenol, Gel chromatography**
On the basic structure of poly(glycerophosphate) lipoteichoic acids	**24 Gram-positive bacteria of the genera Bacillus, Enterococcus, Lactobacillus, Lactococcus, Listeria, Staphylococcus, and the streptococcal pyogenic and oral group**	**(37)**	**HIC**
Structure of macroamphiphiles from several Bifidobacterium strains	**Bifidobacterium bifidum YIT 4007 and YIT 4013, Bifidobacterium breve YIT 4010 and YIT 4014, and Bifidobacterium longum YIT 4021**	**(66)**	**Phenol, Gel chromatography**
Isolation and characterization of lipoteichoic acid, a cell envelope component involved in preventing phage adsorption, from Lactococcus lactis subsp. cremoris SK110	**Lactococcus lactis subsp. cremoris SK110**	**(156)**	**Chloroform-Methanol**
Molecular analysis of lipoteichoic acid from Streptococcus agalactiae	**S. pyogenes 147, S. mutans GS-5, Enterococcus hirae ATCC 9790, S. agalactiae 110,**	**(99)**	**Phenol, Gel chromatography**

Separation of the poly(glycerophosphate) lipoteichoic acids of Enterococcus faecalis Kiel 27738, Enterococcus hirae ATCC 9790 and Leuconostoc mesenteroides DSM 20343 into molecular species by affinity chromatography on concanavalin A	**Enterococcus faecalis Kiel27738, Enterococcus hirae ATCC 9790, and Leuconostoc mesenteroides DSM 20343**	**(90)**	**Phenol, Gel chromatography, HIC**
Small and medium-angle X-ray analysis of bacterial lipoteichoic acid phase structure	**Bifidohacterium bifidum DSM 20239, Enterococcus hirae (Streptococcus faecium) ATCC 9790, Lactococcus garviue NCDO 2155, Listrria welshimeri SLCC 5354, and S. aureus DSM 20233**	**(84)**	**Phenol, Gel chromatography, HIC**
Hydrophobic interaction chromatography fractionates lipoteichoic acid according to the size of the hydrophilic chain: a comparative study with anion-exchange and affinity chromatography for suitability in species analysis	**Enterococcus faecalis Kiel 27738 (DSM 20371)**	**(89)**	**Phenol, Gel chromatography, HIC**
D-alanyl-lipoteichoic acid in Lactobacillus casei: secretion of vesicles in response to benzylpenicillin	**Lactobacillus casei ATCC 7469**	**(132)**	**Phenol, Gel chromatography**
Molecular analysis of lipid macroamphiphiles by hydrophobic interaction chromatography, exemplified with lipoteichoic acids	**Enterococcus hirae NICB 8191, Lactococcus garvieae NCFB 2730, Staphylococcus aureus**	**(34)**	**Phenol, Gel chromatography, HIC**
The structure of pneumococcal lipoteichoic acid. Improved preparation, chemical and mass spectrometric studies	**Streptococcus pneumoniae R6**	**(4)**	**Chloroform-Methanol**
Isomalto-oligosaccharide-containing lipoteichoic acid of Streptococcus sanguis. Basic structure	**Streptococcus sanguis DSM 20567 and of DSM 20068**	**(79)**	**Phenol, Gel chromatography, HIC**

Chemiluminescence of human polymorphonuclear leucocytes after stimulation with whole cells and cell-wall components of Staphylococcus epidermidis	**Staphylococcus epidermidis**	**(97)**	**Phenol, Gel chromatography**
Competitive binding of calcium and magnesium to streptococcal lipoteichoic acid	**Streptococcus sanguis**	**(141)**	**Phenol, Gel chromatography, HIC**
Lipoteichoic acid inhibits remineralization of artificial subsurface lesions and surface-softened enamel	**Lactobacillus casei**	**(18)**	**Phenol, Gel chromatography**
Cytokine-inducing glycolipids in the lipoteichoic acid fraction from Enterococcus hirae ATCC 9790	**Enterococcus hirae ATCC 9790**	**(162)**	**Chloroform-Methanol**
A lipoteichoic acid fraction of Enterococcus hirae activates cultured human monocytic cells via a CD14-independent pathway to promote cytokine production, and the activity is inhibited by serum components	**Enterococcus hirae (ATCC 9790)**	**(1)**	**HIC**
Structure-function relationship of cytokine induction by lipoteichoic acid from Staphylococcus aureus	**S. aureus DSM 20233**	**(108)**	**Butanol-HIC**
Production of IL-12 and IL-18 in human dendritic cells upon infection by Listeria monocytogenes	**L. monocytogenes EGD**	**(80)**	**Phenol, Gel chromatography**
Characterization of lipoteichoic acids as Lactobacillus delbrueckii phage receptor components	**Lactobacillus delbrueckii**	**(137)**	**Phenol, Gel chromatography**
Monoacyl lipoteichoic acid from pneumococci stimulates human cells but not mouse cell	**Streptococcus pneumoniae (strain R36A)**	**(77)**	**Phenol, Gel chromatography, ion-exchange chromatography**
Highly purified lipoteichoic acid from gram-positive bacteria induces in vitro blood-brain barrier disruption through glia activation: role of pro-inflammatory cytokines and nitric oxide	**S. aureus DSM 20233**	**(8)**	**Butanol-HIC**

Comparison of lipoteichoic acid from different serotypes of Streptococcus pneumoniae	S. pneumoniae strain R6, S. pneumoniae strain Fp23 (serotype 4)	(25)	Butanol-HIC
Opsonic antibodies to Enterococcus faecalis strain 12030 are directed against lipoteichoic acid	Enterococcus faecalis strain 12030	(169)	Butanol-HIC
Butanol-extracted lipoteichoic acid induces in vivo leukocyte adhesion	S. aureus DSM 20233	(32)	Butanol-HIC
Lipoteichoic acid isolated from Lactobacillus plantarum inhibits lipopolysaccharide-induced TNF-alpha production in THP-1 cells and endotoxin shock in mice	L. plantarum K8 (KCTC10887BP)	(75)	Butanol-HIC
Macroamphiphilic components of thermophilic actinomycetes: identification of lipoteichoic acid in Thermobifida fusca	Thermobifida fusca and Rubrobacter xylanophilus	(136)	Butanol-HIC
Differential immunostimulatory effects of Gram-positive bacteria due to their lipoteichoic acids	Staphylococcus aureus, Lactobacillus plantarum, Bacillus subtilis	(144)	Butanol-HIC
Phenotypic variation in Streptomyces sp. DSM 40537, a lipoteichoic acid producing actinomycete	Streptomyces sp. DSM 40537	(135)	Butanol-HIC

Table 1: List of publications for isolation of LTA after application of decision tree 1

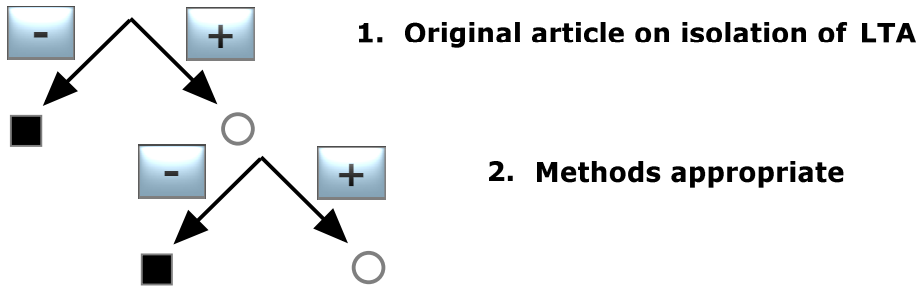

Figure 1: Decision tree 1 (Koch-Dale criterion 1)

3.5 K/D CRITERION 2

3.5.1 Database for K/D criterion 2

The identification of inhibition or even deletion of the synthesis of one of the surface molecules and the respective effect on cytokine induction by the bacteria was the scope for K/D criterion 2. Thus, we searched for deletion mutants of Gram-positive bacteria for LTA or lipoproteins; PGN was excluded in this case being the main component of the Gram-positive cell wall and so we can assume that a mutation in complete biosynthesis of PGN would lead to a failure in growth and in surviving of the Gram-positive bacillus. Hence, we used the keywords “(mutation OR deficiency OR deficient) AND bacterial lipoproteins AND cytokines” or “(mutation OR deficiency OR deficient) AND lipoteichoic acid AND cytokines” revealed 62 publications for bacterial lipoproteins and 58 for LTA from NCBI.

3.5.2 Verification of K/D criterion 2

To identify publications that give information about a deletion mutant of either LTA or bacterial lipoproteins, the pool of publications was selected according to decision tree 2 (fig. 2) and was constructed with the following demands:

1. **Original article on a deletion mutant of LTA or bacterial lipoproteins, respectively**
   Only original articles were included, abstracts and reviews were excluded.
2. **Gram-positive bacteria used?**
   Publication on Gram-negative bacteria were excluded
3. **Experiments done with human primary cells or cell lines?**
   Only studies with primary human monocytes / macrophages and/or cell lines derived from them were included. Publications with only animal experiments and/or animal cells were excluded and in articles with both human and animal models only the human experiments were taken into account for our analysis.
4. **Controls appropriate?**
   The corresponding wildtype should lead to a normal cytokine induction.
5. **Significant influence by deletion of LTA or bacterial lipoproteins**
   Does the mutant lead to an inhibition of cytokine induction?

The demands led to two publications out of 87 (table 2A) for bacterial lipoproteins and two for LTA (table 2B).

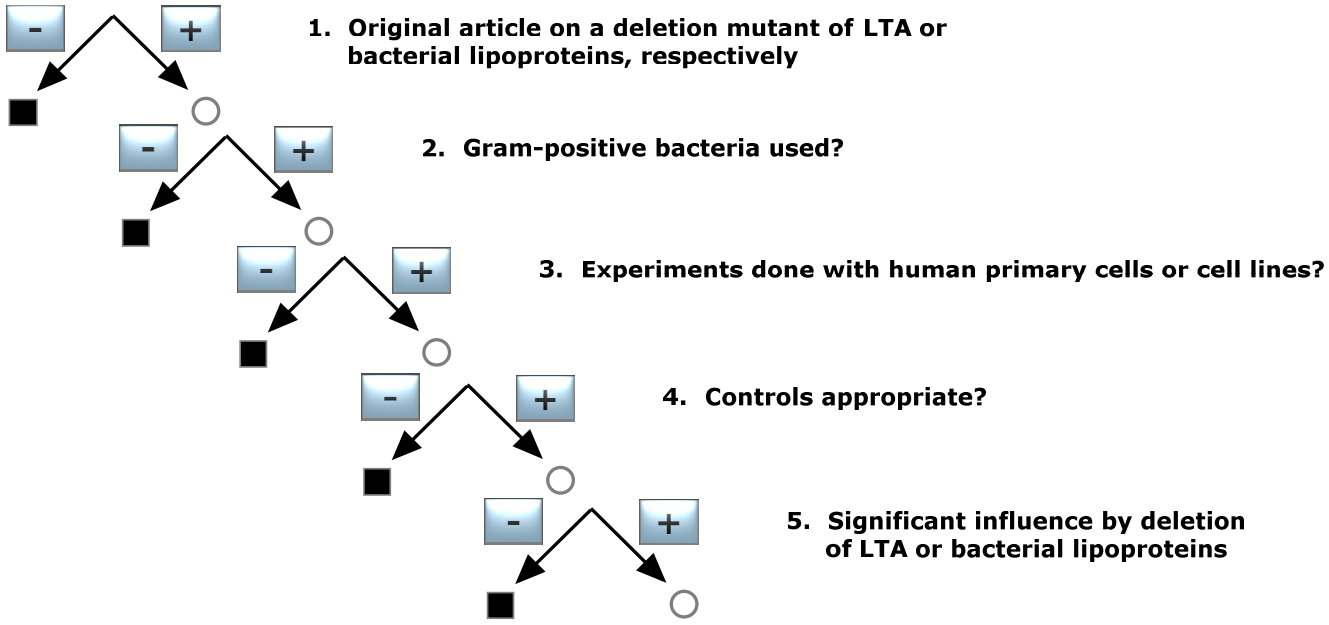

Figure 2: Decision tree 2 (Koch-Dale criterion 2)

reference	bacteria	mutation	cytokines	outcome
(160)	Staphylococcus aureus	diacylglycerol transferase (lgt) deletion	IL-6, IL-8, MCP-1, TNF, IL-10	S.a.Δlgt attenuated in growth in nutrient limitated medium, induction of cytokines is reduced
(186)	Mycobacterium tuberculosis	mutation in Delta19 gene responsible for the production of the 19 kDs lipoprotein	IL-1β, TNF, IL-12	M.t. Δ19 leads to reduced IL-1β induction, but TNF and IL-12 release is unaffected
(143)	Mycobacterium tuberculosis	mutation in Delta19 gene responsible for the production of the 19 kDs lipoprotein	IL-1β, TNF	M.t. Δ19 leads to reduced IL-1β induction, but TNF release is unaffected

Table 2A: Results for Koch-Dale criterion 2 for bacterial lipoproteins

reference	bacteria	mutation	cytokines	outcome
(129)	Lactobacillus ramnosus	deletion of dlt operon responsible for LTA alanylation	IL-10, TNF	IL-10 release significantly reduced for dlt mutant, TNF unaffected compared to wt
(45)	Lactobacillus plantarum NCIMB8826	deletion of dlt operon responsible for LTA alanylation	TNF, IL-12, IFNγ, IL-10	TNF, IL-12 and INFγ significantly reduced for dlt mutant, IL-10 increased

Table 2B: Results for Koch-Dale criterion 2 for LTA

3.6 K/D CRITERIA 3 AND 4

3.6.1 Database for K/D criteria 3 and 4

Using the keywords "(bacteria AND lipoteichoic acid) AND cytokines" or "(bacteria AND peptidoglycan) AND cytokines" or "(bacteria AND lipoproteins) AND cytokines" we obtained 285 publications for lipoteichoic acid, 380 publications for peptidoglycan and 391 publications for lipoproteins from NCBI. All publications were again collected in full length and preselection was done using the following parameters. The preselection and database were used for K/D criteria 3 and 4. Literature cited in these articles constituted a secondary pool of references to complement the systematic analysis.

3.6.2 Preselection of the literature for K/D criteria 3 and 4

In a first step, the pool of publications was preselected according to decision tree 3 as shown in figure 3. This filter was set to concentrate the data on basic quality standards and was constructed with following demands:

1. **Original article on LTA or PGN or bacterial lipoproteins and cytokines**
   Only original articles were included: abstracts and reviews were transferred to pool 2 for background information as mentioned before.

2. **Maximum age of the article must be 10 years**

   The scientific understanding about pattern recognition molecules (pathogen-associated molecular pattern, PAMP) of bacteria and the respective receptors activating the innate immune system has advanced enormously over the last decade as have technologies for structural analysis and preparation. We limited therefore the age of the publications to 10 years. The secondary pool of literature formed by the citations in these articles was meant not to loose valuable supporting information still relevant for today's discussion.

3. **Experiments done with human primary cells or cell lines?**

   To exclude a species discrepancy between the recognition of bacterial cell wall components in human or animals only human studies with primary cells and/or cell lines derived from them were considered. Publications with only animal experiments and/or animal cells were not included and in articles with both human and animal models only the human experiments were taken into account for our analysis.

4. **Number of replicates appropriate?**

   To ensure that the results of the experiments done in the publications were accurate enough for a significant conclusion we included only studies done at least in triplicates and in at least three independent experiments. For primary cell experiments it was necessary to use at least three different donors.

5. **End points appropriate?**

   The following parameters were accepted: Cytokine release measured by ELISA or FACS, luciferase assay to determine NFκB activation, PCR and microarray analysis to determine gene activation.

6. **Endotoxin exclusion (for sub analysis)**

   To ensure that the Gram-positive cell wall components were not contaminated by endotoxin we included only studies who reported a method for endotoxin exclusion like Limulus amebocyte lysate assay, Limulus Anti-LPS Factor (LALF), polymyxin B or other indications for substances without endotoxin content. This criterion did not lead to an exclusion of the publication but was used to analyse whether a possible endotoxin contamination impacts on the results.

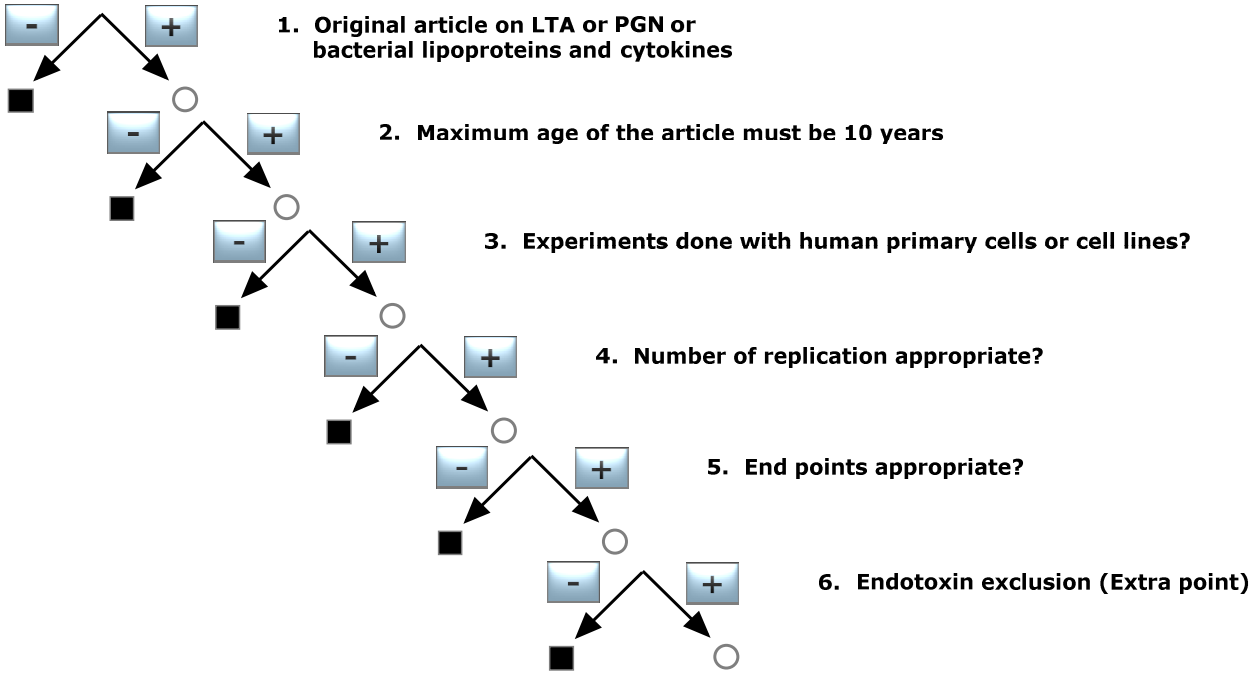

Figure 3: Decision tree 3 (Preselection of the literature for Koch-Dale criteria 3 and 4)

3.6.3 Verification of K/D criterion 3

1. **Determination of cytokine induction after admission of one of the three bacterial surface molecules**
   Did the applied substance induce cytokine release?
2. **Applied substance defined?**
   The employed PGN, LTA or lipoprotein had to be specified with respect to purification grade and source.
3. **Controls appropriate?**
   In this case, controls without admission of the investigated molecules should not result in significant cytokine induction.
4. **Correct dose- or concentration response?**
   Does the purified material respond in a dose- or concentration-dependent manner, e.g. higher cytokine levels at higher concentrations of the stimulus?

