Oliver Dehus

Receptor polymorphisms and non-classical immune stimuli in bacterial immune recognition

Dissertation

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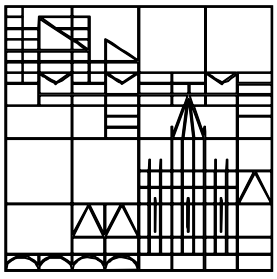


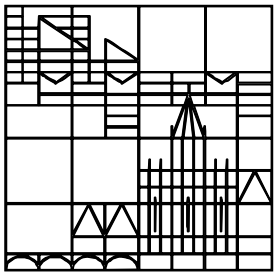
Receptor polymorphisms and non-classical immune stimuli in bacterial immune recognition

Dissertation

Zur Erlangung des akademischen Grades
des Doktors der Naturwissenschaften
an der Universität Konstanz (Fachbereich Biologie)
vorgelegt von

Oliver Dehus

Universität Konstanz
Mai 2008



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List of Publikations

Major parts of this thesis are published or submitted for publication.

- O. Dehus, S. Bunk, S. v. Aulock and C. Hermann: IL-10 release requires stronger toll-like receptor 4-triggering than TNF- a possible explanation for the selective effects of heterozygous TLR4 polymorphism Asp(299)Gly on IL-10 release. *Immunobiol 2008; in press*.

- O. Dehus, G. Rogler and C. Hermann: LPS-inducible anti-inflammatory responses are not diminished in Crohn's disease patients with heterozygous Asp(299)Gly polymorphism. *Submitted to IBD*.

- O. Dehus, T. Hartung and C. Hermann: Endotoxin evaluation of eleven lipopolysaccharide by whole blood assay does not always correlate with Limulus Amebocyte Lysate assay. *J Endotox Res 2006; 12(3):171-80*.

- O. Dehus, M. Pfitzenmaier, S. Maier, N. Fischer, G.Stübs, C. Draing, W. Schwaeble, S. Morath, T. Hartung, A. Geyer and C. Hermann: Reduced immunogenicity of Listeria monozytogenes grown in an intracellular environment is associated with altered LTA expression. *Submitted to JBC*.

Further contributions to publications, not integrated into this thesis:

- Hermann C., von Aulock S., Dehus O., Keller M., Okigami H., Gantner F., Wendel A., Hartung T.: Endogenous cortisol determines the circadian rhythm of lipopolysaccharide-- but not lipoteichoic acid--inducible cytokine release. *Eur J Immunol 2006 36(2):371-9.*

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- Draing, C., Rockel, C., Deininger, S., Sigel, S., Dehus, O., Rupp, T., Ulmer, A., Figueroa-Perez, I., Schmidt, R.R., Götz, F., Hartung, T., Herrmann, C., von Aulock, S.: Lipoteichoic acid from a lipoprotein diacylglycerol transferase deletion mutant is a potent immunobiologically active compound; *To be submitted*

Abbreviations

A	adenosine
AIDS	aquired immune-deficiency syndrome
Asp	asparagine
ATCC	American Type Culture Collection
bp	base pair
CFU	colony forming unit
DC	dendritic cell
DNA	desoxyribonucleic acid
ds	double strand
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
DTT	dithiothreitol
EDTA	ethylendiamine tetra-acetate
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescense-activated cell sorter
FCS	fetal calf serum
FPLC	fast protein liquid chromatography
FSC	forward scatter
G	guanidine
Gly	glycine
HIC	Hydrophobic Interaction Chromatography
IFN	interferon

Ile	isoleucine
Ig	immunoglobulin
IL	interleukin
Inl	internalin
LAL	limulus-amoebocyte-lysate
LALF	limulus anti-LPS factor
LLO	listeriolysin O
LPS	lipopolysaccharide
LTA	lipo-teichoic acid
MOI	multiplicity of infection
mRNA	messenger RNA
MS	mass spectrometry
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
OD	optical density
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Plc	phospholipase C
POD	horse-raddish-peroxidase
PRR	pattern recognition receptor
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA

rpm	rotations per minutes
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfat
SEM	standard error of the mean
Thr	threonine
TLR	toll-like rezeptor
TMB	3,3´5,5´-tetramethylbenzidine
TNF	tumor-necrosis-factor
Tris	Tris-(hydroxymethyl)-aminoethan
UV	ultra violet

Table of content

1 Introduction

1.1 Infection and innate immunity

Infectious diseases are globally the main cause of death. They are caused by micro-organisms that colonize and intrude into the host where they spread and replicate, accompanied with characteristic symptoms of inflammation. Our innate immune system, described over a century ago, is derived from the phylogenetically oldest defence mechanisms and has been conserved in all multicellular organisms, constituting the organism's first line of defence against invading pathogens [1, 2,Hoffmann, 1999 #628]. The microbe-host interaction is a paradigm for co-evolutional events that appear as a competition between infectious agents which are continuously optimising their invasive strategies on the one side and the defence mechanisms of the host on the other side. The immune system needs to balance these mechanisms to efficiently eradicate the pathogens but at the same time avoiding deleterious effects for the body. The white blood cells, which are the major players, derive from myeloid precursors and include competent phagocytes: monocytes/macrophages, dendritic cells (DC) and neutrophilic granulocytes (PMN). Initial detection of pathogens involves the pattern recognition receptors (PRR) of immune cells and also other cell types. These immune receptors are expressed on the cell surface, in organelles and in the cytosol sensing the presence of pathogens by their microbe associated molecular patterns (MAMPs, formerly PAMPs) [3]. MAMPS are indispensable molecules whose functions are sensitive to variations and therefore possess highly conserved structures [4, 5]. Upon stimulation by receptor-ligand

binding, inflammatory effector substances like cytokines, lipid mediators and nitric oxide are released from monocytes/macrophages, stimulating the activity of several populations of target cells [6, 7]. Amongst those, endothelial cells and immune cells respond with the expression of surface adhesion molecules, phagocytes are activated for lysosomal clearance and eliminate pathogens by respiratory burst, DC and monocytes/macrophages express co-stimulatory molecules and differentiate to antigen-presenting cells stimulating the specific immune system involving B- and T-lymphocytes [8]. Apart from the cell-mediated response, an innate humoral defence mechanism involving a variety of serum proteins is known as the complement pathway, which can be activated by three different manners: the classical pathway initiated by IgM and IgG antibodies bound to their peptide antigens and quite similar, the lektin pathway initiated by leucin-rich repeats-carrying recognition molecules like mannose binding lektin bound to certain sugar moieties. The alternative pathway gets activated by spontaneous hydrolysis of effector molecules in the presence of a variety of microbial cell wall components. Activation of complement initiates zymogenic cascades, releasing inflammatory mediators as well as fragments for the opsonisation or direct inactivation of pathogens and toxins. [9)].

Regarding host-pathogen interactions and inflammatory responses, broad inter-individual variations exist and have to be clarified for adequate treatments. However, the courses of diseases are too complex to let us understand completely how the same pathogen causes subclinical, mild, severe or lethal infections. The status of the immune system, which critically depends on the physical condition, determines the outcome of an infection [10]. Deviations from an optimal immune response, which would prevent the invasion and spread of pathogens, might result in an overwhelming inflammatory reaction damaging the body or a diffident, insufficient

defence. Possible explanations for deviations in immune responses are e.g. genetic polymorphisms or regulatory dysbalances occurring for instance during immune suppression. Several individual divergences were already shown to be due to genetic or epi-genetic predisposition like gender, ethnical origin and age [11-14]. Furthermore, the pathogens themselves represent a tremendous source of variability, including structural and strategical variations challenging the immune system. In order to develop effective strategies of prevention and therapy, a much more profound understanding of the molecular mechanisms and its variations involved in immune recognition and signalling on the host's side and virulence on the microbes' side are necessary with intelligent though reductionistic model systems to be the key.

1.2 Cytokines in inflammatory responses

Ineffective recognition of pathogens or inappropriate immune responses may lead to uncontrolled microbial growth or overwhelming systemic inflammatory responses followed by tissue damage, vascular collapse and multiorgan failure, as occurring in severe sepsis and septic shock [15]. However, potent endotoxins alone like lipopolysaccharide (LPS) of Gram-negative bacteria are capable of triggering adverse clinical responses, including procoagulant response and septic shock [16]. Taveira da Silva et al. could demonstrate, that self-administration of Salmonella endotoxin mimics many of the clinical features of septic shock [17]. The most common microbes isolated from patients with severe Gram-negative sepsis are *Escherichia coli*, *Klebsiella* species and *Pseudomonas aeruginosa* [18], whereas *Listeria monocytogenes* is a prominent cause of Gram-positive sepsis [88]. In sepsis, the prevailing cytokines that are involved in a systemic response are tumour necroses factor (TNF), interleukin (IL)-1β and IL-6 which cause hypotension and organ failure associated

with lethal septic shock [19, 20]. Monocytes/macrophages constitute the principal source of proinflammatory cytokines elicited by endotoxins [21].