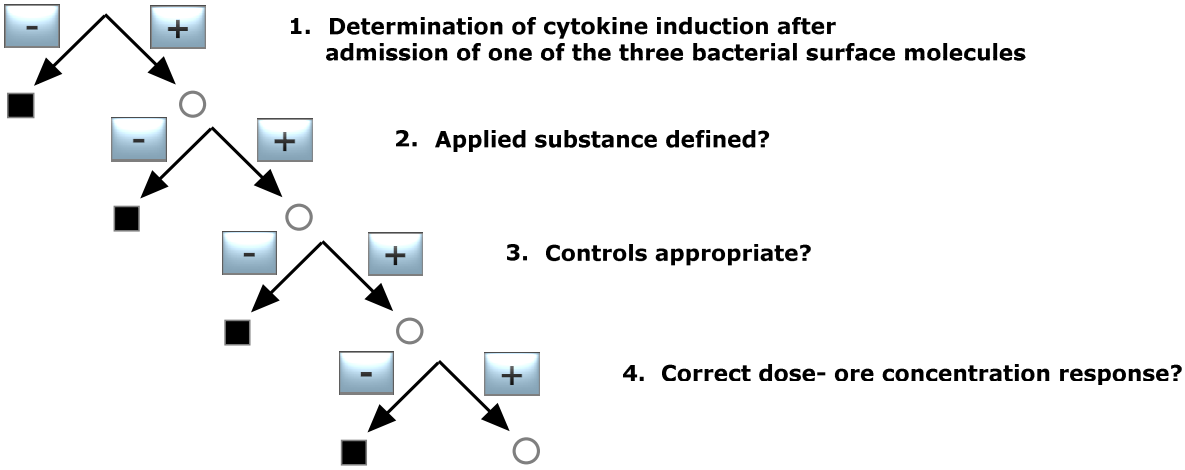

Figure 4: Decision tree 4 (Koch-Dale criterion 3)

3.6.4 Verification of K/D criterion 4

1. **Determination of cytokine induction after admission of one of the three bacterial surface molecules**

   Did the applied substance induce cytokine release?
2. **Exclusion of toxic effects by the antibody or antagonist**

   In this case, the antibody or antagonist of the three bacterial surface molecules should not have a toxic effect on the used cells.
3. **Applied antibody or antagonist defined?**

   The employed antibody or antagonist had to be specified with respect to purification grade and source.
4. **Controls appropriate?**

   In this case, controls with admission of the antibody or antagonist should not result in significant cytokine induction.
5. **Significant change in cytokine release after admission of the antibody or antagonist?**

   The presence of an antibody or antagonist should lead to a significantly reduced cytokine induction.

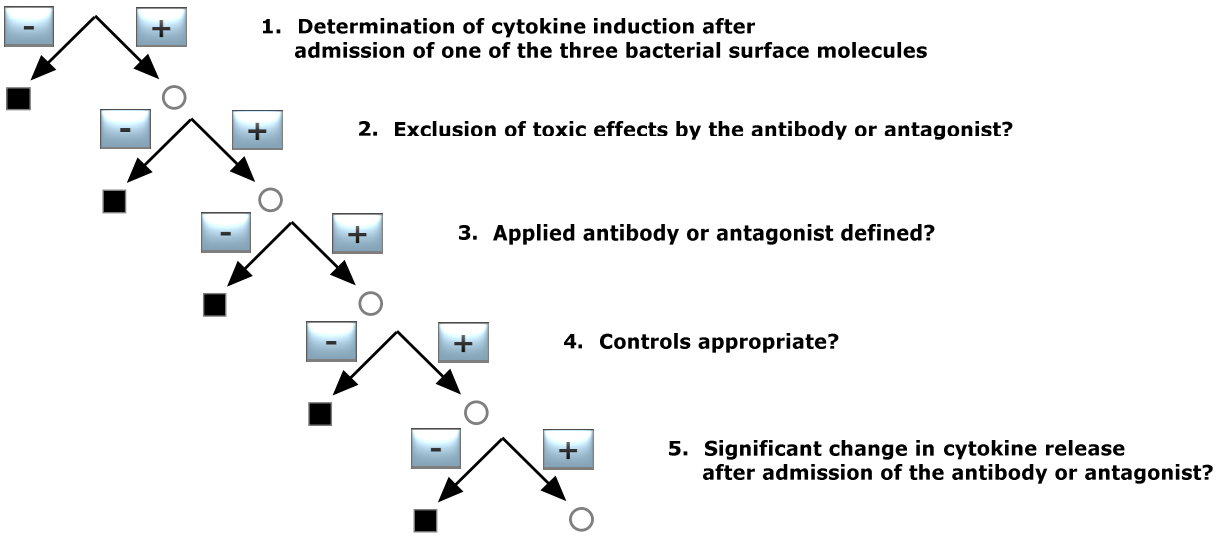

Figure 5: Decision tree 5 (Koch-Dale criterion 4)

If all demands of the decision trees were fulfilled then the probability of correct results is high (best evidence). The possible answers were:

Yes - The study demonstrated that the purified and applied substance leads to a significant cytokine inducing activity in human monocytes / macrophages and can therefore be considered as an important immunostimulatory molecule

No - The substance does not display a sufficient immunostimulatory activity of cytokine-induction in human monocytes / macrophages under controlled conditions.

3.7 RESULTS

3.7.1 Presence of LTA, bacterial lipoproteins or PGN in Gram-positive bacteria (K/D criterion 1)

As mentioned before PGN and bacterial lipoproteins are essential in Gram-positive bacteria; in the absence of a structural identification of a LP which is responsible across a wider variety of bacterial species for cytokine induction, only the general presence of LPs must be assumed, but the criterion can not be considered verified. We identified six reports on LP isolation from five bacterial species, however, largely different in suggested structure as presented in table 3. So our focus was to find publications in which an isolation of LTA was described. 58 publications showed an isolation of LTA using different purification variants and employed 81 different

bacterial strains as shown in table 1. We can therefore conclude that Koch-Dale criterion 1 is fulfilled for PGN and LTA as ubiquitous surface molecules; the limitation that it is not known, which LP should be responsible for cytokine induction, makes it impossible to verify whether the respective specific LP structures are ubiquitous.

name	bacteria	reference	isolation method
Isolation and characterization of a C12-lipopeptide produced by Bacillus subtilis HSO 121	Bacillus subtilis HSO 121	(95)	chromatographic
Isolation and structural analysis of bamylocin A, novel lipopeptide from Bacillus amyloliquefaciens LP03 having antagonistic and crude oil-emulsifying activity	Bacillus amyloliquefaciens LP03	(87)	methanol, silica gel column chromatography, HPLC
Isolation, characterization, and investigation of surface and hemolytic activities of a lipopeptide biosurfactant produced by Bacillus subtilis ATCC 6633	Bacillus subtilis ATCC 6633	(20)	HCl extraction
Lichenysins G, a novel family of lipopeptide biosurfactants from Bacillus licheniformis IM 1307: production, isolation and structural evaluation by NMR and mass spectrometry	Bacillus licheniformis IM 1307	(44)	HPLC
Isolation and partial characterisation of the Triton X-100 solubilised protein antigen from Mycobacterium tuberculosis	Mycobacterium tuberculosis	(76)	Triton X-100
WS1279, a novel lipopeptide isolated from Streptomyces willmorei. Fermentation, isolation and physico-chemical properties	Streptomyces willmorei	(166)	Ethyl Acetate

Table 3: Isolation of different Gram-positive lipoproteins

3.7.2 Deletion of genes involved in biosynthesis of LTA, bacterial lipoproteins and PGN in Gram-positive bacteria (K/D criterion 2)

The structure of Gram-positive bacterial cell walls consists of a large PGN layer. This layer is mainly responsible for the intact composition of the cell wall, so we can hypothesise here, that a Gram-positive mutant lacking PGN would be unable to grow and survive. Yet we must admit, that there is currently no publication available proofing this hypothesis.

The references for the deletion of genes involved in biosynthesis of lipoproteins show a decrease in cytokine induction, using a *S. aureus* mutant (160) and a *M. tuberculosis* one (159, 186) as shown in table 2A. For alteration of LTA, we found two publications showing decrease in IL-10 (129) and TNF, IL-12 and IFNγ (45) release as shown in table 2B.

These findings show the importance of the three surface molecules and the K/D criterion 2 can be considered as fulfilled for LTA, PGN and LPs.

3.7.3 Cytokine induction of LTA, bacterial lipoproteins and PGN in Gram-positive bacteria in the human system (K/D criterion 3)

Isolation or chemical synthesis of PGN, LTA and bacterial lipoproteins and their ability to induce cytokine induction in human immune cells was investigated as the K/D criterion 3. Publications for lipoproteins were divided in two parts, synthetic lipoproteins like Pam_3CysK and isolation and recombinant lipoproteins. As mentioned before we distinguished between publications which provided information about exclusion of endotoxin contamination or did not provide them.

3.7.3.1 Lipoteichoic acid

Fifteen publications with data for endotoxin exclusion revealed a release of pro-inflammatory cytokines like IL-1β, IL-6, IL-8, TNF and G-CSF or the anti-inflammatory cytokine IL-10 in human whole blood, PBMCs, THP-1 cells, human primary monocytes or neutrophils (table 3). Additionally, 9 publications without data for exclusion of endotoxin contaminations found a release of IL-2 in PBMCs, whereas TNF, IL-1β, IL-6, IL-8 and IL-10 release was also found in the papers of this non-

endotoxin group. Since IL-2 is not a monocyte / macrophage product, this result does not add to the conclusions drawn from the higher quality evidence from studies excluding endotoxins. Noteworthy, 15 publications are available describing LTA structures derived by chemical synthesis excluding endotoxin contamination showing cytokine induction in human monocytes / macrophages. No publications were retrieved, in which isolated or synthesized LTA were not capable of inducing cytokine release in human monocytes / macrophages. It is concluded, that there is unambiguous evidence of LTA being capable to activate cytokine release by human monocytes / macrophages.

reference	substance	source of substance	cytokines	cells	endotoxin
(21)	LTA	synthetic	TNF, IL-8	Whole blood	LAL
(53)	PGN, LTA	commercial, isolated	IL-1β	whole blood	LAL
(23)	LTA	isolated	IL-8, IL-1β, IL-6, TNF, G-CSF, IL-10	whole blood	LAL
(74)	LTA	isolated, commercial	IL-10, IL-23	THP-1	Poly B
(175)	LTA, PGN	commercial	IL-8	IFN primed HSC2	Poly B
(12)	LTA	commercial	IL-8	Neutrophils	Manufacturer
(104)	LTA, PAM3	isolated	IL-1β	whole blood, monocytes	LAL
(75)	LTA	isolated	TNF	THP-1	LAL
(26)	LTA	isolated	TNF	whole blood, PBMC	LAL
(25)	LTA	isolated	TNF-, IL-8, IL-10, G-CSF, IL-1β	PBMC, whole blood	LAL

(114)	LTA	isolated	TNF, IL-6	PBMC, Monocytes	TLR4 dependence excluded
(91)	LTA	commercial	TNF	whole blood	LAL (<19ng/mg LTA
(22)	LTA	synthetic	IL-1β, TNF, IL-6, IL-8, IL-10	whole blood	LAL
(109)	LTA	Isolated, synthetic	TNF, IL-1β, IL-6, IL-10	whole blood, PBMC	LAL
(108)	LTA	isolated	TNF	whole blood	LAL
(73)	MDP, LTA	commercial, isolated	TNF-, IL-12 p40, and IL-10	DC	-
(57)	LTA	isolated, commercial	IL-8	PBMC, HEK293	-
(45)	LTA	isolated	IL-1β, TNF, IL-8, IL-6	PBMC	-
(145)	LTA	isolated	TNF	PBMC	-
(69)	LTA	isolated	TNF, IL-1β	THP-1	LTA not TLR4 dependent
(131)	LTA	isolated	IL-2	PBMC	-
(165)	PGN, LTA	commercial	IL-6	HUVEC	Poly B mentioned only in M+M
(111)	LTA	synthetic	TNF	Whole blood	-
(158)	LTA	synthetic	TNF, IL-8	Whole blood	-

Table 4: Publications after fulfilment of decision tree for K/D criterion 3 for LTA

3.7.3.2 Peptidoglycan

If endotoxin-free PGN is used as a stimulus for inducing cytokine release in human immune cells, TNF, IL-1β, IL-6, IL-8, IL-10, GM-CSF and RANTES were detected (table 5). Publications without information about a use of a sufficient endotoxin exclusion revealed similar results, although they mainly used commercially available PGN. However, no synthetic PGN has been reported to induce cytokine release. Furthermore, publications were found in the secondary literature pool showing that further purification of PGN abrogated cytokine induction (171) challenging the capability of PGN to activate monocytes / macrophages. However, they were excluded here since animal cells were used. Noteworthy, several reports showed that the supposed active pattern of PGN, i.e. muropeptides, if free of endotoxin does not activate cytokine release [for review see (170)]. In conclusion, the evidence of PGN inducing cytokine release is poor.

reference	substance	source of substance	cytokines	cells	endotoxin
(82)	MDP	commercial	TNF	whole blood	LAL
(188)	PGN, MDP	manually, commercial	TNF (only PGN or PGN and MDP)	MonoMac 6	Poly B
(100)	LP, PGN	manually, commercial	IL-1β, IL-6, RANTES, GM-CSF	Cord blood-derived mast cells (CBMC)	LAL
(182)	LTA, PGN	manually	TNF, IL-1β, IL-6	whole blood	Poly B
(85)	PGN	manually	TNF	whole blood	Poly B
(118)	PGN	manually	IL-8	THP-1	LAL
(176)	MDP	commercial	IL-8	THP-1	-
(65)	PGN, LTA	commercial	IL-10	THP-1	-

(31)	**PGN**	**commercial**	**IL-12, IL-15**	**THP-1**	**-**
(98)	**DAP**	**manually**	**IL-1β, IL-6**	**MonoMac 6, macrophages**	**-**
(174)	**PGN**	**commercial, manually**	**IL-8, MCP-1, IL-6 and TNF**	**THP-1**	**-**

Table 5: Publications after fulfilment of decision tree for K/D criterion 3 for PGN

3.7.3.3 Bacterial lipoproteins

Isolation and structural analysis of lipoproteins from Gram-positive bacteria was done only in a few cases, so we divided retrieved literature in two subgroups, i.e. five reports on synthetic lipoproteins (table 6A) and three reports on recombinant and in house prepared ones (table 6B). L-OspA from *Borellia burgdorferi* is a commercially available recombinant lipoprotein and leads to a release of IL-1β, IL-6, IL-10, TNF and IL-12 in THP-1 cells (24, 41). Triton extracted lipoproteins were obtained from *Mycoplasma genitalum* (191). The lipoprotein extracted from *Mycoplasma genitalum* was used as a stimulus for THP-1 cells and tested for an endotoxin contamination with polymyxin B led to a release of TNF, IL-1β and IL-6.

Synthetic lipoproteins like PAM_3CysK and PAM_2CysK have biological activity shown as induced release of TNF and IL-6 in publications with data reporting endotoxin exclusion and INF-γ, IL-12, TNF, IL-8 and IL-6 in non-endotoxin tested PAM_2CysK and PAM_3CysK. MALP-2 leads to a release of IL-6 and IL-8 in one publication (47) and was not tested for endotoxin contamination.

reference	substance	source of substance	cytokines	cells	endotoxin
(181)	LP	commercial	TNF, IL-6	THP-1	LAL
(147)	LP	manually	TNF, IL8	Monocytes, HEK293	Poly B
(100)	LP, PGN	manually, commercial	IL-1, IL-6, RANTES, GM-CSF	Cord blood-derived mast cells (CBMC)	LAL
(146)	LP	commercial	TNF	PBMC	-
(154)	LP	commercial	IL-12 p40, GM-CSF, IL-4	PBMC	-

Table 6A: Publications after fulfilment of decision tree for K/D criterion 3 for synthetic LP

reference	substance	source of substance	cytokines	cells	endotoxin
(191)	Mycoplasma LP	commercial, manually	TNF, IL-1β, IL-6	THP-1	Poly B
(24)	L-OSPA	commercial, manually	IL-6, IL-10, IL-12 (p40), TNF, IL-1β	THP-1	LAL
(41)	L-OSPA	commercial, manually	IL-6, IL-10, and IL-12	THP-1	LAL

Table 6B: Publications after fulfilment of decision tree for K/D criterion 3 recombinant or in house prepared LP

3.7.4 Blocking of LTA, bacterial lipoproteins or PGN by antibodies, antagonists or inhibitors prevents or reduces cytokine release (K/D criterion 4)

Only one study was found for LTA (26) where TNF induction was significantly reduced using polypropylene glycol as an inhibitor for LTA. For PGN and bacterial lipoproteins, no paper passed the inclusion criteria. Noteworthy, from the secondary literature pool a paper was identified describing specific anti-LTA antibodies augmenting recognition of LTA and cytokine response hinting also to a structure-specific intervention (96).

3.8 DISCUSSION

This study compiled in a systematic manner the available evidence for the major candidate molecules currently suggested representing in Gram-positive bacteria the functional counterpart to Gram-negative lipopolysaccharides in innate immune activation. Cytokine induction in human monocytes / macrophages and cell lines derived from these was considered a sufficiently broad key event, for which data are available. The K/D criteria gave a generally accepted framework for the analysis of causation of an effect by a given mediator.

A meta-analysis of the three most commonly mentioned Gram-positive bacterial surface structures involved in immunostimulation and cytokine release was more difficult than anticipated: The number of publications is huge, provided information is diverse and the studies offer a variety of different settings, outcomes and results. Our restriction to human studies revealed on the one hand more relevant information to the actual interaction between pathogens and the human immune system but also led to a loss of interesting and important pieces of evidence on the other. Often the used materials were either not tested for endotoxin (at least the information about a relevant test was not provided) or commercial preparations were used without given information about purity and contaminations. Commercial preparations of LTA and PGN have a high risk of being impure or contaminated (40, 92, 109). Furthermore, our own experience with PGN and LTA underlines the importance of purity and exclusion of contaminants in the in-house prepared material. In case of PGN we could confirm findings of Travassos et al (171) that PGN is not able to induce cytokine release when it is highly purified (140). Travassos and his co-workers used

a mouse model for investigation of cytokine induction which led to an exclusion of his studies in this meta-analysis based on the preselection criteria.

In some studies cytokine induction in human cell systems was only a minor aspect of the paper which led to difficulties in drawing conclusions for our analysis. The main problem was to find studies using antagonist of or antibodies to LTA, PGN or bacterial lipoproteins, respectively, which leads to a lack of conclusions for the K/D criterion 4 for all structures but LTA. Such studies would considerably help our understanding of the role of LP and PGN. As a possible substitute to provide evidence for K/D criterion 4 reports could be used, that show how the blockade or abrogation of specific pattern recognition receptors for the different PAMP impairs cell activation. However, this requires the knowledge of these receptors and their specificities as well as it has been the central problem that the predominant techniques used are knock-out-mice, thus restricting us to animal models.