As a counter-player, IL-10 represents one of the most important immune-regulating cytokines and is mainly expressed by monocytes/macrophages, but also in lymphocytes, mast cells and other cell types. It confers its mainly immunosuppressive effects on the immune cells of both the innate and the adaptive immune system. In macrophages, IL-10 release follows TNF production, and down-regulates the proinflammatory reactions [22]. Above all, the inhibition of TNF, IL-1β and IL-6 is crucial, because these cytokines have synergistic activities on inflammatory processes and amplify these responses by inducing secondary mediators such as chemokines, eicosanoids and platelet activating factor. The IL-10 induced inhibition of inflammation is mediated by modulation of transcription and reduction of the stability of mRNA, characterized by AU-rich elements in the 3'-untranslated regions [23, 24]. Furthermore, IL-10 leads to inhibition of NFκB, which plays a key role as transcription factor for many inflammatory genes, via suppressing both inhibitor κ B kinase and DNA binding of NFκB [25]. IL-10 also enhances the production of the antagonists of some proinflammatory effectors, e.g. of soluble p55 and p75 TNF receptor [26, 27] as well as IL-1 receptor antagonist [28]. The anti-inflammatory potential of IL-10 has been demonstrated by preventing experimental endotoxaemia [29, 30] and suppressing experimental intestinal inflammation in the mouse [31]. The severe consequences of a misbalance of circulating proinflammatory and anti-inflammatory cytokines become evident in trauma and sepsis patients where the IL-10 to TNF ratio is high [32-34]. The other extreme, i.e. a low IL-10 to TNF ratio, is associated with an imbalance in favour of proinflammatory cytokines, as observed in case of autoimmune diseases, e.g. of systemic lupus erythematodus [35] or

inflammatory bowel disease which is characterized by chronic mucosal inflammation, a possible consequence of a dysbalance of proinflammatory and regulatory cytokines [36, 37]. In some cases, the benefit of IL-10 therapies is discussed [38, 39].

1.3 Immune recognition by toll-like receptors

With at least 11 different members identified, the toll-like receptors (TLRs) form the greatest family of PRRs and are of major significance for the detection of MAMPs in mammals [3, 40, 41]. In 1988, the Toll protein was first described in Drosophila, where it initiates immune responses against fungal infections [42, 43]. Subsequently, a set of mammalian proteins containing an extracellular c-terminal leucin-rich repeat and an intracellular N-terminal toll/interleukin-1 receptor (TIR) domain were found to be structural related to Drosophila toll and called TLRs [44]. A general signalling pathway of human toll is depicted in figure 1. The TLRs detect a variety of different PAMPs, including e.g. tri-acyl lipopeptides from bacteria and mycobacteria (TLR1) [45, 46], lipoproteins and lipo-teichoic acid (LTA) from Gram-positive bacteria (TLR2) [47], double-stranded viral RNA (TLR3) [48], LPS from Gram-negative bacteria (TLR4) [49, 50], flagellin (TLR5) [51], di-acyl lipopeptides from mycoplasma (TLR6) [52], GU rich single strand RNA (TLR7 and TLR8) [53] and bacterial DNA (TLR9) [54]. TLR10 [55] and recently TLR11 [41] have also been identified, but their ligands are unknown so far. The first human TLR described was TLR4 [56] and the *tlr4* gene was identified in 1998 [57]. A defect of TLR4 mediated signalling in C3H/HeJ mice due to a point mutation was found to result in an incapability of responding to LPS [49, 50]. Human TLR4, located on chromosome 9, is expressed by monocytes/macrophages, DC, PMN, mast cells and at organ-specific levels by epithelial cells [58-61]. During the initiation of an immune response which is in first

place initiated by the PRRs, the pattern of cytokines released by immune cells is crucial for a successful host defence and varies depending not only on the pathogen involved, but also on the individual host. In the latter context, mutations in form of single nucleotide polymorphisms (SNP) entailing an altered immune recognition are currently a major matter of research since they might account for inter-individual susceptibilities towards certain diseases and for differences in immune reactions; one prominent example are the polymorphisms of TLR4.

1.4 The polymorphism Asp(299)Gly of TLR4

Genetic polymorphisms are allelic variants within a population occurring by definition at a frequency of over 1%. Their most common appearances are SNPs, which can either be silent or result in a functional aberration. If located in a promotor region, SNPs may affect gene expression or in case of an amino acid exchange alter the protein structure. According to the hypothesis of mutations emerging in genomic regions of strong selective pressure, genes involved in immunity and particular in immune recognition exhibit a relatively high number of SNPs [62]. In this respect, the question why significant inter-individual differences in susceptibility to infection and its severe outcomes exist, is freshly discussed [31, 63].

Since Gram-negative infections are still of outstanding clinical relevance, many efforts have been undertaken to precisely elucidate the role of the LPS recognition receptor TLR4 and its polymorphisms. Arbour et al. screened the coding region of the *tlr4* gene and detected two co-segregating missense mutations that affect the extracellular domain [64], which is considered the most conserved one [65].

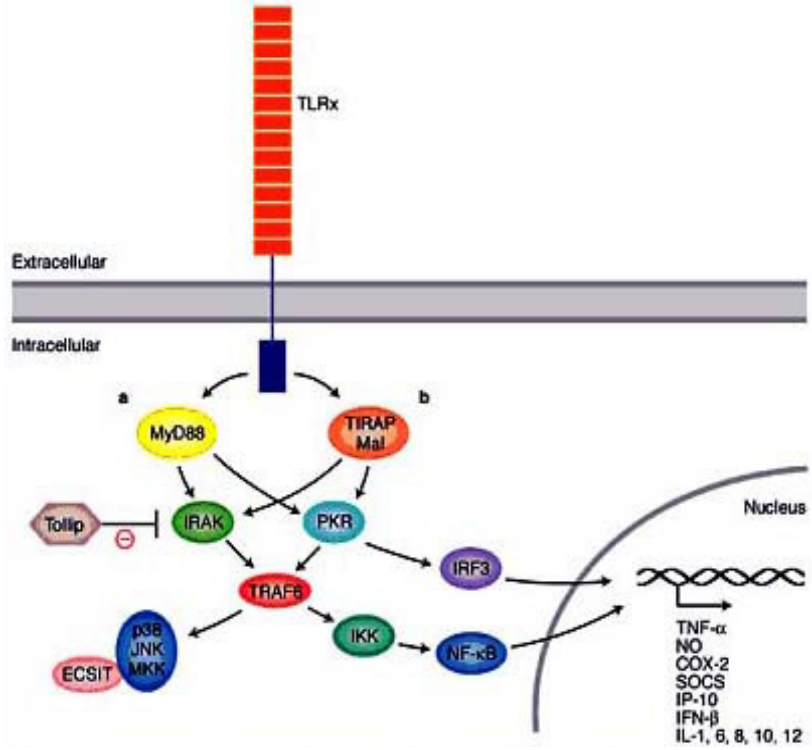

Figure 1 TLR signalling is mediated by at least two distinct pathways (adopted with slight modifications from Expert reviews in Molecular Medicine©2003 Cambridge University Press)

After recognition of a MAMP, TLRs are capable of differentially activating distinct downstream signalling events via different cofactors and adaptor proteins mediating diverse immune responses. (a) The 'classical' MyD88-dependent TLR signalling pathway is activated via the conserved, cytoplasmic TIR domain [for 'Toll/interleukin 1 receptor (IL-1R)], which provides a scaffold for recruitment of the adaptor molecule MyD88 and serine/threonine kinases of the IL-1R-associated kinase (IRAK) family. Following IRAK auto-phosphorylation, the TRAF6 adaptor protein interacts and induces translocation of the transcription factor NF-kB to the nucleus, resulting in transcriptional activation of genes encoding cytokines and chemokines. In addition, TLRs bridge the signalling pathway via ECSIT to TRAF6 for p42/p44 mitogen-activated protein kinase (MAPK) kinase (MKK), p38 and JNK in response to specific bacterial products. Toll-interacting protein (Tollip) plays an inhibitory role in TLR2/4-mediated cell activation by suppressing the activity of IRAK. (b) The MyD88-independent TLR signalling pathway is activated via the TIR-domain-containing adaptor protein (TIRAP; also designated Mal for 'MyD88-adapter-like') and results in activation of the dsRNA-binding protein kinase PKR. This protein has been proposed to be a central downstream component of both the TIRAP- and MyD88-dependent signalling pathways and could mediate potential crosstalk between them. The MyD88-independent pathway appears to utilise both IFN-regulatory factor 3 (IRF3) and NF-kB, and results in the expression of IFNγ-inducible genes including IP-10.

In one case, an A to G transition at position +896 downstream of the translation start site results in the replacement of an aspartic acid residue (Asp) by glycine (Gly) at amino acid 299. The second polymorphism was found to be in complete linkage disequilibrium with *tlr4*/A(896)G and was a C to T transition at position +1196, resulting in an exchange of threonine (Thr) by isoleucine (Ile) at amino acid 399. In transfection experiments, the Asp(299)Gly but not the Thr(399)Ile mutation was found to interrupt LPS-induced TLR4 signalling [64]. In numerous studies with patients, some associations of the Asp(299)Gly polymorphism with several diseases have already been reported, suggesting that the mutation might in deed alter inflammatory responses [62]. Moreover, variations of the sensitively balanced inflammatory actions during immune responses are believed to be linked with an increased susceptibility towards the development of chronic diseases like e.g. autoimmune disorders, asthma or inflammatory bowel disease; in the latter case, patients suffering from Crohn's disease or ulcerative colitis have been found to carry the heterozygous TLR4 Asp(299)Gly polymorphism at a increased frequency [66, 67]. However, no functional studies concerning the effect of this mutation for LPS-induced immune responses have been performed so far.