Taken together, our results indicate that among the pertinent candidate molecules under discussion, i.e. LTA, PGN and bacterial lipoproteins, only for LTA the retrievable data support all K/D criteria in a satisfactory manner. It can therefore be considered as a PAMP, certainly not excluding others. Furthermore, data available are not sufficient to consider if the Gram-positive endotoxin until endotoxic shock like reactions can be produced applying this molecule. So far only reports on liver injury in galactosamine-sensitized mice (58) are available. It still remains to be elucidated, however, which receptors are involved in this activation and whether and how other PAMP modulate the process. Collecting more data, especially in human cell systems will provide better information and more knowledge of the interaction between LTA and the human innate immune system. Furthermore it will be important to find out if LTA is a prerequisite for cytokine induction using bacteria which have atypical LTAs or even replacing structures. Little is known about these bacteria and their contribution to cytokine release with the exception of Mycobacteria like *M. leprae* and *M. tuberculosis* where it is known, that the lipomannan structures play an important role in cytokine induction (3, 19). Propionibacteria, Bifidobacteria and Micrococci have no LTA (163), but only little is known about their cytokine inducing capacity. Finally, strong evidence is available from the fact that synthetic LTA shows a similar cytokine profile compared to the natural isolated substance which supports the importance of LTA in cytokine induction in humans (22, 110).

Bacterial lipoproteins seem also to be important for cytokine release, but it still seems to be difficult to isolate and purify them. The majority of research is done with synthetic lipoproteins such as $PAM_2Cys\text{-}K_4$ or $PAM_3Cys\text{-}K_4$ and still it remains unclear whether this reflects the stimulation by bacterial components. For the studies analysed in this meta-analysis the outcome is that bacterial lipoproteins fulfil only partially the first three K/D criteria due to the fact that a ubiquitous general structure has not been identified nor made available by purification or synthesis; the lack of studies using antibodies or antagonists for these lipoproteins makes it impossible to assess criterion 4.

PGN and its contribution as a PAMP, however, is a subject of a long ongoing discussion with regard to its contribution to cytokine release and interaction with the human immune system. Especially in Gram-positive bacteria it appeared to be important for detection of the immune system simply for the reason that it is a major constituent of the total content of the Gram-positive cell wall. PGN was first discussed to signal via TLR2 (149). NOD however (42) recognises PGN breakdown products. According to more recent studies, PGN has to be internalized and digested for an immune reaction (190) and highly purified PGN does not seem to be a trigger for cytokine release (171). However, these reports were not included in our investigation due to the restriction to the human system and the mentioned studies were done in mice or in murine cell systems. As mentioned before, our own studies support the findings of Travassos (171) using human whole blood in this case (140). These results are also not included in this analysis due to the fact that they are not yet published.

A key question not addressed in this systematic review due to the limited number of studies available is the one of specific activity, i.e. whether the amount of the respective PAMP per bacterium can account for the immunostimulating activity. Until recently, no structure fulfilled this requirement, i.e. in contrast to the pg quantities of LPS per ml required, ng per ml quantities of the Gram-positive PAMP are needed. However, Deininger and collaborators (23) were able to show that presentation of LTA on a surface augments its stimulatory activity by 3-4 log orders. Similar findings are not available for PGN and LP.

	LTA	PGN	LP
K/D 1	+++	+++	-
K/D 2	+++	+++	+++
K/D 3	+++	+++	++
K/D 4	+	-	-

Table 7: Fulfilment of the four K/D criteria for LTA, PGN and LP

Finally, based on this meta-analysis, bacterial lipoproteins and PGN might play a role in cytokine induction in humans as no contradictory results were found for human monocytes / macrophages, but LTA is the only Gram-positive membrane compound, which fulfils all of the four Koch/Dale Criteria making it a prime candidate for the Gram-positive counterpart to LPS in Gram-negative bacteria (table 7).

4 Different *S. aureus* whole bacteria mutated in putative pro-inflammatory membrane components have similar cytokine-inducing activity

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Summary sentence: Deletion mutants of *S. aureus* and their purified cell wall components exclude peptidoglycan and show redundant pattern recognition to lipoteichoic acids for macrophage activation.

Running title: Pro-inflammatory cell wall components of *S. aureus* mutants

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4.1 ABSTRACT

The role of the different major cell wall components of Gram-positive bacteria for immune stimulation is controversial. We thus compared the cytokine inducing capacity of different *S. aureus* (SA) mutants lacking either lipoproteins (SA 113 Δlgt), or wall teichoic acids (WTA) (ΔTA), or possessing a reduced D-alanine content in lipoteichoic acid (LTA) (SA 113 Δdlt) to its corresponding wildtype (SA 113 wt). Inactivated whole bacteria and their purified cell wall components peptidoglycan and LTA, were used to stimulate human whole blood and macrophages from TLR2 knock-out mice. We found that all *S. aureus* strains induced similar amounts of TNF, IL-8 and IL-10 and none of them was dependent on the presence of TLR2. Surprisingly, in case of SA 113 Δlgt a significant attenuated release of only IL-1β protein and mRNA in human whole blood was observed. Highly purified peptidoglycan from all strains in contrast to LTA had a very low activity in stimulating cytokine release. Taken together these results demonstrate that major cell wall alterations like lack of WTA, lipoproteins or alterations in L-alanine content do not affect the overall cytokine inducing potential of whole bacteria and thus cytokine induction is initiated by redundant mechanisms.

4.2 INTRODUCTION

The role of different cell wall components of Gram-positive bacteria to activate the immune system is highly controversial. Most studies are done with either (partially) purified components with often unknown contaminations, with cell models reflecting only some of the activation pathways of the innate immune system, combining human and murine models or studying a single bacterial mutation. It has been proven to be largely impossible to sort these different findings out. Here, we aimed to study a series of the most relevant bacterial mutants emerging in recent years, using human whole blood as the most physiological primary human model for cytokine release by the innate immune system and combined this with the high-purity isolation of lipoteichoic acids (LTA) and peptidoglycan (PGN) from the same mutants in the very same experimental model.

Despite medical advances in treatment of bacterial infections, the frequency of *S. aureus* infections is steadily increasing and becoming even more complicated with the emergence of antibiotic-resistant strains. So far the interaction of the host immune system with Gram-positive bacteria is not completely understood, and a key immunostimulatory component like lipopolysaccharide (LPS) in Gram-negative bacteria has not been identified. In contrast to Gram-negative bacteria, where LPS is a major molecule of the outer membrane (39), in Gram-positive bacteria other major molecules are found, like lipoproteins, considerably larger amounts of peptidoglycan, wall teichoic acids (WTA) and lipoteichoic acid (LTA) (11, 26, 106, 117). Peptidoglycan of Gram-positive bacteria is decorated with LTA on its external surface (2) whereas lipoproteins are incorporated in the peptidoglycan-layer (56). In case of *S. aureus* and many other Gram-positive bacteria, LTA and lipoproteins are recognised via Toll-like receptor (TLR) 2/TLR6 or TLR2/TLR1 (70). While on one hand both lipoproteins and LTA are reported to play an important role in immune activation (11, 62, 150), their potency to induce cytokine release is questioned by others. Hashimoto et al. (52) reported that LTA isolated from a lipoprotein diacylglycerol transferase deletion (SA 113 Δlgt) mutant is 100-fold less active in inducing cytokine release from a monocytic cell line than LTA from the respective wildtype and suggest that not LTA but lipoproteins are the dominant immunobiologically active compound in *S. aureus* (51). However, von Aulock et al. showed that LTA from the *S. aureus* mutant strain used by Hashimoto's group exerts

similar cytokine inducing activity as LTA from the corresponding wildtype strain using human whole blood (178). A key problem in this discussion is, that the putative lipoprotein responsible for activation of the innate immune system by *S. aureus* or other relevant Gram-positive pathogens has not been identified, purified or finally synthesized leaving room for speculation.

Likewise the role of peptidoglycan was controversially discussed (7, 29, 93, 171). While first studies indicated that it signals via TLR2 (29, 61), these results were confuted by Travassos et al. (171) who showed that highly purified peptidoglycan does not possess cytokine inducing activity. Furthermore they showed that a contamination with LTA and lipoproteins was responsible for the TLR2 signalling.

To gain more information about the role of the different membrane components of S*. aureus* for immune recognition of whole bacteria we compare here the immune stimulatory activity of three different *S. aureus* mutant strains: SA 113 *lgt*::*ermB* (SA 113 Δlgt) lacking the lipoprotein diacylglycerol transferase resulting in a lack of lipoproteins in the bacterial cell wall (160). Next, SA 113 ΔdltA (SA 113 Δdlt) lacking the dlt operon and thus leading to a reduced alanine content of the LTA (130), and SA 113 ΔtagO (SA 113 ΔTA) lacking wall teichoic acids (WTA) (81) plus the corresponding wildtype strain. We used the human whole blood cytokine release model (50) since it uniquely gives access to primary human cells in their natural environment without preparation artefacts. It is evident that for the study of human pathogens primary cell human models are favoured and that cell lines or even worse cells transfected with a single receptor system will give false indications of the relative role of individual components. It is also necessary to use a sufficiently large number of leukocyte donors to exclude an effect of unknown polymorphisms. We also included the measurement of several cytokines since they might be affected in a differential way by the structural mutations.

4.3 MATERIALS AND METHODS

Bacterial strains and cultivation

S. aureus strain SA 113 wildtype (SA 113 wt), mutant strain SA 113 Δlgt, mutant strain SA 113 Δdlt and mutant strain SA 113 ΔTA were grown aerobically in a medium containing tryptic soy broth (25 g/l), beef extract (5 g/l) (both from BD Biosciences, Heidelberg, Germany) and glucose (0.8% v/v). Bacteria were kindly

provided by Andreas Peschel (Dep. of Medical Microbiology and Hygiene, Tübingen, Germany) and Ulrich Zähringer (Dep. of Immunochemistry, Borstel, Germany) and were harvested after 18 h stirring at 37 °C, 150 rpm. Integrity of bacteria and potential contaminations by Gram–negative bacterial species were tested by Gram staining. For stimulation of human and murine cells the harvested bacteria were washed three times with PBS (PAA Laboratories GmbH) at 1100 x g for 10 min (centrifuge CL-GP, Beckman Coulter, Fullerton, CA, USA) and the pellet was resuspended in PBS to OD_{600}=1 (i.e. about 10^9 bacteria/ml). For UV-inactivation and subsequent whole blood stimulation, 1 ml of 10^9 bacteria was irradiated on ice (UV-Stratalinker 1800, Stratagene, La Jolla, CA, USA) 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). For heat-inactivation, 1 ml of 10^9 bacteria was incubated at 95°C for 10 min. To confirm inactivation of bacteria colony growth on blood agar plates (Columbia-blood agar, Heipha Diagnostika, Eppelheim, Germany) was followed for 24 h at 37°C and 5% CO_2.

Preparation of peptidoglycan

Peptidoglycan was purified from *S. aureus* 113 wt, Δlgt, Δdlt and ΔTA according to Girardin et al. (43). Briefly, bacteria were grown in 10-liters MRS-culture, harvested after centrifugation at 4°C and resuspended in pyrogen-free water. This solution was added in drops to 8% boiling Sodium Dodecyl Sulphate (SDS, Applichem, Darmstadt, Germany) and boiled for 30 minutes. After cooling down, the solution was centrifuged for 20 min at 100,000 x g and the pellet was washed with pyrogen-free water until no SDS could be detected according to the method described by Hayashi et al. (54). Bacterial samples were physically disrupted with acid-washed glass-beads (100 µm, Sigma Aldrich, Germany) and PGN was then recovered by differential centrifugation (5 min at 2300 rpm). The pellet was successively digested for 2 h at 37°C with 1 mg/ml α-amylase (from pig pancreas, Roche, Mannheim, Germany), 1 mg/ml DNAse I (from bovine pancreas, Roche, Germany), 1 mg/ml RNAse A (Roche, Germany) and 1 mg/ml lipase (from *Canida rugose*, Sigma, Germany) in 25 ml $MgCl_2$ (200 mM) for 2 h at 37°C and with 100 µg/ml of trypsin (Sigma) in 10 mM $CaCl_2$ for 18 h at 37°C. Samples were washed with pyrogen-free water and boiled in 2% SDS. After SDS removal, the resulting pellet had a quantity of 250 to 500 mg of PGN and was treated 49% hydrofluoric acid (HF, Merck, Darmstadt, Germany) for 48-72 h at 4°C.

After removal of the HF, the pellet was washed five times with pyrogen-free water and the solution was lyophilised and stored at -20°C.

LTA preparation

The harvested bacteria underwent butanol extraction and hydrophobic interaction chromatography as described (25, 108). The isolated LTAs were tested by kinetic Limulus amoebocyte lysate assay (Charles River, Charleston, SC, USA) and contained <1.0 EU/mg LTA, i.e. less than 100 pg LPS equivalents per mg LTA.

Human whole blood incubation

Human whole blood from healthy volunteers was taken at 9 am, using heparinized S-monovettes® (Sarstedt, Numbrecht, Germany) and diluted 1:5 with RPMI 1640 in polypropylene reaction vials (Eppendorf-Nethler-Hinz GmbH, Hamburg, Germany). All stimuli were sonified and stimulation was performed for 24 h, at 37°C and 5% CO_2 in humidified air. After incubation, the vials were shaken to resuspend pellets and centrifuged for 2 min at 14,000 x g. The cell-free supernatants were stored at -80°C until cytokine measurement by ELISA. Differential blood counts were routinely performed on the blood of all volunteers with a Pentra60 haematology analyzer (ABX Diagnostics, Montpellier, France) to rule out acute infections. LPS from *Salmonella abortus equi* (S.a.e.) as control stimulus was purchased from Sigma, Deisenhofen, Germany.

Murine bone marrow cells

Bone marrow wash out cells from TLR2-knock out mice (kindly provided by Tularik, South San Francisco, CA, USA) and TLR2 wildtype mice were prepared. Therefore, mice were killed by injection i.v. of 150 mg/kg pentobarbital (Narcoren®, Merial, Halbergmoos, Germany). Femur and humerus were removed and rinsed out with 10 ml ice-cold Dulbecco's Phosphate Buffered Saline (PBS; Life Technologies, Karlsruhe, Germany). Cells were then washed, transferred into siliconized glass tubes (Vacutainer®, Bioscience, Heidelberg, Germany), resuspended in RPMI 1640 (Cambrex, Verviers, Belgium) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% penicillin and streptomycin in flat-bottom 96-well-plates at a density of 5 x 10^6 cells/well, stimulated, and incubated over night at 37°C and 5%

CO_2. Supernatants were collected and stored at -80°C or were directly used for cytokine measurements. LTA and LPS were used as control stimuli in each experiment to prove the vitality of TLR deficient cells (TLR2 $^{-/-}$ / LPS) and to prove the receptor defect (TLR2 $^{-/-}$ / LTA).

Enzyme-linked immunosorbent assay

Cytokines were determined by sandwich enzyme-linked immunosorbent assay (ELISA) based on commercial antibody pairs against human TNF, IL-8, IFNγ (Thermo Scientific, Perbio Science, Bonn, Germany), IL-10 (Pharmingen, Becton-Dickenson, Sparks, USA), IL-1β and IL-6 (R&D Systems, Minneapolis, USA). Antibodies against murine TNF were purchased from R&D (Wiesbaden, Germany). Binding of biotinylated antibody was quantified using streptavidin-peroxidase (Jackson Immuno Research, West Grove, PA, USA) and the substrate TMB (3,3', 5,5'-tetramethylbenzidine, Sigma). The reaction was stopped by adding 50 µl of 1 M H_2SO_4. Absorptions were measured at a wavelength of λ = 450/690 nm in an ELISA reader (Rainbow, Tecan, Crailsheim, Germany). The amount of cytokines was calculated from a standard curve by the Easyfit program (SLT-Lab instruments, Crailsheim, Germany). Recombinant cytokines used as standards were obtained from the National Institute of biological Standards and Controls, UK (hu-TNF, mu-TNF, and hu-IL-1β), BD Biosciences, Pharmingen (hu-IL-10), PeproTech, Frankfurt, Germany (hu-IL-8) and TEBU-BIO, Offenbach, Germany (hu-IFNγ). Assays were carried out in flat bottom, ultrasorbent 96-well plates (Nalge-Nunc).

Preparation of human peripheral blood mononuclear cells for IL-1β mRNA analysis

Human whole blood incubations were performed as described above with 1, 3, 7, 10, 13 h of incubation time. Samples were resuspended by gentle shaking, centrifuged at 400 x g for 2 min and cell-free supernatants were stored at -80°C until cytokine measurement. The remaining cell pellet was resuspended and transferred into a 15 ml centrifugation tube (Greiner Bio-One) on ice. 5 ml of Erythrocytes lysis buffer (EL buffer, Qiagen, Hilden, Germany) was added to each sample and incubated for 10 min on ice. Samples were briefly vortexted twice during the incubation. The samples were centrifuged at 400 x g for 10 min at 4°C and the supernatant was discarded. The remaining leukocyte pellet was washed twice with PBS and stored at -80°C until

RNA preparation. RNA was extracted using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) and used for reverse transcription.

Quantitative Real-Time PCR

cDNA was quantified by Real-Time PCR via the LightCycler system (Roche) with LightCycler FastStart DNA Master SYBR Green (Roche) using specific primers from ThermoScientific (ThermoScientific, Hamburg, Germany) : IL-1β: forward 5´-AAACAGATGAAGTGCTCCTTCCAGG-3´, reverse: 3´-TGGAGAACACCACTTGTTGCTCCA-5´ GAPDH: forward 5´-GAAGGTGAAGGTCGGAGTC-3´, reverse: 5´-GAAGATGGTGATGGGATTTC-3´.

Statistics

Statistical analyses and graphs were performed using the Graph Pad Prism software, Version 4.0 (Graph Pad Software, San Diego, USA). All data are given as mean ± SEM. P values were considered significant with * for $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ in all graphs and were tested with a one-way ANOVA and Bonferroni's Multiple Comparison test.

4.4 RESULTS

Whole SA 113 wt, SA 113 Δlgt, SA *113 ∆dlt* and SA *113 ∆TA bacteria induce a comparable cytokine profile except for IL-1β*

The SA 113 Δlgt strain, which lacks the diacyl-glycerol transferase and therefore contains no lipoproteins in its cell wall is described to be less immunostimulatory in human monocytic (MonoMac6), epithelial (pulmonary A549) and umbilical vein endothelial cells than SA 113 wt (160), whereas nothing has been published up to now about the immunostimulatory activity of whole SA 113 ∆dlt, which has a reduced alanine content in the LTA and SA 113 ∆TA that lacks WTAs. To gain more information about the role of the respective cell wall components for the cytokine releasing activity of whole *S. aureus* bacteria, we compared the four different strains using primary human immune cells. Human whole blood was stimulated with increasing concentrations of UV- or heat-inactivated bacteria from wildtype and

mutant strains and the release of TNF, IL-1β, IL-8 and IL-10 was measured. For TNF, all mutant strains showed the same immunostimulatory potency as the wildtype strain (Fig. 1A) independently whether the bacteria were UV- or heat-inactivated. Heat inactivation of whole bacteria resulted in a stronger impairment of the immune stimulatory potential compared to UV-inactivation, which can be explained by the fact that UV-inactivation only destroys the bacterial DNA and has no or only minor effects on the bacterial membranes in comparison to heat-inactivation.

Similar results were found for IL-10 and IL-8 (data not shown). For IL-1ß release we found variations, which were most pronounced for the SA 113 Δlgt strain and which resulted in a significant reduction of IL-1β compared to the other *S. aureus* strains. In general, heat inactivation of whole bacteria led to a steeper concentration response curve than UV inactivation. No differences in bacterial growth were observed for the bacteria except for SA 113 ΔTA, which was attenuated and needed twice as much time to reach an optical density of 1 compared to the other strains (data not shown).