1.5 Major bacterial immune stimuli

1.5.1 Bacterial pathogens

The majority of infectious agents relevant for humans are found in the domain of the prokaryotes. Despite the introduction of antibiotics, infections with extracellular or intracellular replicating bacteria are steadily increasing [68] and mortality of bacteraemia remains high with 25-40% [69-71]. Especially in non-industrialized

countries, infectious diseases like gut infections are still the main cause of mortality and morbidity. In general, symbiotic bacteria constitute the individual human body flora with 500 to 1000 species, performing indispensable metabolic tasks and avoiding the establishment of pathogenic micro-organisms. However, amongst these commensals opportunistic pathogens exist that may cause an infection when they get the chance to become invasive, like in immunocompromised individuals [72]. Invasion involves a complex, and in many cases poorly understood activation of virulence factors; some of those are also responsible for the adaptation to a physically and chemically different environment and for immune evasion. One typical regulatory mechanism is the two-component system which senses extracellular changes like temperature or osmolarity and induces the display of defined genetic programs organised in regulons. Variability and horizontal gene transfer fosters the efficiency and spread of virulence genes [73]. Furthermore, both pathogenic bacteria and even non-pathogenic symbiotics possess the ability to express molecules that cause after entering the blood stream, inflammation and provoke symptoms of sepsis. Such immunogenic substances can either be secreted (e.g. Listeriolysin O, Staphylococcal enterotoxin B) or released after cell death, or cell-renewal and -division. Thus, some of the most immunogenic compounds recognized by TLRs are expressed in the cell wall (e.g. lipoproteins, LPS, LTA). The cell wall of the prokaryotes is a flexible but robust building which withstands the turgor and shields the organism from many substances with antibiotic activities. For that reason, its turnover is carefully regulated to ensure growth and cell division without damage. These circumstances as well as the fact that the cell wall contains the bacteria's outermost components contacting and interacting with the host, have made the bacterial cell wall an intensively studied subject–probably harbouring the key to at

least transiently overcome the massive health problems due to increasing antibiotic resistances. Within the domain of prokaryotes, a common classification is done by the feasibility of Gram staining, thus discriminating Gram-negative from Gram- positive bacteria due to differences in the cell wall. While Gram-negatives possess two phospho-lipid membranes with a thin layer of peptidoglycan in between, Gram-positives have only one phospho- lipid membrane surrounded by a thick layer of peptidoglycan (LIT). Besides the immunogenic components of the peptidoglycan and lipoproteins, Gram- negative bacteria express the highly potent LPS while Gram-positives express LTA which provokes a moderate inflammatory response [74, 75]. Typical for MAMPs, both LPS and LTA show a highly conserved structure building up a repetitive hydrophilic chain participating in forming the cell surface connected to a lipid moiety which is embedded in the membrane [76].

1.5.2 Lipopolysaccharide

The basic structure of classical, "smooth" LPS as examined mostly for enterobacteriaceae consists of a repetitive polysaccharide chain (O-antigen) with a high variability determining the serological specificity, the core oligosaccharide and a lipid moiety, also named lipid A (Fig. 2 A). This β-1,6-linked D-glucosamine disaccharide bearing two phosphate groups in position 1' and 4', substituted with six fatty acids 12 to 14 carbons in length, is alone sufficient for the activation of TLR4 mediated signalling and full toxic activity *in vivo* and *in vitro* [77-80]. LPS induces the expression of a cytokine pattern similar to stimulation with whole bacteria and also activates the complement system [80, 81]. Deviations from the architecture of the prototypical LPS have been identified only recently, like the phosphorylation pattern of the disaccharide or the number and nature of the acyl chains. Such deviations occur only in a limited range, but bear significant consequences for the immune

recognition, concerning the recognition by specific PRR and the induction of cytokine patterns [77, 82, 83,Erridge, 2002 #784]. Such exceptions from the rule are the penta-acyl lipid A with partially unsaturated carboxylic acid residues from *Rhodobacter sphaeroides* (LPS-receptor TLR4 antagonist, [84]), the O-methylized monophosphorylated lipid A from *Leptospira interrogans* (TLR2 antagonist, [85] or the monophosphorylated penta-acyl LPS from *Porphyromonas gingivalis* (signalling via lipoprotein receptor TLR2; [86]). For the opportunistic pathogen *Pseudomonas aeruginosa* it was shown recently that the acylation of the lipid A can differ between isolates from the environment or from a source of cystic fibrosis, associated with different immune stimulatory potencies [87]. In all cases of non-classical lipid A structures, the induction of cytokine release from blood leukocytes is less potent. Taken together, the system of PRR sensing LPS seems to display a very specific receptor-ligand interaction which is sensitive towards even small sterical modifications. Still, many questions concerning the association between non-classical LPS architectures and immune recognition have to be solved in order to understand the species-specific infection strategies and inflammatory responses.

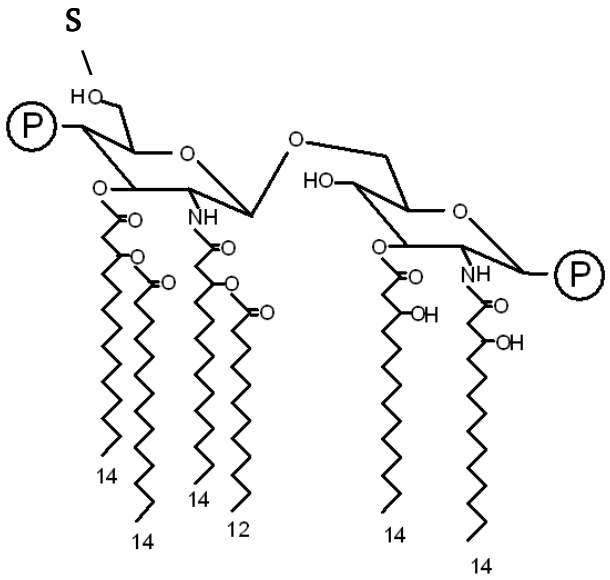

Figure 2 A Lipid A from *E. coli.* (adopted from R. Darveau, University of Washington School of Dentistry) Prototypical enterococcae-LPS consisting of the hexa-acylated disaccharide (Lipid A) connected to the polysaccharide backbone (S) consiting of a core oligosaccharide and an O-specific polysaccharide antigen. Numbers indicate carbon chain-length. P=phospho-esther

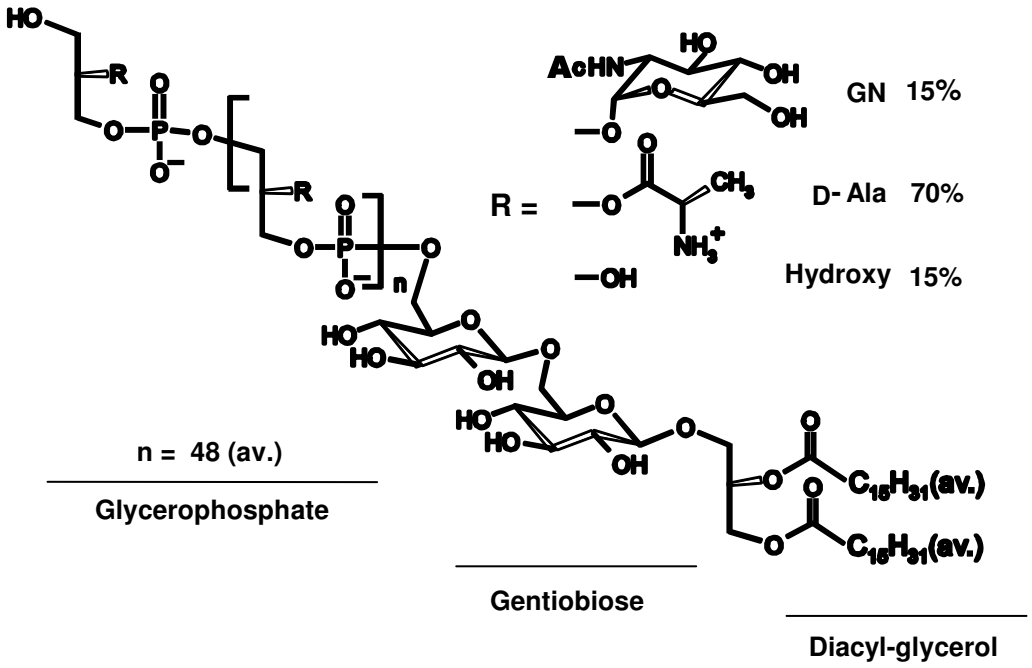

Figure 2 B LTA from *Staphylococcus aureus* (adopted from S. Morath, 2001, J Exp Med). The glycerophosphate backbone with an average (av) length of n=48 units is substituted with R as indicated (GN= α-D-N-acetylglucosamine; D-Ala=D-alanine) and connected via a gentiobiose with a diacyl-glycerol.