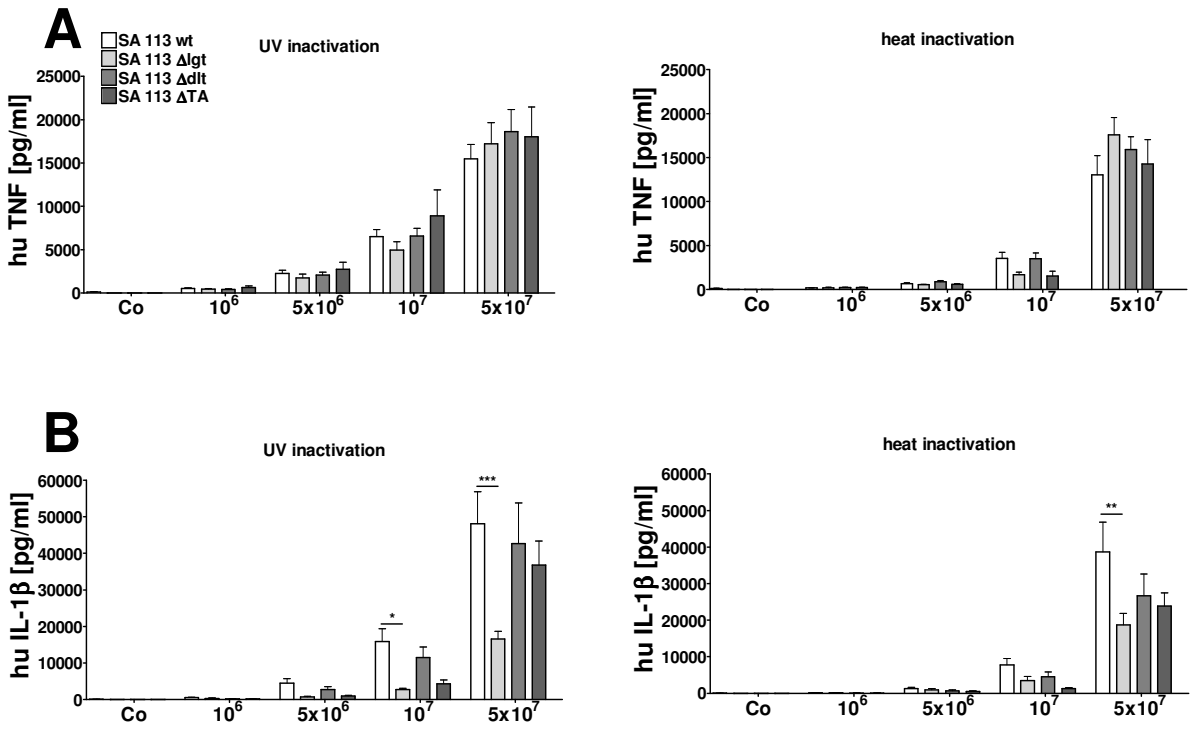

Fig. 1: Comparison of cytokine induction by UV- and heat-inactivated bacteria from SA 113 wt and SA 113 Δlgt in human whole blood.

Human whole blood from 16 healthy volunteers was stimulated with whole UV-inactivated (left) or heat-inactivated (right) SA 113 wt, SA 113 Δlgt, SA 113 Δdlt or SA 113ΔTA bacteria for 22 h. TNF (A) and IL-1β (B) release was measured in the cell-free supernatants by ELISA. Data are means ± SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Cytokine induction by whole SA 113 wt and mutant bacteria does not depend on TLR2

To test whether the immunostimulatory properties of whole bacteria depend on the TLR2 receptor, we stimulated murine bone marrow and spleen derived macrophages from wildtype and TLR2 knock-out mice with different concentrations of UV- or heat-inactivated bacteria from the four strains. Stimulation with LPS from *Salmonella abortus equi* (S.a.e.) and LTA prepared from *S. aureus* strain DSM 20233, served as control for the TLR2 knock-out. As shown in Fig. 2, the TNF release of bone marrow derived macrophages from wildtype and TLR2 knock-out mice was similar and thus indicating that TLR2 is not a prerequisite for any of the four *S. aureus* strains to stimulate cytokine induction in murine macrophages. Heat inactivation led to a slightly weaker immune activation compared to UV-inactivated bacteria**.**

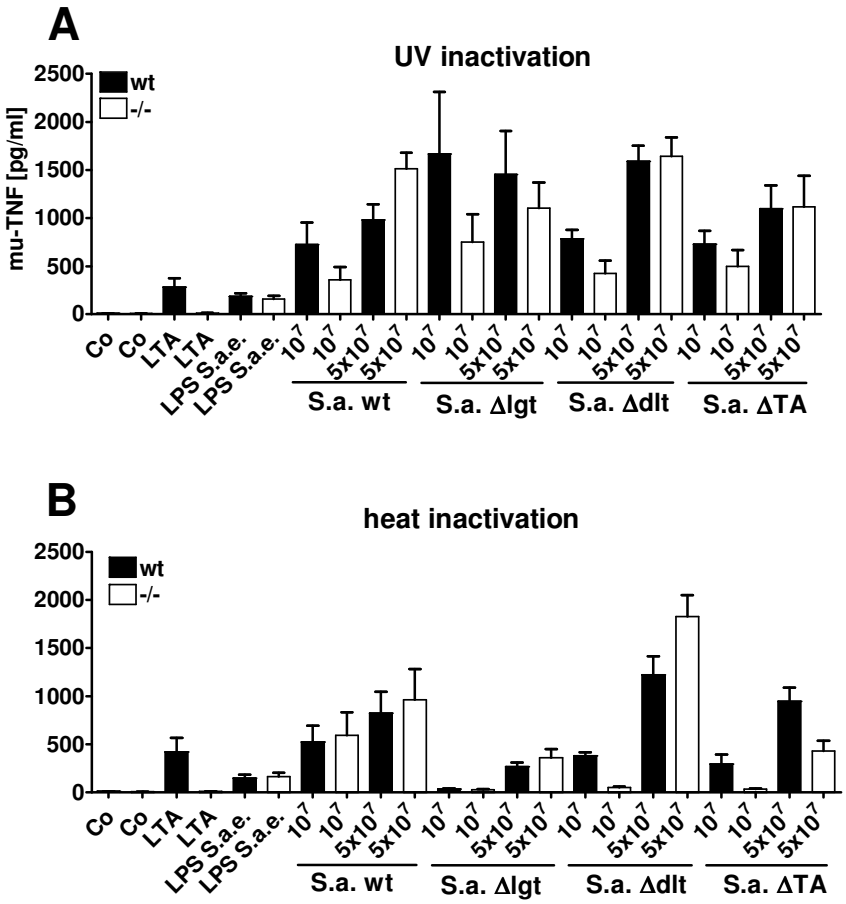

Fig. 2: Comparison of cytokine induction by UV- and heat-inactivated bacteria in bone marrow cells from wild-type and TLR-2 knock-out mice.

Bone marrow derived macrophages from 8 different wild-type and TLR2$^{-/-}$ mice, respectively, were stimulated with LTA (10 µg/ml), LPS (LPS 10 ng/ml), whole UV-inactivated (A) or whole heat-

inactivated (B) SA 113 wt, SA 113 ∆lgt, SA 113 Δdlt or SA 113ΔTA bacteria for 22 h. Murine TNF release was measured in the cell-free supernatants by ELISA. Data are means ± SEM.

Purified peptidoglycan from SA 113 wt and its mutant strains does not induce significant cytokine release in human whole blood.

To identify the structure responsible for the immunostimulation, we isolated peptidoglycan according to Girardin et al. (43) and used it for stimulation of human whole blood. In line with the findings of Girardin et al., purified peptidoglycan of all strains failed to induce significant amounts of IL-1β (Fig. 3). Though, 1 µg of the different PGN preparations induced only few percent of the amount of IL-1β inducible by the same amount of LTA. Induction of TNF was also very low, but revealed borderline significant amounts compared to the unstimulated control (data not shown).

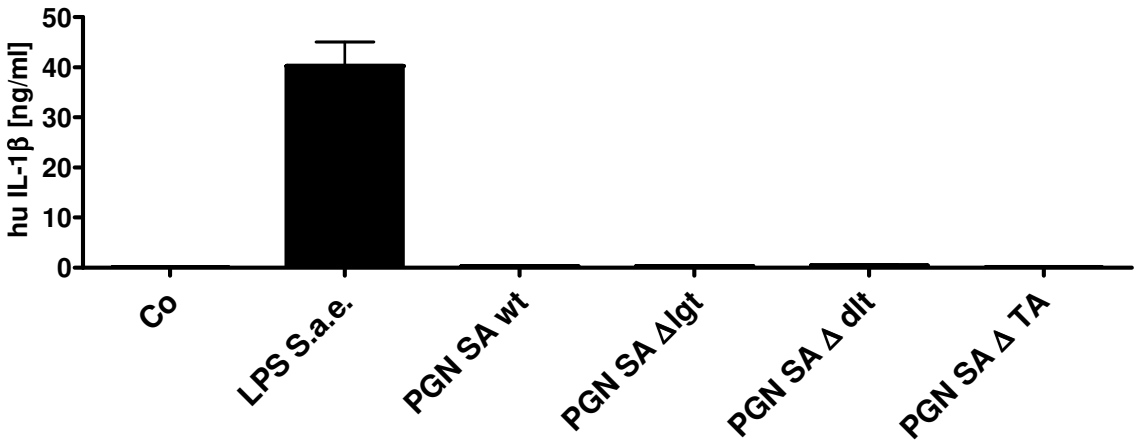

Fig. 3: Comparison of cytokine induction by peptidoglycan from four different *S. aureus* strains
Human whole blood from 8 different donors was stimulated with 10 ng LPS, or 1 µg of peptidoglycan from *S. aureus* 113 wt, Δlgt, Δdlt and ΔTA. IL-1β release was measured by ELISA. Data are means ± SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test.

LTA from SA 113 Δlgt results in attenuated IL-1β release and from Δdlt for both TNF and IL-1 compared to LTA from SA wildtype

Next we prepared LTA from the four SA 113 strains according to Morath et al. (108) and compared their cytokine inducing activity in human whole blood. While the

cytokine inducing activity of LTA from SA 113 Δdlt was slightly attenuated (Fig. 4A) for both TNF and IL-1, the release of IL-1β (Fig. 4B), was strongly diminished in case of LTA from SA 113 Δlgt compared to LTA from SA 113 wt as we observed when whole bacteria from this strain were used (Fig. 1B). For IL-8, no differences in cytokine release from human whole blood could be detected (data not shown). This confirms the role of alanine substituents for cytokine induction (108), but it is unclear why only one of the cytokines is influenced significantly by the Δlgt mutation and IL-8 not at all.

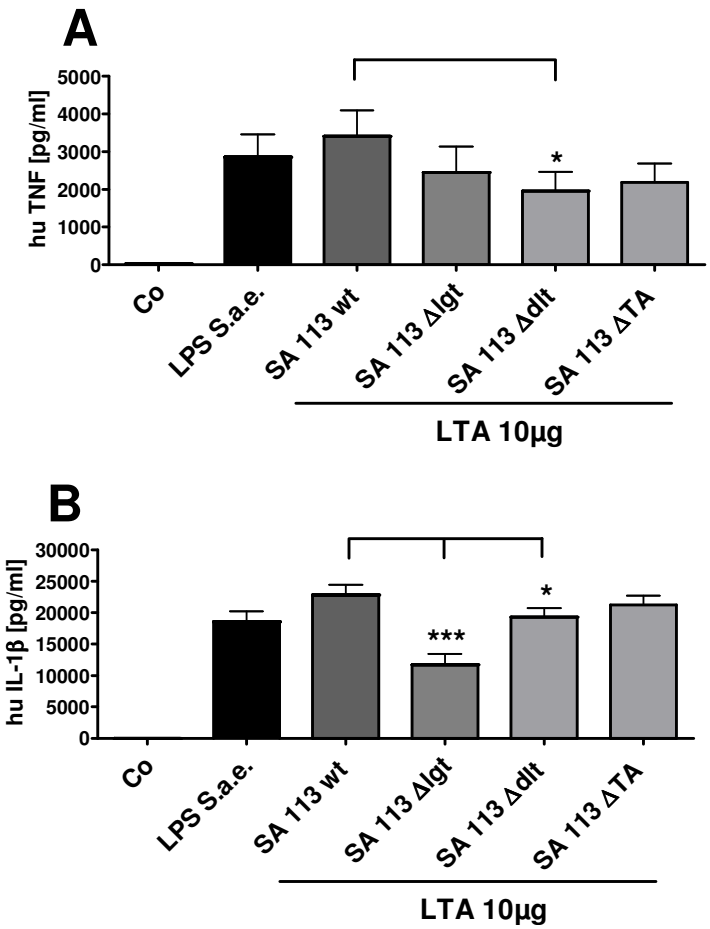

Fig. 4: Comparison of cytokine induction by LTA from four different *S. aureus* strains

Human whole blood from 8 different donors was stimulated with 10 ng LPS or 10 µg of LTA from *S. aureus* 113 wt, Δlgt, Δdlt and ΔTA. TNF and IL-1β release was measured by ELISA. Data are means ± SEM. One-way ANOVA with Bonferroni's Multiple Comparison Test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Attenuation in IL-1β release is correlated with lower mRNA levels.

We have shown that human whole blood stimulated with whole bacteria or with LTA from SA 113 Δlgt released significant lower amounts of IL-1β compared to LTA from wildtype bacteria. To test whether this is due to differences on the mRNA level we performed quantitative Real-Time PCR. Human whole blood was stimulated with LTA from SA 113 wt or Δlgt and incubated for 1, 4, 7, 10, 13 and 24 hours. Erythrocytes were lysed and the remaining PBMC were used for RNA preparation. The resulting mRNA kinetics showed significant higher levels of IL-1β mRNA for samples stimulated with LTA from SA 113 wt compared to Δlgt (Fig. 5) confirming the result on protein level.

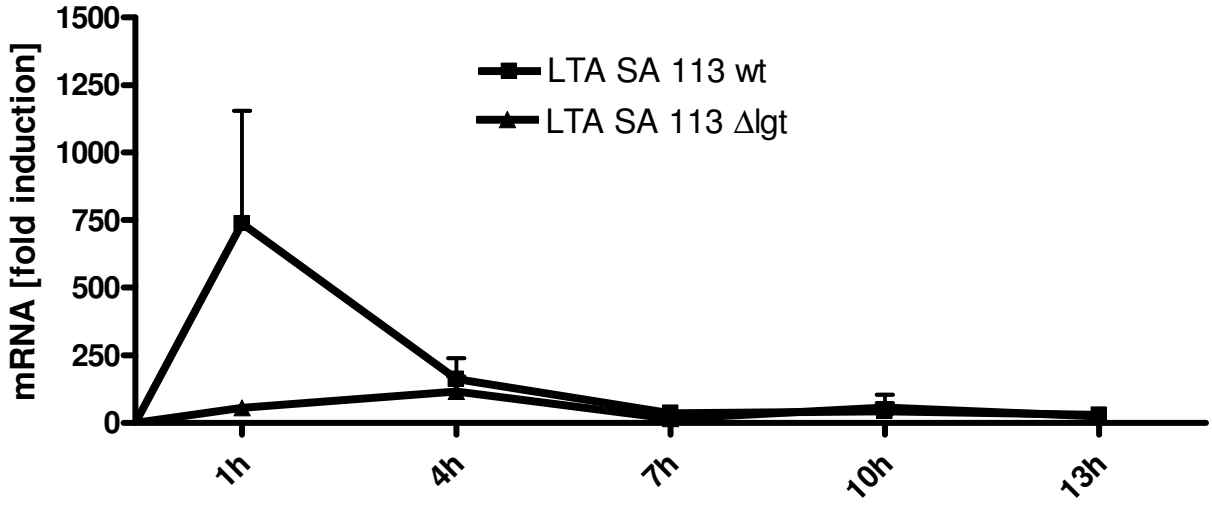

Fig. 5: Comparison of IL-1β mRNA induction by human whole blood stimulated with either LTA from SA 113 wt or Δlgt

Human whole blood from four different donors was stimulated with 10 µg/ml of LTA from SA 113 wt or Δlgt for the give time periods. Erythrocytes were lysed, RNA prepared and IL-1β mRNA levels were measured by Real-Time PCR. Data are means ± SEM.

4.5 DISCUSSION

In contrast to Gram-negative bacteria, where the major immune stimulatory component could be attributed to LPS finally by chemical synthesis (39), a counterpart in Gram-positive bacteria is still under debate. Regarding the differences in cell wall structure between the two types of bacteria, three different cell wall molecules are in focus, that is peptidoglycan, the major component of Gram-positive cell walls, LTA, an amphiphilic molecule with structural similarities to LPS and cell wall incorporated lipoproteins of unknown structure activity relationship. All of them

have been reported to interact with the human immune system (11, 25, 106, 117), but there are also reports who doubt especially the importance of PGN as an immunostimulatory molecule (171).

In this study we aimed to investigate the role of the different cell wall components for the immunostimulatory properties of *S. aureus.* We compared SA 113 wt to three different mutants, which were lacking lipoproteins (Δlgt) or wall teichoic acids (ΔTA) or possessed a reduced alanine content of the LTA (Δdlt). Different to previous attempts, we used a human primary, physiological cell model, with sufficient donor numbers and studied also adequately purified and endotoxin contamination controlled preparations of PGN and LTA of the same mutants.

First, we characterised the immunostimulatory properties of whole UV- or heat-inactivated bacteria of the four strains in human whole blood. Similar TNF release was induced by all strains indicating that there is no effect of the mutations on immune recognition of whole bacteria. This does not mean that these structures are not involved in immune recognition: if we remove a tine from a fork you can still pick something. It appears that the different recognition measures are redundant to an extent that the lack of one is not even quantitatively affecting immune cell activation. The observed significantly lower induction of IL-1β for the Δlgt mutant leads to the suggestion, that lipoproteins might play a role in induction of IL-1β; however, it is most difficult to explain why this affects only certain cytokines. It can also not be excluded that such a major defect in lipid metabolism is not effecting for example the fatty acid composition of the LTA, where structure/function relationships are not yet established. The finding clearly indicates that studies focussing on one cytokine only and interpreting this as activation / no-activation of the monocyte / macrophage are missing the complexity of the recognition system. Similarly, as done in many publications (including from our own group) studying isolated bacterial structures or using cell models with limited pattern recognition capabilities is endangered to overemphasize the relative contribution of single PAMP and PAMP receptors. We likely would not have survived without developing redundancy in the recognition of pathogens.

Next, we investigated the TLR2-dependence of the different *S. aureus* strains using bone marrow derived macrophages from wildtype and TLR2 knock-out mice. Cytokine release from murine macrophages of wildtype and TLR2 knock-out mice was similar for all four bacterial strains, indicating that TLR2 is not at all important for

cytokine release induced by wildtype S. aureus and not for any of its mutants. This is in line with previous findings showing that heterozygous TLR2 polymorphism does not affect whole bacteria induced cytokine responses (180). However, this should similar to above reasoning not mean that TLR2 is not involved in recognition (and studies with individual bacterial components or cell models limited to TLR2-based recognition show this) but obviously there is redundancy again.