1.5.3 Lipo-teichoic acid

Until recently, the majority of infectious diseases were referred to the Gram-negative bacteria and research had been focusing on them and on their highly pyrogenic LPS and its lipid A moiety. However, today Gram-positive infections are increasing, first of all in immunocompromised individuals [88] and consequently those cell wall components of Gram-positives that are indispensably involved in bacterial life and pathogenity are being examined more closely. LTA is a molecule apparently combining those two aspects: Its heterogenous functions comprise colonisation, cell division and virulence [89-91]- involving the regulation of autolytical activity, homeostasis of physiochemical surface properties [92], cation homeostasis [93] and resistance to antimicrobial cationic molecules [94]. In the opportunistic intracellular pathogen *Listeria monocytogenes*, LTA is reported to be the scaffold for non-covalently bound proteins like internalin B (InlB) which alone is able to confer invasiveness into host cells [95, 96]. At the same time, LTA is a unique stimulus of cytokines, inducing a strong chemokine expression but almost no IL-12 or IFNγ [74, 97]. Furthermore, LTA activates the L-ficolin dependent C4 turnover of complement [98]. Not only the immune stimulatory capacity, but also the amphiphilic structure of LTA resembles its Gram-negative "counterpart" LPS. The well characterized LTA from *Staphylococcus aureus* is made up of a polyglycerophosphate backbone with in average 48 repeating units, substituted with D-alanine (70%) and α-D-N-acetylglucosamine (15%). This backbone, protruding the cell wall, is connected via a gentiobiose (α 1-6 glucose β) to a membrane-anchored diacylglycerol, containing 50% methylated fatty acid residues with an average length of 14 carbons (Figure 2 B; [99]). Structural deviations concerning the backbone length, its substituents, the disaccharide and the length of the fatty acid residues are already known for the LTA

from *S. pneumoniae* ([100], *B. subilits* [97] or Lactobacilli species [101], but are not associated with significant differences in immune stimulation [102]. By the use of synthetic LTA derivatives it could be shown that the diacylglycerol alone displays weak biological activity, while a complete cytokine release compared to native LTA was induced when six glycerophosphate units substituted with four D-alanine and one D-N-acetylglucosamine were connected to the diacylglycerol via a gentiobiose [74, 103]. Since until now no natural LTA mutants are known, the importance of LTA for the bacteria is obvious. Its functional variety and its immune stimulatory potency make it a promising molecule for investigating pathogen-host interactions and adaptation strategies in order to develop of bactericidal treatments, possibly interfering with LTA synthesis

1.5.4 *Listeria monocytogenes* as an intracellular pathogen

Intracellular bacteria independent whether they are facultative or obligatory pathogens, are in contrast to the obligate extracellular ones not limited to the epithelium of the host but become invasive. The intracellular immune recognition and responses are poorly elucidated until now. However, the cytosolic PRRs NOD1 and NOD2 have been reported recently to sense the presence of muropeptides, fragments from the cell wall peptidoglycan [104-106]. In some cases, like *Listeria monocytogenes* and *Shigella spp.*, the attacks of the humoral immune response are avoided by direct cell to cell spread, making a protective host defence depending on the T-cell responses necessary. They become internalized into the host cells via zipper- or trigger mechanisms and thus are localised in endosoms or phagosomes. To overcome the bactericidal medium of those vacuoles, different strategies have evolved to either escape into the cytosol (e.g. *L. monocytogenes*, *Shigella spp.*), become resistant (e.g. *Francisella tularensis*, *Salmonella typhimurium*) or render the vacuoles

harmless (e.g. *Legionella pneumophila*, *Mycobacteria spp.*). After having crossed the human epithelial barrier they may infiltrate lymphatic tissue and from there be transported to the spleen and the liver. Via the lymph- and the blood-stream virtually all organs like kidneys, lung, heart or brain can become infected. Whereas some pathogens are specialized for colonizing certain organs (e.g. *Shigella flexneri* in the colon), others lead to general systemic infections. A prominent example that has become a model organism for studying pathogen-host interactions is the opportunistic intracellular Gram-positive rod *L. monocytogenes* [107]. Being the only known human pathogenic strain of the *Listeria* genus, *L. monocytogenes* is detected on 15% of the foodstuff including vegetables, meat and seafood, making it a transient inhabitant of the human and animal gastrointestinal tract with estimated five to nine exposures per person and year, therewith providing the basis for gut invasive listeriosis [88, 108-110]. Compared to other food born diseases, systemic listeriosis is relatively rare: 1< 100.000 per year in Germany but the lethality of 25-30% is much higher than for other gut infections like salmonellosis. For hosts with a non-competent immune system, the risk is high: AIDS patients are 300 times more susceptible than the average population and unborn children have almost no change for survival if therapy is delayed [111, 112].