To link the cytokine inducing potential of the whole *S. aureus* and its mutants in to the immune stimulatory components of their membranes we isolated peptidoglycan and LTA, the only components for which isolation protocols exist so far. Peptidoglycan was prepared from the four SA 113 strains according to Girardin et al. (43) and stimulated human whole blood with the prepared PGN of either of the strains. HF treatment in all cases diminished IL-1β and led to marginal and borderline significant amounts of TNF, indicating that a remaining contamination and not PGN, which this treatment does not affect, is responsible for most of the activities typically observed. This important finding has been reproduced here for four *S. aureus* strains in two individual preparations. Thus, PGN seems to have a minor role in immunostimulation of human whole blood not only for *S. aureus* wt, but also for its mutants. Moreover, it is remarkable that the resulting pellet after peptidoglycan purification of SA 113 ΔTA lost its compact and compressed composition compared to the other peptidoglycan preparations of SA 113 wt, Δlgt and Δdlt. This might be due to the fact, that WTAs are important for the structural integrity of Gram-positive cell walls and underlines the findings that this mutant shows a decreased colony spreading ability (72) and a reduced capacity in nasal colonisation in rats (81).

Next we aimed to investigate the role of LTA in immunostimulation of human whole blood. For this purpose we prepared LTA from all strains and measured TNF, IL-8 and IL-1β release. We observed significantly lower TNF and IL-1β levels when LTA from SA 113 Δdlt was used as stimulus, which corresponds to the reduced D-alanine content in the LTA, earlier shown to be important for biological activity of LTA (108) also shown for synthetic variants (Morath et al., 2002). These findings underline previous studies with LTA from *Lactobacillus plantarum* Δdlt, which also showed reduced cytokine inducing activity (45). This finding clearly links a structural change in one component of a pattern recognition molecule with its biological activity.

For IL-1β we also observed a significant reduction of cytokine inducing activity of LTA from SA 113 Δlgt compared to its corresponding wildtype, which is in line with our

finding for whole bacteria from SA 113 Δlgt. mRNA analysis by quantitative Real-Time PCR revealed significantly higher levels of mRNA from PBMCs after stimulation with LTA from the wildtype than from SA 113 Δlgt, indicating that the Δlgt mutation in *S. aureus* somehow specifically affects the IL-1β release. To our knowledge this is the first report of such a specific consequence of the Δlgt mutation, and the underlying mechanism needs to be further investigated. It implies a fine-tuning of the pattern of cytokine release in response to structural changes.

Taken together, we have shown that except for the release of IL-1β, major cell wall alterations like lack of WTA, lipoproteins or changes in LTA structure which have consequences on the cell wall integrity and bacterial growth, do not affect the overall cytokine stimulating capacity of whole bacteria in human or murine primary immune cells. It appears that after the exclusion of PGN as a cytokine inducer on its own, besides LTA, the only purified and synthesized component reproducing large parts of the innate immune stimulation by Gram-positive bacteria, other components must exist, which can activate very similar responses. The chemical nature of these compounds still needs to be established. This challenges the numerous reports of the importance of more or less isolated components of this multiple pattern recognition process.

4.6 ACKNOWLEDGEMENT

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5 Lipoteichoic acid from a Staphylococcus aureus wildtype or a lipoprotein diacylglycerol transferase deletion mutant possess similar immune stimulatory activity in human monocytes but not cell lines

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Running title: Lipoteichoic acid is a potent immune stimulus

(prepared for submission)

5.1 ABSTRACT

Lipoteichoic acid (LTA) is a potent immunostimulatory surface component of Gram-positive bacteria as shown by preparation of the full native structure by optimized isolation procedures in the absence of contaminations, e.g. lipopolysaccharide, and by chemical synthesis of a full structure based on the LTA of *Staphylococcus aureus*. This was recently challenged by an LTA preparation from a lipoprotein diacylglycerol transferase deletion (Δlgt) mutant, reported to lack palmitate-labeled lipoproteins and immunostimulation. In the present study, however, whole bacteria as well as the LTA from the SA 113 Δlgt mutant and from the respective wildtype SA 113 strain induced a comparable release of TNF and IL-8 in incubations with human whole blood or peripheral blood mononuclear cells though not in a monocytic human cell line. Analysis of the LTA structure by NMR and GC-MS confirmed that the content and fatty acid composition of the anchor of both LTA was similar. In case of isolated human primary cells we could show that the presence of human serum was crucial for full cytokine inducing activity of LTA, but serum did not enable cytokine induction by Δlgt LTA in the monocytic cell line. However, the comparable cytokine inducing potential of both LTA for primary human immune cells was furthermore confirmed by analysis of gene expression profiling which showed a significant concordance of gene induction between wildtype and Δlgt LTA, but major differences in comparison to $Pam_2Cys\text{-}SK_4$. In conclusion, lipoprotein deficiency does not affect LTA structure and activity as well cytokine induction by whole bacteria in human monocytes. However, in dedifferentiated cell lines or under artificial absence of serum proteins, different biological activities can be demonstrated.

5.2 INTRODUCTION

Sepsis caused by Gram-positive and Gram-negative bacteria is approximately equally frequent and is associated with similar symptoms of disease. While the major immunostimulatory component of Gram-negative bacteria, endotoxin or lipopolysaccharide (LPS), has been known for a long time and is well recognised, the nature of its Gram-positive counterpart is by far less understood.

Lipoteichoic acid (LTA) bears structural similarities to LPS in that it is an amphiphile integrated into the bacterial membrane. The role of LTA as an immune stimulus was controversial for a long time, since the conventional preparation procedure resulted in a loss of activity caused by partial degradation and often brought in substantial contaminations with LPS (109). However, some years ago, the preparation procedure for LTA was modified in such a way that the complete structure could be isolated under LPS-free conditions (108). LTA prepared in this manner displayed potent immunostimulatory properties, inducing a wide spectrum of cytokines and large amounts of chemokines and chemoattractants (25, 179). Final proof that LTA was indeed able to induce cytokine release came with the successful synthesis of a fully synthetic LTA based on the LTA from *Staphylococcus aureus* (110). In contrast to a previous attempt to synthesize a partial structure of LTA (115), this structure showed immunostimulatory potency in human whole blood and induced a similar cytokine pattern compared to native LTA (22). With a series of synthetic partial structures even a minimal active pattern to activate human monocytes could be deduced (21).

The role of LTA as cytokine inducing stimulus was questioned by an article of Hashimoto et al. (52). It described that LTA isolated from a lipoprotein diacylglycerol transferase deletion (Δlgt) mutant was 100-fold less active in inducing cytokine release from a monocytic cell line than LTA from the respective wildtype. Furthermore, it was suggested that not LTA but lipoproteins are the dominant immunobiologically active compound in *Staphylococcus aureus* (51). However, von Aulock et al. have shown that in case of human whole blood, i.e. stimulation of human primary monocytic cells both LTA posses comparable cytokine inducing potency (178). In the present study, we stimulated human whole blood with the same wildtype bacteria and mutant strains described above and observed a comparable potency to induce cytokine release. Next we prepared LTA from either strain and incubated it with primary human cells as well as with cell lines and measured the

induced cytokine release. We observed that the LTA from the mutant strain did stimulate cytokine release in primary human cells though not in human cell lines. Furthermore, both LTA and the synthetic diacylated lipopeptide $Pam_2Cys\text{-}SK_4$ were used to stimulate human whole blood and microarray analysis was done. In this case, no significant difference could be observed between LTA from SA113 wt and SA113 Δlgt whereas $Pam_2Cys\text{-}SK_4$ showed a different pattern of gene induction. These results underline the key role of LTA as an immune stimulus of Gram-positive bacteria in primary human leukocytes. Only in reductionistic model systems other cell wall components can display differential properties.

5.3 MATERIALS AND METHODS

Bacterial strains and cultivation

Staphylococcus aureus strain SA 113 wildtype (SA 113 wt), mutant strain SA 113 *lgt*::*ermB* (SA 113 Δlgt) lacking the lipoprotein diacylglycerol transferase were grown aerobically in a medium containing tryptic soy broth (25 g/l), beef extract (5 g/l) (both from BD Biosciences, Heidelberg, Germany) and glucose (0.8% v/v). The bacteria were harvested after 18 h stirring at 37°C, 150 rpm. Integrity of bacteria and potential contaminations by Gram–negative bacterial species were tested by Gram staining. The bacteria were frozen at –20°C until LTA extraction. For stimulation of human and murine cells the harvested bacteria were washed three times with PBS (PAA Laboratories GmbH) at 1100 x g for 10 min (centrifuge CL-GP, Beckman Coulter, Fullerton, CA, USA) and the pellet was resuspended in PBS to OD_{600}=1 (i.e. about 10^9 bacteria/ml). For UV-inactivation and subsequent whole blood stimulation, 1ml of 10^9 bacteria per well was irradiated on ice (UV-Stratalinker 1800, Stratagene, La Jolla, CA, USA) 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). For heat-inactivation, 1ml of 10^9 bacteria was incubated for 5 min at 95°C in Eppendorf cups Eppendorf, Hamburg, Germany). No colonies grew on blood agar plates (Columbia-blood agar, Heipa Diagnostika, Eppelheim, Germany) after 24 h at 37°C and 5% CO_2.

LTA preparation

The harvested bacteria underwent butanol extraction and hydrophobic interaction chromatography as described (25, 108). The isolated LTAs were tested by kinetic Limulus amoebocyte lysate assay (Charles River, Charleston, SC, U.S.A.) and contained <1.0 EU/mg LTA, i.e. less than 100 pg LPS equivalents per mg LTA.

Human whole blood incubation

Human whole blood incubations were performed as described previously (49). Briefly, human blood was drawn from healthy volunteers into heparinized S-monovettes® (Sarstedt, Nümbrecht, Germany) and diluted fivefold in RPMI 1640 medium (Biochrom, Berlin, Germany). LTAs or whole bacteria were used as stimuli. The final volume was adjusted to 500 µl and the incubations were carried out in open polypropylene vials over night for 22 h at 37°C and 5% CO_2. The pelleted blood cells were then resuspended by gentle shaking and centrifuged at 400 x g for 2 min. The cell-free supernatants were stored at -80°C until cytokine measurement by ELISA.

Preparation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) of healthy volunteers were prepared with CPT™ Cell Preparation Tubes (BD Biosciences). After centrifugation at 1,600 x g for 20 min, PBMCs 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) for at least four times. RPMI 1640 and the stimuli were incubated for 30 min at RT and then the cells were plated at a density of $4x10^5$/tube in the absence of any serum supplement or in the presence of 10% autologous serum or FCS (Biochrom). PBMCs were stimulated in the presence of 5% CO_2 at 37°C for 22 h. The cell-free supernatants were stored at -80°C until cytokine measurement by ELISA.

Preparation of human peripheral blood mononuclear cells for microarray analysis

Human whole blood incubations were performed as described above with 7h of incubation time. Samples were resuspended by gentle shaking, centrifuged at 400 x

g for 2 min and cell-free supernatants were stored at -80°C until cytokine measurement. The remaining cell pellet was resuspended and transferred in a 15 ml centrifugation tube (Greiner Bio-One) on ice. 5 ml of Erythrocytes lysis buffer (EL buffer, cat. no. 79217, Qiagen, Hilden, Germany) was added to each sample and incubated for 10 min on ice. Samples were briefly vortexted twice during the incubation. The samples were centrifuged at 400 x g for 10 min at 4°C and the supernatant was discarded. The remaining leukocyte pellet was washed twice with PBS and stored at -80°C until RNA preparation.

Cell lines

The human monocyte / macrophage cell line THP-1, obtained from the American Type Culture Collection (ATCC, Manassas, VA) was maintained in RPMI 1640 supplemented with 10% FCS (Biochrom) and 100 IU/ml penicillin/streptomycin at 37°C with 5% CO_2. Before stimulation, cells were seeded at $1x10^5$ cells/well into 96-well cell culture plates (Greiner Bio-One) in the presence of PMA (10 ng/ml) for 24 h to induce differentiation to macrophages. Fresh medium was added and cells were incubated for another 24 h before stimulation without any serum contend.

Cytokine assays

Cytokines released by human whole blood and cell lines were measured by sandwich ELISA using commercially available antibody pairs and recombinant standards. Monoclonal antibody pairs against human TNF and IL-8 were purchased from Endogen (Perbio Science, Bonn, Germany) and IL-6 from R&D (Wiesbaden, Germany). Recombinant standard for TNF and IL-6 were a kind gift from S. Poole (NIBSC, Herts, UK) and rIL-8 was from PeproTech (Tebu, Frankfurt, Germany). Assays were carried out in flat-bottom, ultrasorbant 96-well plates (MaxiSorp, Nunc, Wiesbaden, Germany). The secondary biotinylated antibodies were detected by horseradish-peroxidase-conjugated streptavidin (Biosource, Camarillo, CA, USA) and TMB (3,3',5,5'-tetramethyl-benzidine, Sigma) was used as substrate. The reaction was stopped with 1 M H_2SO_4 and the absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm.

Analytical methods

LTA samples were analysed by ^{1}H- and two-dimensional nuclear magnetic resonance (NMR). All measurements were performed on a Bruker Avance DRX 600 spectrometer at 300 K using a 5 mm BBI probe head. Samples were prepared as solutions in 0.6 mL D_2O with sodium 3-trimethylsilyl-3,3,2,2-tetradeuteropropanoate (TSP-d_4) added as internal standard for ^{1}H-NMR (δ_H = 0.00 ppm) and acetone for ^{13}C-NMR (δ_C = 30.02 ppm), respectively. Structure elucidation was accomplished using gradient-enhanced HSQC spectra and two-dimensional total correlated spectroscopy (TOCSY) with pre-saturation for water suppression. TOCSY experiments were carried out in the phase-sensitive mode applying mixing times of 100 ms. Data acquisition and processing were performed with standard Bruker software. The average number of repeating units in the *poly*-glycerophosphate backbone and the percentage of substitution were determined by integration of the pertinent peak volumes in the ^{1}H-NMR. The outcome of glycerophosphate measuring has an error of +/- 5 units. Further analysis of the membrane anchor regarding the fatty acid composition is based on GC-MS measurements.
For the determination of the fatty acid composition of the LTA, gas chromatography-mass spectroscopy (GC-MS; Hewlett-Packard, Böblingen, Germany) was used. LTA (550 µg SA 113 wt, 470 µg SA 113 Δlgt) was dissolved in 100 µl 6 M hydrochloric acid and 500 µl methanol. After addition of 1 ml heptane the samples were incubated at 85°C for 8 h. The organic-phase was evaporated and dissolved in hexane before measurement. For normalisation, the C_{15} carboxylic acid methyl ester (Merck, Darmstadt, Germany) was used as an internal standard. The peaks of the resulting chromatogram were quantified relatively and the MS scans of the peaks were matched with the NBS75K library (Hewlett Packard).

RNA isolation, amplification and labelling, Microarray production and data analysis

RNA isolation and its amplification and labeling as well as the production of microarrays and the following array hybridization and data analysis were kindly provided by Miltenyi Biotec (Miltenyi Biotech, Bergisch-Gladbach, Germany).

Statistics

Statistical analysis was performed using the Graph Pad Prism program (Graph Pad Software, San Diego, USA). Repeated-measure analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison test was assessed. For statistical analysis of two unpaired groups of non-parametric data, the Mann Whitney test was used, whereas Wilcoxon matched pairs test was chosen for two paired groups of non-parametric data. Data are given as means ± SEM. A p-value <0.05 was considered significant. In the figures *, ** and *** represent p-values <0.05, <0.01 and <0.001, respectively; ns, not significant. Cytokine levels given per millilitre blood were corrected for the dilution factor 5 in the 20% blood incubation.

5.4 RESULTS

Whole bacteria from SA 113 Δlgt are as potent as SA 113 wt in inducing cytokine release in human whole blood.

The SA 113 Δlgt strain, which lacks the diacylglycerol transferase, is described to be less immunostimulatory in human monocytic (MonoMac6), epithelial (pulmonary A549) and umbilical vein endothelial cells than SA 113 wt (160). We were interested whether this effect also translates to primary human cells. Therefore, we stimulated human whole blood with increasing concentrations of UV-inactivated bacteria from these wildtype and mutant strains and measured the release of TNF, IL-8 and IL-6. The mutant strain showed the same immunostimulatory potency as the wildtype strain (Fig. 1A and B). Similar results were found for IL-6 (data not shown). It is concluded that Δlgt mutant bacteria do not lack a key component to induce human whole blood cytokine release.

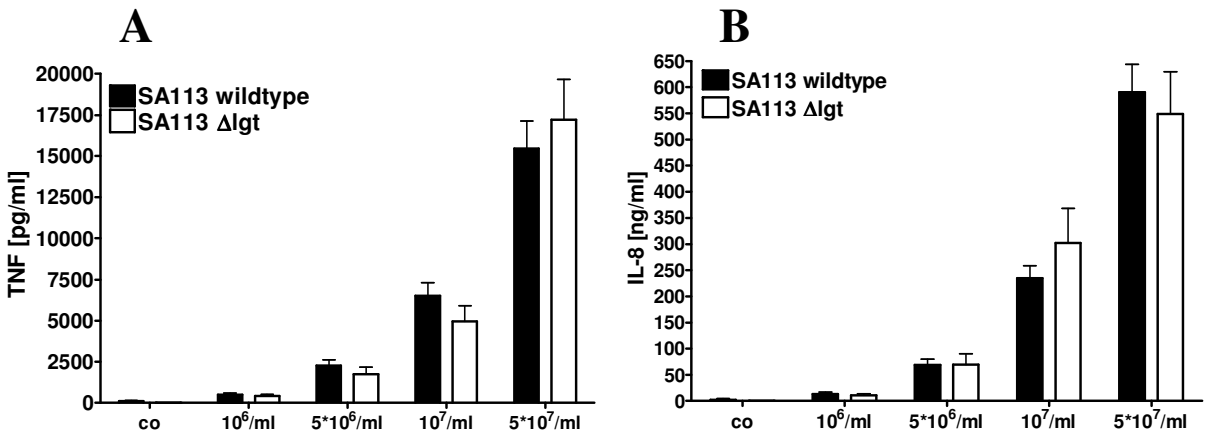

Fig. 1: Comparison of cytokine induction by UV-inactivated bacteria from SA 113 wt and SA 113 Δlgt in human whole blood.

Human whole blood from 16 healthy volunteers was stimulated with whole UV-inactivated SA 113 wt or SA 113 Δlgt bacteria for 22 h. TNF (A) and IL-8 (B) release was measured in the cell-free supernatants by ELISA. Data are means ± SEM.

LTA from wildtype and mutant strains induce the same amount of TNF and IL-8 in human whole blood and PBMC, but not in human THP-1 cells.

We have previously reported that LTA from SA 113 Δlgt and SA 113 wt displays equal immunostimulatory potency in human whole blood (178). We further stimulated human PBMC with increasing concentrations of LTA from SA 113 wt and SA 113 Δlgt and measured the induced TNF and IL-8 release. Similar to the results of the stimulation of whole blood, both LTA induced comparable cytokine release (Fig. 2 A, B). However, when THP-1 cells were stimulated with LTA from SA 113 Δlgt and SA 113 wt, the cells responded only to the wildtype LTA, not to the Δlgt LTA (Fig. 2C). We can conclude that differential properties of the LTA preparations must depend on either the cell type or different incubation conditions.

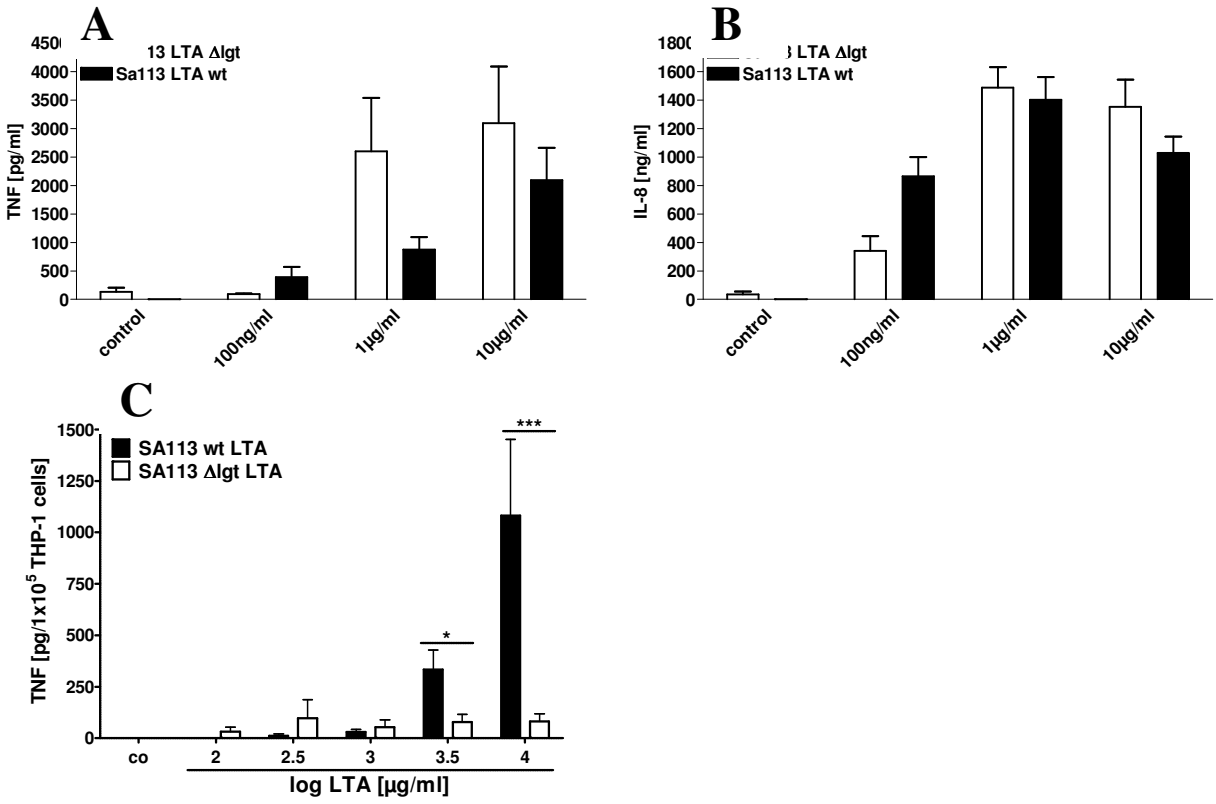

Fig. 2: Comparison of cytokine release by LTA from SA 113 wt and SA 113 Δlgt in human PBMC and THP-1 cells.

PBMC from 4 healthy volunteers (A and B) and THP-1 cells (C) in 9 replicates in two independent experiments were stimulated with LTA from SA 113 wt or SA 113 Δlgt for 22 h. IL-8 (A) and TNF (B and C) release were measured in the cell-free supernatants by ELISA. Data are means ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (A and B, Wilcoxon matched pairs test; C, Mann-Whitney test).

Immune stimulation by SA 113 Δlgt LTA is increased in the presence of human serum

We have shown that LTA from SA 113 wt and SA 113 Δlgt are equipotent in stimulating cytokine release in PBMC, but not in a human monocytic cell line. Besides the fact that PBMCs are primary cells, the experiments contain a major difference. PBMCs were stimulated with 10% autologous serum whereas THP-1 cells contained 10% FCS. To determine to what extend the presence of serum influences the cytokine induction by LTA we stimulated PBMCs with LTA in the presence of either 10% autologous serum, 10% FCS or no serum supplement. Both LTAs were equipotent in inducing TNF in the presence of autologous serum. However, cytokine release induced by LTA from both species was strongly reduced in the absence of a

serum supplement. FCS supplement led to a significant increase in TNF release in case of wt LTA but only to a slight increase in cytokine release in case of Δlgt LTA (Fig. 3). However, the response of human THP-1 cells to LTA SA 113 Δlgt could not be restored if FCS was replaced by human serum (data not shown) indicating a difference in the ability of the two types of LTA to activate primary cells or cell lines. It is concluded, that serum-free conditions can exhibit different activities of the two preparations, but appear not to account for the differences seen in the THP-1 cell line.

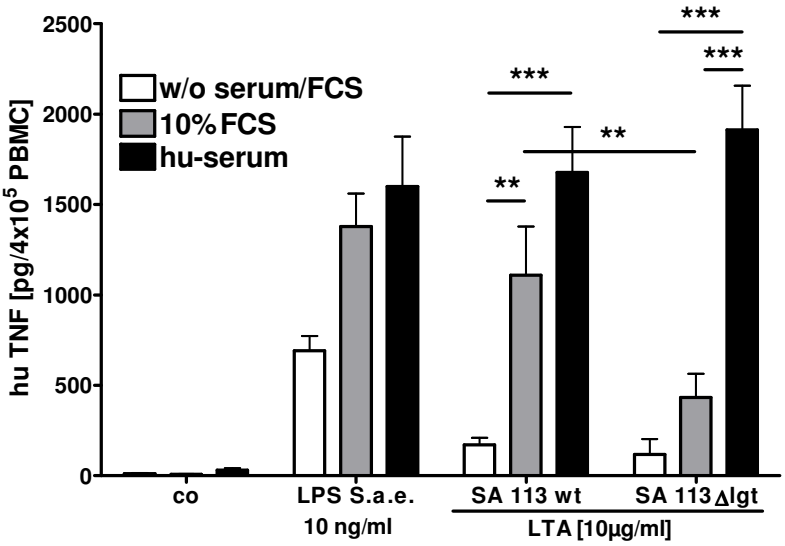

Fig. 3: Influence of serum and FCS on the LTA induced cytokine release in PBMC.

PBMC from 8 healthy volunteers were stimulated with 10 ng/ml LPS from *Salmonella abortus equi* or with 10 µg/ml LTA from SA 113 wt and SA 113 Δlgt for 22 h in the presence or absence of FCS and heat inactivated serum. TNF release was measured in the cell-free supernatants by ELISA. Data are means ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (repeated-measure ANOVA with Newman-Keuls Multiple Comparison Test).

LTA from SA 113 wt and Δlgt have a similar chemical structure

To determine the chemical structures of both LTAs we analysed two different lots each of LTA from SA 113 wildtype and Δlgt by NMR. Table 1 show that all LTA preparations contained similar amounts of glycerophosphate units, similar alanine levels and that the rate of GlcNAc is below 1% for both LTA.

Composition	LTA SA 113 wildtype		LTA SA 113 delta lgt	
	Lot 3	Lot 4	Lot 6	Lot 7
Glycerophosphate units	44-45	41	40	34
Fatty acids	C17	C15-C16	C17	C14-C15
D-Ala	77-80%	82-85%	76-79%	79-84%
GlcNAc	0.3%	0.2%	0.5%	0.2%

Table 1: NMR analysis of the chemical structure of LTA from SA 113 wt and SA 113 Δlgt.

The LTA anchor from SA 113 Δlgt contains the same composition of fatty acids compared to LTA from SA 113 wt.

Since it has been described that differences in the fatty acid content of LTA influence its immunostimulatory potency (77, 151), we investigated the fatty acid composition of the LTA anchor from both bacterial strains by GC-MS. The LTAs underwent an acidic methanolysis and the released methyl esters of fatty acids were determined by GC-MS. LTA from SA 113 wt and SA 113 Δlgt mainly consisted of C_{14}, C_{16}, C_{17} and C_{18} fatty acids (Table 2) and no significant difference could be detected between wt and lgt LTA. The peak at 15.6 min was only present in two out of the three preparations and is represented by a C_{14} with an additional methyl-group. In conclusion, no major structural difference between the preparations was observed. However, due to the micro-heterogeneity of LTA, more subtle changes and lack of certain subspecies can not be excluded.

Peak at	Fatty acid	Area under the curve (%)	
		SA 113 wt	SA 113 Δlgt
15.5 min	Methyltetradecanoate	121.6 ± 2.3	73.43 ± 1.01
15.6 min	C_{14} + CH_3	185.5 ± 5.5	154.3 ± 1.3
16.8 min	Hexadecanoic acid	139.9 ± 4.9	113.4 ± 0.97
18.7 min	Hepta-/ Octadecanoic acid	140.95 ± 14.05	132.6 ± 4.23

Table 2: Analyses of the fatty acid content of LTA from SA 113 wt and SA 113 Δlgt after acidic methanolysis by GC-MS.

The C_{15} carboxylic acid methylester (Merck, Darmstadt, Germany) was used as an internal standard and its area under the curve was set to 100%. The areas for the other peaks were correlated to this value. Data represent means ± SEM of one lot of wt or lgt LTA, measured at least in duplicates and is one representative out of three independent preparations. One-way ANOVA with Kruskal-Wallis test did not reveal statistical differences.

LTA from SA 113 wt and Δlgt but not PAM_2Cysk induce a similar gene expression profile of inflammatory genes

Stimulation of human whole blood and of human PBMC by SA 113 wt LTA and LTA from the Δlgt mutant resulted in comparable cytokine release levels. To compare the immunostimulatory activity of both LTA in a broader attempt, an immunology microarray comprising 1076 genes was performed. The genes belonged to different immunological gene families such as apoptosis and signal transduction, cell cycle, chemokines and cytokines and their receptors, extracellular matrix proteins, inflammation and complement system. Human whole blood was stimulated with both LTAs and the synthetic diacylated lipoprotein $Pam_2Cys\text{-}SK_4$ for 7 hours. After lysation of the erythrocytes the RNA was extracted for microarray analysis on the immunochip. In Fig. 4 it is shown that the clustered heat-maps of SA113 wt and Δlgt showed strong overlaps, but was different from the cluster of $Pam_2Cys\text{-}SK_4$. Furthermore, only four out of 1,076 genes were significantly different regulated after stimulation with either of the LTAs, whereas stimulation with $Pam_2Cys\text{-}SK_4$ led to a considerably different pattern and a significant differently regulation of 43 genes compared to SA 113 wt LTA and 39 compared to Δlgt (data not shown).

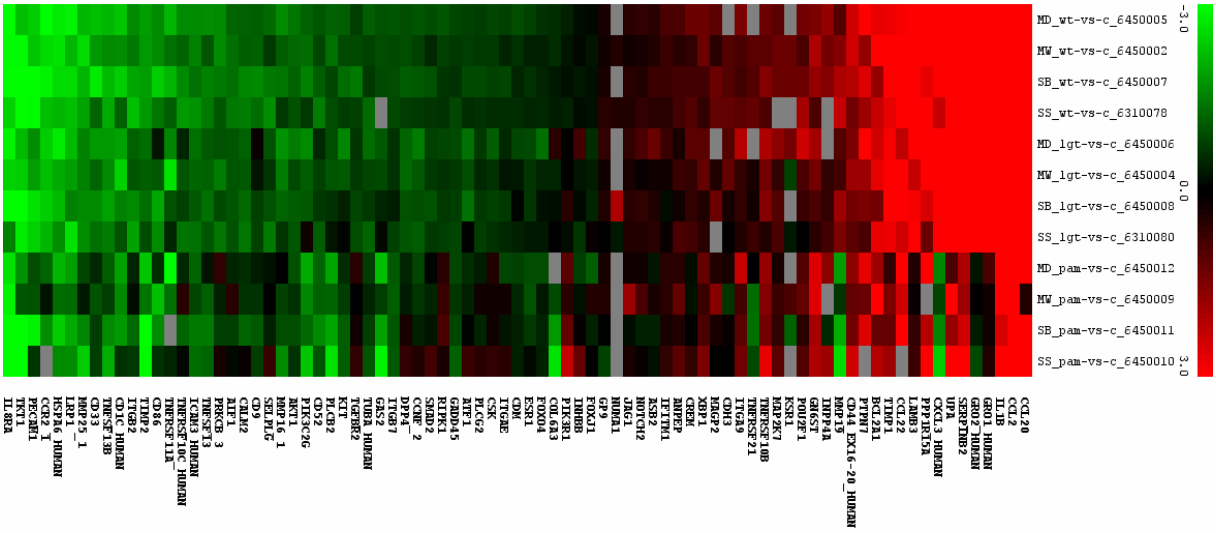

Fig. 4: Microarray analysis of LTA from SA113 wt and Δlgt plus $Pam_2Cys\text{-}SK_4$

mRNA expression pattern obtained with mRNA microarrays of human whole blood from 4 healthy volunteers stimulated with either LTA from SA 113 wildtype (wt), LTA from SA 113 Δlgt (lgt) or $Pam_2Cys\text{-}SK_4$ (pam) or left untreated (c) for 7h. Erythrocytes were lysed and RNA was prepared from the remaining PBMC.

5.5 DISCUSSION

The immune stimulatory potential of LTA, a major component of the Gram-positive cell wall is still under debate. A new isolation procedure for LTA was published using butanol for the extraction instead of hot phenol or chloroform-methanol resulting in highly pure and intact LTA (108). The previously used methods were demonstrated to result in a decomposition of the LTA accompanied with the reduction or loss of its immunostimulatory potency, thus explaining previous contradictory data (109). Since then, LTA from several species have been isolated and characterized. Despite structural variations they all show conserved cytokine induction patterns (25, 45, 57, 58). Finally, a fully synthetic LTA representing LTA from *Staphylococcus aureus* was shown to be a potent stimulus of cytokine release (110).

Recently, Hashimoto et al. (52) isolated an LTA inactive in cytokine induction in human pro-monocytic cell line from a *Staphylococcus aureus* mutant, which is characterized by the loss of the lipoprotein diacylglycerol transferase resulting in the absence of lipoproteins. Upon performing some further experiments this group concluded that LTA in general has no immunostimulatory potency and that the activity in LTA preparations stems from contaminations with highly potent lipoproteins. As this conclusion cannot explain the immunostimulatory activity of

synthetic LTA, which shows a cytokine induction pattern which is identical to that of highly purified native LTA (21, 22), we have explored alternative explanations for the reported lack of immunostimulatory activity of the LTA of this mutant in the present study.

First we characterised the immunostimulatory properties of whole, UV-inactivated bacteria from SA 113 wt and SA 113 Δlgt in human whole blood. The bacteria were equipotent in inducing cytokine release, indicating that despite the lack of lipoproteins in the mutant, the main immunostimulatory structure must be present. However, whole bacteria of the mutant strain have been described to induce lower levels of pro-inflammatory cytokines than the wildtype bacteria in MonoMac cells and other cell lines (160). This appears to indicate a difference between the ability of the bacteria to activate primary cells in comparison to these cell lines. Such a difference is not surprising given the reduced differentiation of pro-monocytic leukemic proliferating cells versus non-proliferating health human monocytes.

Next we isolated LTA from SA 113 wt and SA 113 Δlgt according to Morath et al. (108). The stimulation of human PBMC resulted in the release of similar amounts of TNF and IL-8 by both LTA. This is congruent with our results of the stimulation of human whole blood (178). It has to be noted that the experiments with human whole blood and PBMC have been performed independently in laboratories of von Aulock (University of Konstanz) and Ulmer (Research Center Borstel). However, when we stimulated PMA differentiated THP-1 cells with either of the two LTA, the THP responded only to wt LTA but not to Δlgt LTA (Fig. 2C). In our experimental setting, PBMC are stimulated in the presence of autologous serum, while THP are cultured and stimulated with FCS. As shown in figure 3, the presence of human serum is a prerequisite for LTA to potently induce cytokine release. As recently shown by our group this is due to the presence of a human immunoglobulin that augments cytokine induction by LTA. Surprisingly, this is more pronounced for Δlgt LTA than for wt LTA (155). However, the addition of human serum did not enable human monocytic cell lines to respond to Δlgt LTA, as we would have expected. This might be due to the fact that LTA must be coated to surfaces to potently induce cytokine release (23), what might be hampered if working with adherent cells. These recent results might also explain why Hashimoto et al. observed low cytokine release by LTA from SA 113 Δlgt when stimulating cell lines.

To exclude that our observations are due to structural differences of the two LTA, NMR and GC-MS analysis were done NMR analysis of two different lots of either of the LTAs revealed in similar amounts of glycerophosphate units and similar alanine levels, which indicates no major differences between the two molecules. However, measurement of composition and length of fatty acids is relatively inaccurate via NMR and thus we decided to determine this manner by GC-MS measurement. Acidic methanolysis led to a release of methyl esters of fatty acids which were further analysed by GC-MS. The results demonstrate again no major difference in the fatty acid composition in the preparation of LTA from SA 113 Δlgt compared to the wildtype strain (Table 2), indicating that the deletion of the lipoprotein diacylglycerol transferase has no influence on the fatty acid content and its composition of SA 113 Δlgt LTA. Taken together, these data do not demonstrate a major structural difference between both LTAs. Differences seem to be not larger than between different batches of wt-LTA. However, due to the micro-heterogeneity of LTA the analytical methodologies do not allow to distinguish more subtle differences such as location of substitutes, combinations of fatty acids, place of fatty acid methylations etc.. Also the absence or presence of certain variants in the mixture of LTA molecules cannot be shown. It can thus not be excluded that such variants account for the differences seen in systems other than human monocytes in the presence of autologous serum. However, a small fraction of lipoprotein contamination exerting its effect only under conditions, where TA does not, represents the alternative explanation.

To further compare immune stimulatory potential of wt and Δlgt LTA, we investigated the effect of immune stimulation by either wt or Δlgt LTA and the synthetic lipoprotein $Pam_2Cys\text{-}SK_4$, which was used as a broadly used lipoprotein substitute for the unknown natural one of *S. aureus*, in an RNA level by microarray analysis. The clustered heat-maps of SA113 wt and Δlgt showed strong overlaps whereas stimulation with $Pam_2Cys\text{-}SK_4$ resulted in a different pattern. Comparison of wt and Δlgt LTA resulted in only 4 significantly different regulated genes, whereas stimulation with $Pam_2Cys\text{-}SK_4$ showed 43 significantly different regulated genes compared to SA 113 wt LTA and 39 compared to Δlgt (data not shown). Taking a closer look on the regulated genes mainly chemokines are upregulated in whole blood stimulations with both LTAs, which is consistent with previous findings (179) and cannot be found in stimulations with $Pam_2Cys\text{-}SK_4$. These gene array results

support our protein data obtained from LTA-stimulated primary human cells and point to a significant concordance between the two LTA preparations. In contrast a distinct difference is observed between both LTA and the synthetic lipoprotein $Pam_2Cys\text{-}SK_4$. Taken together, Δlgt mutants contain similar LTA inducing potential and identical mRNA response by human blood monocytes. Even in the absence of lipoproteins, like in SA 113 Δlgt, LTA is a major immunostimulatory structure of Gram-positive bacteria and induces cytokine release in human whole blood and peripheral blood mononuclear cells in a comparable manner. Only artificial situations, such as the absence of human serum or dedifferentiated cell lines, allow to demonstrating differential activities of the mutant strain.

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6 Summarising discussion

In contrast to Gram-negative bacteria, where the major immune stimulatory component could be finally attributed to LPS by chemical synthesis (39), a counterpart in Gram-positive bacteria is still under debate. Regarding the differences in cell wall structure between the two types of bacteria, three different cell wall molecules are in focus, which are PGN, the major component of Gram-positive cell walls, LTA, an amphiphilic molecule with structural similarities to LPS and cell wall incorporated lipoproteins of unknown structure activity relationship. All of them have been reported to interact with the human immune system (11, 25, 106, 117), but there are also reports who doubt especially the importance of PGN as an immunostimulatory molecule (171).

In this study, we aimed to investigate the role of each of these structures using different approaches. First a meta-analysis and therefore a systematic review of the published literature was performed checking for a possible fulfilment of the four well-known criteria of Koch and Dale for LTA, PGN or bacterial lipoproteins as major immune stimuli. The meta-analysis turned out to be extensive: The number of publications about cytokine release and immunostimulation of these three surface molecules is huge, the provided information is diverse and the studies offer a large variety of different settings, outcomes and results. We decided to restrict our search to human studies what revealed on the one hand more relevant information of the actual interaction between pathogens and the human immune system but might also have led to a loss of interesting and important pieces of evidence. The first step (and therefore the first criterion) in this review was to verify if the three analyzed surface molecules were found or even purified from cytokine inducing Gram-positive bacteria. PGN is well known to be ubiquitous in Gram-positive bacteria (177), whereas some Gram-positive bacteria lack LTA i.e. several Micrococci (*M. luteus, M. flavus, M. sodonensis*), *Bifidobacterium bifidum* and Mycobacteria (127, 163). Bacterial lipoproteins are also essential for Gram-positive bacteria (6, 119) and we could also find publications mentioning lipoprotein isolation from different Gram-positive bacteria. However, the absence of a structural identification of a LP which is responsible across a wider variety of bacterial species for cytokine induction leads to the conclusion, that only a general presence of LPs must be assumed, but the K/D criterion 1 can not be considered verified. We therefore focused on finding

publications that were able to isolate LTA from Gram-positive bacteria and we revealed 58 original articles mentioning LTA isolation with appropriate methods. This led to the conclusion that the K/D criterion 1 is fulfilled for LTA and PGN. Next, the identification of inhibition or even deletion of genes involved in the synthesis of one of the surface molecules and the respective effect was the aim for K/D criterion 2. Thus, we searched for deletion mutants of Gram-positive bacteria for LTA or lipoproteins; PGN was excluded in this case, because one can hypothesise that a mutant lacking PGN would be unable to grow or survive due to the responsibility of PGN for the integrity of the Gram-positive cell wall. After application of our predefined demands for the specific selection of the literature we were able to find three publications for LP and two in the case of LTA showing that deletion or modification of either of the molecules leads to a significantly reduced cytokine induction and immunostimulatory properties of these bacteria. Work presented in this thesis complements these findings showing that LP deletion mutants are as capable to induce cytokine release, while modifications of alanine content in LTA reduces cytokine induction. Hence, we can consider the Koch/Dale criterion 2 being fulfilled for LTA only, with controversial findings for LP.

The database for Koch/Dale criterion 3, addressing the question weather exposure to LTA, PGN or bacterial lipoproteins induces cytokine induction, was the most voluminous compared to the other criteria. Using the keywords “(bacteria and lipoteichoic acid) and cytokines” or “(bacteria and peptidoglycan) and cytokines” or “(bacteria and lipoproteins) and cytokines” we obtained 285 publications for LTA, 380 publications for PGN and 391 publications for lipoproteins from NCBI. The publications were preselected according to a filter in order to set basic quality standards and they were then further analysed for the fulfilment of the Koch/Dale criterion 3 and 4, respectively. These decisions resulted then in 24 publications for LTA, 12 for PGN and 8 for LP. In all cases, induction of different cytokines could be shown which led to a fulfilment of Koch/Dale criterion 3. The last part of this meta-analysis was the test for publications which provide information about an antibody, antagonist or inhibitor for LTA, PGN or LP and its role in cytokine induction in humans. Unfortunately, no publication could be found for either PGN or LP and only one for LTA (26). This publication shows a significant reduction in cytokine release when applying the inhibitor polypropylene glycol and based on this, the Koch/Dale criterion 4 can be considered as fulfilled for LTA with the limitation of one identified

paper only. Noteworthy, from the secondary literature pool a paper was identified describing specific anti-LTA antibodies augmenting recognition of LTA and cytokine response hinting also to a structure-specific intervention (96). The lack of a sufficient amount of publications in this case leads to the need of collecting more data especially in human cell systems, which will provide better information and more knowledge about the interaction between LTA and the human innate immune system. Furthermore it will be important to find out if LTA is a prerequisite for cytokine induction using bacteria which have atypical LTAs or even replacing structures. Taken together, based on this meta-analysis, bacterial lipoproteins and PGN might play a role in cytokine induction in humans as no contradictory results were found for human monocytes / macrophages, but LTA is the only Gram-positive membrane compound, which fulfils all of the four Koch/Dale criteria making it a prime candidate for the Gram-positive counterpart to LPS in Gram-negative bacteria. Therefore, the next parts of this thesis addressed the role of PGN, LTA and LP on an experimental basis.

For this purpose we compared SA 113 wt to three SA different mutants, which were lacking lipoproteins (Δlgt) or wall teichoic acids (ΔTA) or possessed a reduced alanine content of the LTA (Δdlt). First, we characterised the immunostimulatory properties of whole UV- or heat-inactivated bacteria of the four strains in human whole blood. Similar TNF release was induced by all strains indicating that there is no effect of the mutations on immune recognition of whole bacteria. Additionally, we observed a significantly lower induction of IL-1β for the Δlgt mutant. This observation leads to the suggestion, that lipoproteins might play a role in induction of IL-1β; however, it is most difficult to explain why this affects only certain cytokines. These results show that it is important to studying more than one cytokine because a focus on one cytokine leads to missing of the complexity of the recognition system. A further problem is the focus on isolated bacterial structures or cell models with limited pattern recognition capabilities which might lead to different conclusions concerning the relative contribution of single PAMPs or receptors. Next, the question arose if and how the important pattern recognition receptor TLR2 contributes to the recognition of whole bacteria. The results after testing murine bone marrow derived macrophages from wild type and TLR2 knock-out mice showed no difference in cytokine release from all mutants. This indicates that TLR2 is not important for cytokine release of SA 113 wt and the three mutants, but this does not mean, that TLR2 is not involved in

recognition and shows again a hint for redundancy. To link the cytokine inducing potential of the whole *S. aureus* and its mutants to the immune stimulatory components of their membranes we isolated PGN and LTA, the only components for which isolation protocols exist so far. PGN was prepared from the four SA 113 strains according to Girardin et al. (Girardin et al., 2003) and human whole blood was stimulated with the prepared PGN of either of the strains. The last step of the purification (HF treatment) diminished IL-1β induction in all cases and led to marginal and borderline significant amounts of TNF, indicating that a remaining contamination and not PGN, which is not affected by this treatment, is responsible for most of the activities typically observed. This important finding stands in contrast to studies cited in the meta-analysis. Thus, PGN seems to have a minor role in immunostimulation of human whole blood not only for *S. aureus* wt, but also for its mutants. The reason for this contradiction of the meta-analysis and the experimental findings is the limitation on publications using human systems, whereas Travassos et al. (171) showed the abolished cytokine release and immunostimulation of PGN only in mice. Since our own findings are not yet published, they could therefore not be included in the meta-analysis. As PGN does not contribute to immunostimulation as an isolated structure according to these results we next aimed to investigate the role of purified LTA of SA 113 wt and the three mutants. Significantly lower TNF and IL-1β release were observed after stimulating whole blood with LTA from SA 113 Δdlt, this corresponds to the reduced D-alanine content in the LTA as shown before (Morath 2001; Grangette, 2005). LTA from SA 113 Δlgt also induced significantly lower amounts of IL-1β compared to the wild type LTA indicating again a contribution of lipoproteins in IL-1β induction as observed before with whole bacteria. mRNA analysis also revealed lower levels of IL-1β mRNA in PBMCs stimulated with LTA from SA 113 Δlgt compared to SA 113 wt, indicating that the Δlgt mutation specifically affects the IL-1β release. To our knowledge this is the first report of such a specific consequence of the Δlgt mutation, and the underlying mechanism needs to be further investigated. It implies a fine-tuning of the pattern of cytokine release in response to structural changes.

These structural changes and the accompanying loss of lipoprotein content in the cell wall of SA 113 Δlgt and the resulting consequences were investigated in the last part of the thesis. The establishment of a method for highly pure and immunostimulatory active LTA (108) lighted the possibility to investigate its contribution to the recognition

of Gram-positive bacteria by the immune system. Since then, LTA from several species have been isolated and characterised (25, 45, 57, 58), but recently, Hashimoto et al. (52) challenged these findings with an isolation of an LTA being inactive in cytokine induction, namely from SA 113 Δlgt. Contrary to the findings of Hashimoto et al., we observed a similar cytokine pattern of LTA from SA 113 Δlgt compared to wt LTA, only in IL-1β induction we observed significantly lower cytokine release of SA 113 Δlgt both in stimulation with whole bacteria and LTA. One major difference in the experimental setting of Hashimotos and our group is the complexities of the cell system used for the experiments. Stoll et al. (160) showed a lower induction of pro-inflammatory cytokines using whole bacteria of the mutant strain in MonoMac cells compared to the wild type, whereas our results in human whole blood do not show this effect. This might indicate a difference between the ability of the bacteria to activate primary cells in comparison to these cell lines. This difference is not surprising given the reduced differentiation of monocytic leukemic proliferating cells versus non-proliferating healthy human cells. However, when we stimulated PMA-differentiated THP-1 cells with either of the LTAs, we could confirm the results from Hashimotos group with LTA from SA 113 Δlgt being inactive in a cell line system. Again, we found a difference in our experimental settings comparing primary cells (PBMCs) and cell lines (THP-1), which is the use of autologous serum for PBMCs and FCS for THP-1 cells. As recently shown by our group (155) the presence of human serum is a prerequisite for LTA to induce cytokine release due to the presence of a human immunoglobulin that augments cytokine induction by LTA. Unfortunately, the addition of human serum did not enable THP-1 cells to sufficiently respond to LTA form SA 113 Δlgt, as we expected. A possible explanation is the fact that LTA must be coated to surfaces to potently induce cytokine release (23, 155), what might be hampered if working with adherent cells. Additionally this might explain why Hashimoto et al. observed low cytokine release by stimulating cell lines with LTA from SA 113 Δlgt. Another explanation for the observed problems in inducing cytokine release in cell lines using LTA from SA 113 Δlgt could be a difference in the chemical structure of this LTA compared to the wild type. For this purpose we analysed the structures of wt and Δlgt LTA by NMR and GC-MS. NMR analysis revealed similar amounts of glycerophosphate units and alanine levels, indicating no major differences between the two molecules. Additionally, GC-MS analysis of the released methyl esters of fatty acid after acid methanolysis also demonstrated no

major differences in fatty acid composition. Taken together, the deletion of the lipoprotein diacylglycerol transferase has no influence on the fatty acid content and its composition, these differences seem not to be larger than between different batches of SA 113 wt LTA. This also confirms the findings of Hashimoto (52). However, due to the micro-heterogeneity of LTA the analytical methodologies do not allow to distinguish more subtle differences like location of substitutes, combinations of fatty acids, place of fatty acid methylations etc. Supposable, these minor differences do not play a role in the observed different recognition of LTA from SA 113 wt and Δlgt, but it would be inaccurate to exclude it.

So far we investigated the role of different stimuli from *S. aureus* mainly on a protein basis measuring cytokine release. To gain more insight of the complex signal transduction and the underlying genomic alterations we investigated the effect of immune stimulation by either LTA from SA 113 wt or Δlgt plus the synthetic lipoprotein $Pam_2Cys\text{-}SK_4$ in an RNA level by micro array analysis. $Pam_2Cys\text{-}SK_4$ was in this case applied as a broadly used lipoprotein substitute for the unknown natural one of *S. aureus*. Clustered heat-maps of LTA from SA 113 wt and Δlgt strongly overlap whereas stimulation with $Pam_2Cys\text{-}SK_4$ resulted in a different pattern. Further evidence for a different pattern of LTA and lipoprotein recognition is that only four significant differently regulated genes were found comparing SA 113 wt and Δlgt LTA, whereas stimulation with $Pam_2Cys\text{-}SK_4$ resulted in 43 significant differently regulated genes compared to SA 113 wt LTA and 39 compared to LTA from SA 113 Δlgt. In the end, mainly chemokines are upregulated in whole blood stimulations with both LTAs, which is in line with previous findings (179) and cannot be found in stimulations with $Pam_2Cys\text{-}SK_4$. Hence, we can conclude that the gene array results support our cytokine protein data from LTA-stimulated primary human cells and points again to a concordance between the two LTA preparations. Therefore, we can say that we could not find evidence doubting the role of LTA as being a major immune stimulus and that LTA contributes to the recognition of Gram-positive bacteria even in the absence of lipoproteins (Δlgt) in complex systems like human whole blood or PBMCs. Otherwise, in artificial situations such as the absence of human serum or dedifferentiated cell lines, demonstrates differential activities of the mutant strain and points therefore to a contribution of lipoproteins in this case.

In addition it is possible to clarify the role of the three surface molecules in their interaction with the human innate immune system with an alternative and more

objective approach. Sir Austin Bradford Hill described criteria for assessing evidence of causation in 1965 (60 58 (1965), 295-300). The criteria are as following:

- **Strength:** The stronger an association between cause and effect the more likely a causal interpretation, but a small association does not mean that there is not a causal effect.
- **Consistency:** Consistent findings of different persons in different places with different samples increase the causal role of a factor and its effect.
- **Specificity:** The more specific an association is between factor and effect, the bigger the probability of a causal relationship.
- **Temporality:** The effect has to occur after the cause.
- **Biological gradient:** Greater exposure should lead to greater incidence of the effect with the exception that it can also be inverse meaning greater exposure leads to lower incidence of the effect.
- **Plausibility:** A possible mechanism between factor and effect increases the causal relationship with the limitation, that knowledge of the mechanism is limited by best available current knowledge.
- **Coherence:** A coherence between epidemiological and laboratory findings leads to an increase of the likelihood of this effect. However, the lack of laboratory evidence cannot nullify the epidemiological affect on the associations.
- **Experiment:** Similar factors which lead to similar effects increase the causal relationship of factor and effect.

Even as Sir Austin Bradford Hill did not intent to describe a checklist it is here used as one for the three Gram-positive membrane components LTA, PGN and LP.

- **Strength**

For this thesis the strength of an association between factor and effect was addressed by comparing cytokine induction of the stimulus versus the non stimulated cells. For LTA cytokine induction occurred in all cases at high levels compared to the control. This was the case for TNF, IL-1β and IL-8. The absence of lipoproteins using LTA from SA 113 Δlgt led to a significant decrease of IL-1β induction in human whole blood, but cytokine levels remained higher than the unstimulated control. This might show that LP have a role in IL-1β induction, but it seems not to be the major one. The lack of WTA did not alter cytokine release, whereas a reduced alanine content in the LTA from the Δdlt mutant led to significant reduction of TNF and IL-8 induction still

being higher than the unstimulated control. This result confirms previous findings (108). PGN is in this thesis reported not to be an immune stimulus as the PGN preparations failed to induce significant amounts of IL-1β and only borderline significant amounts of TNF in human whole blood. Even that the strength of evidence is very low for the purified component its role in immune recognition cannot be excluded as PGN breakdown products like MDP signal intracellularly via NOD (43), which also confirms Bradford Hills warning that a small association does not mean that there is not a causal effect (60). For LP it is more difficult to address this question as there is currently no purified LP from Gram-positive bacteria available. The synthetic derivates $Pam_3Cys\text{-}SK_4$ and $Pam_2Cys\text{-}SK_4$ are a very potent inducer of cytokine release in cell lines (13), but fail to induce higher cytokine amounts in complex human systems like PBMCs or human whole blood (26).

- **Consistency**

LTA was prepared from many different species by different groups at different places; the database for analysis of cytokine induction by different LTAs is large. Even in this thesis alone LTA was prepared from different SA 113 mutants and was tested for cytokine release in different laboratories; here mentioning Arthur Ulmers Lab in Borstel, Germany. Again, evidence for LTA is strong as results are consistent over different places and different experimenters. The findings for PGN not contributing to cytokine release when highly purified from this thesis confirmed previous findings of Travassos et al (171) also leading to a strong evidence for the absence of a causal relationship in this case. For LP however, findings may be considered as consistent for different persons and places, but the lack of LP preparations lead to the lack of different samples as only synthetic artificial derivates are being used. It is still not clear whether these synthetic molecules really reflect the in vivo situation.

- **Specificity**

A high specificity of the effect would lead to a strong evidence for a causal effect. The readout systems in this thesis are based on reactions of the human innate immune system. This system needs to react very quickly on threats and comes with a decreased specificity as the intruder has to be distinguished between endogenous and pathogen quickly without a priority on its identification. Hence, cytokine release in complex human systems is an unspecific, but fast reaction. Here it is only possible to draw conclusions based on the cytokine profile of the three cell wall components. LTA induces a broad spectrum of cytokines, different from the spectra of PGN and

LP. This is also the case comparing LTA and the synthetic lipoprotein $PAM_2Cysk\text{-}SK_4$ on RNA level in microarray analysis as done in one of the articles of this thesis. The widest cytokine pattern is the one from whole bacteria as they are able to induce the whole range of human cytokines as expected without major differences when comparing different SA 113 mutants and the wild-type except for IL-1β using SA 113 Δlgt as a stimulus. It has also been taken into account, that synthetic LTA is able to induce cytokines in humans in a similar manner to native LTA (21, 22) which is a hint for a specific reaction. As synthetic lipoproteins also exist and are able to induce cytokine induction at least in cell lines (13). We can conclude here, that the specificity is low for PGN and slightly higher for LP and LTA.

- **Temporality**

As the effect has to occur after the stimulation to provide evidence for a causal effect, it is here possible to fulfil this sub-point for LTA PGN and LP as the cytokines were measured 22h post stimulation. Also the RNA kinetics show an increase of IL-1β levels only at the during the first 3 hours post stimulation with either LTA from SA 113 wt or Δlgt, so we can consider a time dependent effect. Although this kinetic was not tested for LP or PGN we can clearly say that cytokine release always occurs after the stimulation.

- **Biological gradient**

Increase of the stimulus concentration leads in all cases to an increase in cytokine release independent of the stimulus being whole bacteria, LTA or PGN. It was also previously shown for synthetic lipoproteins (13). The biological gradient exists for all stimuli used in this thesis and leads therefore to evidence of a causal relationship.

- **Plausibility**

Next it is important to find a plausible mechanism for the interaction of LTA, PGN and LP with the human innate immune system, which would provide more evidence for a causal relationship between stimulus and cytokine release. Historically, LTA was first considered to signal via TLR4 (164), but this was found to be due to contaminations with LPS (109). Purified LTA was then considered to signal via TLR2 (149) with confirmation by using a synthetic LTA (111). This was questioned again by using a LTA from a SA Δlgt mutant lacking lipoproteins (52) with a decreased cytokine inducing activity and loss of TLR2 dependence. Our own findings however show that this mutant LTA is not able to induce cytokines in cell lines (155) but this thesis also displays similar cytokine inducing activity in human whole blood with the already

mentioned exception in IL-1β release, which is also found in experiments using whole bacteria of this strain. This underlines the importance of Bradford Hill's statement that the knowledge of a mechanism is limited by current knowledge (60). The understanding of this mechanism is likely to develop further in the future. The history shows the importance of this limitation also for PGN, which was first described to be a TLR2 stimulus (164), but later found to not be immunostimulatory when highly purified (140, 171). At least for synthetic lipoproteins the mechanism is clear by signaling via TLR2 (128). In addition to this it is important to mention that the three surface molecules are widespread and highly conserved in Gram-positive bacteria. The fact that synthetic molecules of LTA and LP display a somehow similar cytokine inducing activity leads to a prediction of a possible specific mechanism for immune recognition even though these mechanisms are still not fully understood and somehow controversial. We can here conclude that the mechanism and therefore the specificity is highly dependent on current general knowledge of purification procedures, experimental settings and ligand-receptor interactions. At least for LTA and lipoproteins the causal relationship is probable in this case as well as for PGN being recognised intracellularly.

- **Coherence**

A coherence between epidemiological and laboratory finding would increase the causal connection between the stimuli and the effect on cytokine release. Gram-positive bacteria are known to have the possibility to induce severe infections and sepsis in humans, so it is necessary to address here the comparison between cytokine release of whole bacteria and the purified components LTA, PGN and LP. Using whole bacteria as a stimulus we found a wide range of cytokine release. LTA was similar to this cytokine pattern, but less potent in total. PGN and LP do not induce as high amount of cytokines, so we can see here mainly coherence between laboratory and epidemiological findings in case of LTA. Additionally, LTA was found to be detectable in patients with *Streptococcus pneumoniae* induced meningitis (161) which leads to a higher probability of evidence for a causal relationship between stimulus and effect. A high coherence between epidemiological and laboratory finding exists only for LTA.

- **Experiment**

The most similar factor to the three surface molecules LTA, PGN and LP is the Gram-negative molecule LPS. It was used as a positive control in all experiments and possesses a structural amphiphilic similarity to LTA and lipoproteins. The potency of LTA in cytokine release compared to LPS is about 100 to 1000 fold lower, but LTA potency is still 100 to 10,000 fold higher than PGN or LP. The difference in potency is further reduced, when LTA is presented on a surface (23), a phenomenon not observed for other stimuli.

In summary LTA delivers most evidence for a causal relationship of stimulus and effect based on Bradford Hill's criteria. Seven out of eight criteria are fulfilled, only the specificity is missing. For PGN, four out of eight criteria are fulfilled with the exception of strength, specificity, coherence and experiment. For strength it has to be noted that the weak association here does not mean that there is no causal connection between stimulus and effect looking on the fact that PGN breakdown products are recognised by the immune system. For bacterial lipoproteins only four criteria are fulfilled, namely temporality, biological gradient, plausibility and experiment with a weaker evidence for a plausible mechanism as only synthetic lipoproteins are available with a clearly investigated specific mechanism via TLR2. The response is weak, results are consistent but are not coming from different samples, the coherence is missing, but the experiments can partially be shown to be similar to other stimuli as LP show a structural similarity to LPS. Based on this one can consider only LTA to have a proven causal role in cytokine release. PGN and LP have based on current available knowledge as much of a proven causal role in cytokine induction as a non-causal one.

	LTA	PGN	LP
Strength	+++	-	-
Consistency	+++	+++	-
Specificity	+	-	+
Temporality	+++	+++	+++
Biological Gradient	+++	+++	+++

Plausibility	+++	+	++
Coherence	+++	-	-
Experiment	+++	-	++

Table 1: Fulfilment of Bradford Hill criteria for LTA, PGN and LP

Summarising this thesis we can conclude, that we were able to gain considerable information about possible counterparts in Gram-positive bacteria to LPS, which is the main immune stimulus in Gram-negative bacteria. We were able to exclude the contribution of PGN to cytokine induction at least when it occurs as a purified molecule, which does not mean that it does not contribute at all in the recognition of whole bacteria. Furthermore, we could show that mutations of single parts of the Gram-positive cell wall structure do not lead to major differences in cytokine release when stimulating human primary cells with whole bacteria. This indicates a system of redundancy of the human innate immune system in order to make sure that unknown bacteria with alterations in appearance or cell wall structures are still recognised quickly. Additionally, lipoproteins seem to have a minor role in immunostimulation in complex systems like whole blood or PBMCs, but simultaneously they might have an effect on signal transduction and release of a single cytokine named IL-1β. Certainly this needs to be further investigated.

Finally we can say that LTA remains a major immunostimulatory molecule of Gram-positive bacteria, but there is still a lot work to do to further clarify the roles of PGN and especially lipoproteins in the recognition process of Gram-positive bacteria.

7 Summary

Lipopolysaccharide (LPS) is the major immunostimulatory component of Gram-negative bacteria, but its counterpart in Gram-positive bacteria is still under discussion. Looking on the Gram-positive cell wall, three components are considered to be recognised by cells of the human innate immune system: Peptidoglycan (PGN), quantitatively the main component, lipoteichoic acid (LTA) as a similar amphiphile structure compared to LPS and lipoproteins (LP). To find out more about the immunostimulatory capacity of these membrane components, the first approach in this thesis was to screen public available literature for evidence, that cytokine release in humans is connected with one or more of the named components. This research was done systematically as a meta-analysis with the four well-known Koch-Dale (K/D) criteria with a restriction to human studies: 1.) Evidence of LTA, PGN or LP in cytokine inducing Gram-positive bacteria, 2.) Synthesis inhibition of LTA, PGN or LP impairs cytokine release, 3.) Exposure to purified LTA, PGN or LP induces cytokine release, 4.) Blocking of LTA, PGN or LP prevents or reduces cytokine release:

- PGN and LP were found to be ubiquitous in Gram-positive bacteria, but the absence of a structural identification of a LP, which is responsible across a wider variety of bacterial species for cytokine induction, leads to the conclusion that only a general presence of LPs must be assumed. Hence, the K/D criterion 1 can not be considered as fulfilled for LP. Despite that, 58 original articles were found mentioning LTA isolation concluding that K/D criterion 1 can be considered as fulfilled for LTA and PGN but not LP. Searching for deletion of genes involved in the synthesis of LTA and lipoproteins three publications for LP and two in the case of LTA, which were showing reduced cytokine induction. PGN had to be excluded in this case, because we are not aware of a PGN deletion mutant - probably a Gram-positive mutant lacking PGN would be unable to grow and survive. Hence, K/D criterion 2 was LTA, PGN and LP. Cytokine induction of the isolated structures was found for any of the three investigated molecules and therefore K/D criterion 3 could also be considered as verified. Finally, the search for inhibitors resulted in a failure of finding publications for PGN and LP and only one publication for LTA, which shows a significantly reduced cytokine release when applying the inhibitor polypropylene glycol. Thus, K/D criterion 4 is only

fulfilled for LTA with the limitation of just one paper found showing this evidence. Taken together, the results of the meta-analysis indicated that PGN and LPs might play a role in cytokine induction and therefore in immune recognition of Gram-positive bacteria in humans, but the evidence for LTA being the major immune stimulus in Gram-positive bacteria is strong as it is the only investigated molecule fulfilling all K/D criteria.

The interesting findings of this meta-analysis needed to be investigated experimentally. The model organism for these investigations was *Staphylococcus aureus* (SA), which is a frequent human pathogen and often colonises humans asymptomatically, but is also able to induce severe infections in tissue or even spreading into the blood. In the second part of the thesis, three different SA 113 mutants, which were lacking lipoproteins (Δlgt) or wall teichoic acids (ΔTA) or possessed a reduced alanine content of the LTA (Δdlt) were compared to its corresponding wildtype with respect to their immunostimulatory capacity in human primary cells and murine bone marrow (BM) derived macrophages.

- Whole bacteria (UV- and heat-inactivated) of all strains induced similar TNF release in human whole blood. IL-1β release was found to be significantly reduced only for SA 113 Δlgt. Also no major difference in cytokine release could be found using whole bacteria as a stimulus for murine BM macrophages derived from Toll-like receptor (TLR) 2 wt and knock-out mice. Isolation of PGN from the four SA strains and using these isolated molecules as a stimulus in human whole blood resulted in a loss of IL-1β induction and led to marginal and borderline significant amounts of TNF release compared to the unstimulated control. This stands in contrast to the studies cited in the meta-analysis. LTA of these four strains was however a potent inducer of cytokines in human whole blood, but LTA from SA 113 Δdlt was found to induce significantly lower amounts of TNF and LTA of SA 113 Δlgt had the same effect on IL-1β induction. The reduced induction of IL-1β induction of Δlgt LTA seemed to be based on a reduced mRNA production.

Despite the strong evidence that LTA is a major immunostimulatory principle of Gram-positive bacteria, recent reports suggested that not LTA but lipoproteins are the dominant immunostimulatory structures of SA. Therefore we compared the LTA from SA 113 Δlgt and its corresponding wildtype in more detail:

- Whole bacteria and LTA from SA 113 Δlgt were equipotent in human whole blood with the previously reported exception of the significant reduction in IL-1β release compared to the wildtype. THP-1 cells failed to release cytokines when stimulated with SA 113 Δlgt LTA, whereas they responded to wt LTA. Coating to surfaces and/or replacement of FCS to autologous human serum did not enable THP-1 cells to respond. Structural differences between SA 113 wt and Δlgt LTA could not be found using NMR and GC-MS technology and also microarray analysis of stimulated human whole blood revealed a very similar pattern of these two stimuli on mRNA level. However, comparison of these two LTA with a synthetic lipoprotein named $Pam_2Cys\text{-}SK_4$ resulted in a different RNA pattern. These results suggest no major differences between the LTAs and underline the importance of LTA being a major immunostimulatory molecule.

In summary, the results of this thesis contribute to the understanding of the innate immune response with the focus on cell wall components of Gram-positive bacteria. This may lead to new approaches to treatments against Gram-positive bacterial infections in the future.

8 Zusammenfassung

Gram-negative Bakterien haben eine Struktur, die hauptsächlich für die Stimulation des menschlichen Immunsystems bei diesen Bakterien verantwortlich ist und Lipopolysaccharid (LPS) genannt wird. Das entsprechende Gegenstück in Gram-positiven Bakterien wird hingegen kontrovers diskutiert. Die Gram-positive Zellwand besteht im Wesentlichen aus drei Komponenten, die als immunstimulatorisch angesehen werden: Peptidoglykan (PGN), die quantitative Hauptkomponente, Lipoteichonsäure (LTA), welche eine ähnlich amphiphile Struktur wie LPS hat, und Lipoproteine. Um ein besseres Verständnis für die immunstimulatorischen Eigenschaften dieser Membrankomponenten zu erhalten, wurde zunächst bereits vorhandene Literatur nach Hinweisen analysiert, die auf eine Verbindung zwischen Zytokinausschüttung im Menschen und einer oder mehrerer dieser Verbindungen hinweist. Hierfür wurde eine systematische Meta-Analyse unter Zuhilfenahme der vier wohlbekannten Postulate von Koch/Dale (K/D) durchgeführt, die auf humane Studien begrenzt wurde: 1.) Vorhandensein von LTA, PGN oder Lipoproteinen in zytokin-induzierenden Bakterien, 2.) Syntheseinhibition von LTA, PGN oder Lipoproteinen beeinträchtigt die Zytokinausschüttung, 3.) Applikation der jeweils aufgereinigten Zellwandkomponenten führt zu Zytokinausschüttung, 4.) Blockierung von LTA, PGN oder Lipoproteinen reduziert oder verhindert Zytokinausschüttung.

- PGN und LP sind ubiquitär in Gram-positiven Bakterien vorhanden. Die Abwesenheit der genauen Identifikation der Lipoproteinstruktur, welche dann für die Zytokinausschüttung verantwortlich wäre, führt zu dem Schluss, dass man nur eine generelle Präsenz von Lipoproteinen annehmen kann. Somit ist das erste Postulat für Lipoproteine nicht erfüllt. Abgesehen davon wurden 58 Originalpublikationen gefunden, welche eine Aufreinigung von LTA beschreiben. Dies führt zu dem Schluss, dass das erste Postulat für LTA und PGN, nicht aber für LP erfüllt ist. Die Suche nach Publikationen, die eine Deletion in den Genen, die in der Synthese von LTA oder Lipoproteinen involviert sind, beschreiben, resultierte in drei Artikeln für Lipoproteine und deren zwei für LTA, welche alle eine reduzierte Zytokinausschüttung zeigen. PGN konnte in diesem Fall nicht mit einbezogen werden, da es nach bisherigem Wissen keine Deletionsmutante von PGN gibt - es ist aber sehr wahrscheinlich, dass eine solche Mutante nicht in der Lage wäre zu

überleben. Somit kann auch das zweite Postulat für alle drei Membrankomponenten als erfüllt angesehen werden. Da die isolierten Strukturen jeweils zu Zytokinausschüttung im Menschen führten, kann das dritte K/D Postulat für alle drei Oberflächenmoleküle als verifiziert angesehen werden. Die Suche nach Inhibitoren der drei Komponenten ergab nur eine Publikation für LTA, die eine signifikant reduzierte Zytokinausschüttung nach der Applikation des Inhibitors Polypropylenglycol zeigt. Für PGN und Lipoproteine wurde in diesem Zusammenhang keine Puplikation gefunden. Daher ist das Postulat 4 nur für LTA mit der Einschränkung, dass nur eine Publikation in diese Richtung gefunden werden konnte, erfüllt. Zusammenfassend weisen die Resultate dieser Meta-Analyse darauf hin, dass PGN und Lipoproteine eine Rolle in der Zytokinduktion und somit in der Immunerkennung von Gram-positiven Bakterien im Menschen spielen könnten. Jedoch gibt es profunde Hinweise, dass LTA die wichtigste immunstimulatorische Komponente von Gram-positiven Bakterien ist, da LTA als einziges Molekül alle vier Postulate erfüllt.

Diese interessanten Befunde der Meta-Analyse wurden im Folgenden experimentell untersucht. Als Modellorganismus wurde *Staphylococcus aureus* (SA) herangezogen, welches ein häufiges menschliches Pathogen ist und zwar meist asymptomatisch auftritt, jedoch ebenfalls in der Lage ist, schwerwiegende Infektionen hervorzurufen. Im zweiten Teil dieser Arbeit wurden drei verschiedene SA 113 Mutanten im Vergleich zum korrespondierenden Wildtyp und ihrer immunstimulatorischen Kapazität in menschlichen Primärzellen sowie in murinen Knochenmarksmakrophagen untersucht. Diese Mutanten zeichneten sich durch das Fehlen von Lipoproteinen (Δlgt) oder Wandteichonsäuren (ΔTA) bzw. eines reduzierten Alanin-Gehalts der LTA (Δdlt) aus.

- Ganze Bakterien (UV- und Hitze-inaktiviert) aller Stämme induzierten vergleichbare Mengen an TNF in humanem Vollblut. IL-1β Ausschüttung hingegen war signifikant reduziert für SA 113 Δlgt. Ausserdem konnte kein signifikanter Unterschied in der Zytokinausschüttung nach Stimulation von Knochenmarksmakrophagen von Toll-like-Rezeptor (TLR)-2-Wildtyp und ihren knock-out Mäusen mit ganzen Bakterien festgestellt werden. Aufreinigung von PGN aus allen vier SA-Stämmen und Verwendung dieser isolierten Moleküle

als Stimulus in humanem Vollblut resultierte in einem Ausbleiben der IL-1β Induktion. Ausserdem führte diese Stimulation zu marginalen, aber grenzwertig signifikanten Mengen an TNF-Ausschüttung verglichen mit der unbehandelten Kontrolle. Dieses Resultat steht im Kontrast zu den Ergebnissen der Meta-Analyse für PGN. Aufgereinigte LTA dieser vier Stämme war hingegen in der Lage, Zytokinausschüttung im humanen Vollblut zu induzieren. LTA von SA 113 Δdlt zeigte eine signifikant schwächere Induktion von TNF und LTA von SA 113 Δlgt hatte einen ähnlich reduzierenden Effekt auf die Induktion von IL-1β. Diese reduzierte Zytokinausschüttung scheint auf eine reduzierte Produktion von IL1β-mRNA zurückzuführen zu sein.

Trotz der eindeutigen Hinweise, dass LTA die wichtigste immunstimulatorische Komponente der Gram-positiven Bakterien sei, zeigen jüngere Studien, dass Lipoproteine und nicht LTA die dominanten immunstimulatorischen Zellwandkomponenten seien. Daher haben wir LTA von SA 113 Δlgt und dem korrespondierenden Wildtyp detaillierter untersucht:

- Ganze Bakterien und LTA von SA 113 Δlgt waren equipotent in humanem Vollblut mit der schon vorherig festgestellten Ausnahme der signifikant reduzierten IL-1β-Ausschüttung im Vergleich zur Wildtyp-LTA. THP-1 Zellen schütteten keine Zytokine nach Stimulation mit Sa 113 Δlgt LTA aus, während sie auf SA 113 wt LTA reagierten. Auch das Coaten auf die Zellkulturplattenoberfläche und/oder das Austausch von FCS mit autologem, humanem Serum befähigten THP-1-Zellen nicht zu einer Reaktion auf SA 113 Δlgt LTA. Strukturelle Unterschiede zwischen den beiden LTAs konnten weder im NMR noch im GC-MS gefunden werden. Microarray-Analysen von mit den beiden LTAs stimuliertem Vollblut zeigten ein ähnliches Muster dieser beiden Stimuli auf mRNA Ebene. Wie auch immer, der Vergleich dieser beiden LTAs mit einem synthetischen Liporotein ($Pam_2Cys\text{-}SK_4$) resultierte in einem deutlich unterschiedlichen mRNA-Muster. Die Ergebnisse legen nahe, dass es keine wesentlichen Unterschiede zwischen den beiden LTAs gibt und unterstreichen die Wichtigkeit der LTA als wichtigste immunstimulatorische Komponente.

Zusammenfassend tragen die Resutate dieser Studie zu dem Verständnis der angeborenene Immunantwort im Hinblick auf Zellwandkomponenten von Gram-positiven Bakterien bei. Dies könnte in Zukunft zu neuen Ansätzen in der Behandlung von Infektionen mit Gram-positiven Bakterien führen.

9 Declaration of author's contribution

- **Rockel, C.**, and Hartung, T. (2009): "A systematic review of Gram-positive membrane components inducing human monocyte / macrophage cytokine release". *Prepared for submission*.

The search for publications, the selection of publications following decision trees and the analysis and discussion of results was done by Christoph Rockel. The study was designed by Thomas Hartung. The manuscript was written by Christoph Rockel with contributions by T. Hartung.

- **Rockel, C.**, S., Sigel, Deininger, S., Draing, C., Dehus, O., Rupp, T., Ulmer, A., Götz, F., Hartung, T., Hermann, C., and von Aulock, S. (2009): "Lipoteichoic acid from a *Staphylococcus aureus* wildtype or a lipoprotein diacylglycerol transferase deletion mutant possess similar immune stimulatory activity in human monocytes but not cell lines". *Prepared for submission*.

The experiments were done mainly by Christoph Rockel and Stefanie Sigel. Susanne Deininger, Christian Draing, Tamara Rupp and Oliver Dehus contributed to the experiments. All experiments were done under the supervision of Corinna Hermann and Sonja von Aulock. Key experiments were repeated in the laboratories of Artur Ulmer. Wild type and mutant strains were kind gifts from Friedrich Götz. The study was designed by Thomas Hartung, Corinna Hermann and Sonja von Aulock. The manuscript was written by Christoph Rockel.

- **Rockel, C.**, Hermann, C., and Hartung, T., (2009): "Different *S. aureus* whole bacteria mutated in putative pro-inflammatory membrane components have similar cytokine-inducing activity." *Submitted.*

The experiments were done by Christoph Rockel under the supervision of Corinna Hermann. The study was designed by Thomas Hartung and Corinna Hermann. The manuscript was written by Christoph Rockel.

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