The mechanisms of infection and intracellular growth have been investigated in several cellines, including epithelial cells, macrophages and hepatocytes, showing a relatively similar replication cycle of *L. monocytogenes*. The bacteria get internalized by macrophages via phagocytosis or by non-phagocytes via induced phagocytosis involving first of all the internalins (Inl)A and InlB. Within minutes, the phagosomal membrane gets lysed involving the cytolysin Listeriolysin O (LLO) and the phaphatidylinositol-specific phospholipase C (PlcA). In the host cytosol, the Listeria

replicate with an average generation time of 40 minutes [88]. Motility is provided by the membrane bound protein ActA which recruits the host protein VASP and katalyses the polarized polymerisation of monomeric G-actin, resulting in the protrution of the bacteria through the host cell. Reaching the plasma-membrane, pseudopodes, also called listeriopodes, trigger the internalization into the vicinal cell. Escaping the vacuole surrounded by a double-membrane, involving additionally the phospholipase C (PlcB), initiates a new cycle (Figure 3). By this cell-to-cell spread, the humoral immune response is avoided. If the innate immune system is not capable of containing invasive Listeria, clearance of infection is then dependent on a T-cell mediated resistance, one explanation for the increased susceptibility towards systemic infections in the case of immunocompromised individuals, pregnant women and their foetuses or newborns. Most of the known genes involved in virulence are regulated by the transcription factor PrfA whose transcriptional and translational expression depends on both physical and chemical factors of the surrounding [88]. The genes that are directly or indirectly affected by PrfA encode for a variety of proteins which mediate into virulence (e.g. host cell entry, phagosomal escape, actin-based motility, hexose-phosphate transport, ABC transport, cell wall modification, secretion [88, 113, 114]. According to what is known about the virulence of Listeria, their immune evasion strategy so far involve the lysosomal escape and the cell to cell spread avoiding humoral defence mechanisms. It is still unclear, what role PRRs play for the sensing of intra- and extracellular Listeria and whether structural modifications of the bacterial cell wall during infection bear further benefits of immune evasion. The capability of sensing and reacting towards changes in the environment is the basis of the bivalent nature of *L. monocytogenes* occurring as an extracellular harmless saprophyte or as an intracellular pathogen. Understanding these regulatory

mechanisms and the associated consequences for immune recognition build up a basis for efficient listericidal therapies.

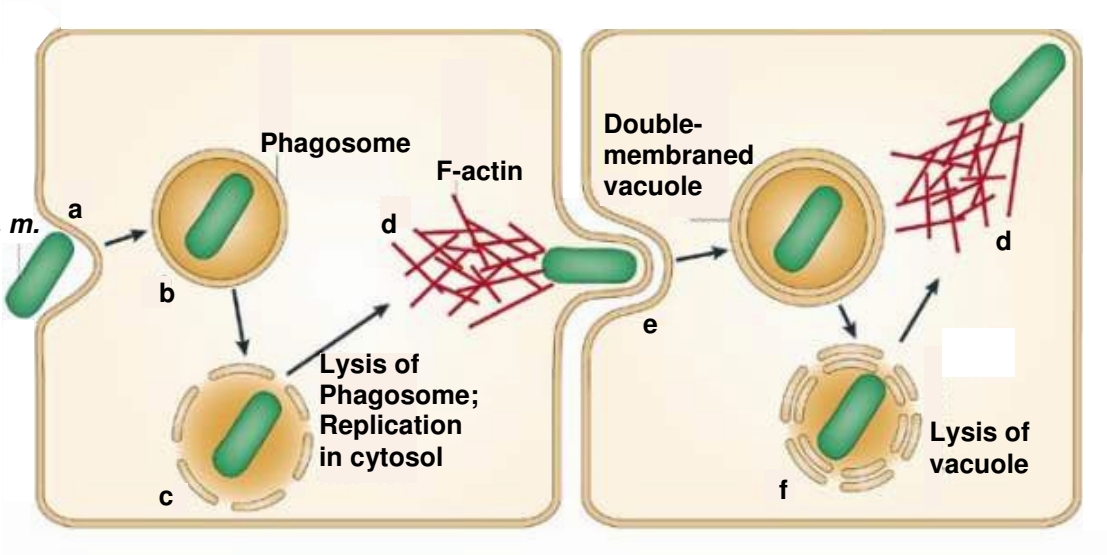

Figure 3 Infection and cell to cell spread by *Listeria monocytogenes* (*L.m.*) (adopted with slight modifications from Nature reviews microbiology 2006, Vol.4) a) active or induced phagocytosis by mammalian host cell. b) inside the phagosome, *L.m.* express Listeriolysin O (LLO) and phospholipase C (PlcA). c) Phagosome gets disintegrated and L.m. starts doubling in the cytosol every 40 minutes. d) Expressing actA and recruiting VASP, L.m. uses actin polymerisation to form listeriopodes contacting neighbouring cells. e) After internalisation by the adjacened cell, L.m. is enclosed in a double-membraned vacuole. f) Lysis of the vacuole expressing LLO, PlcA and PlcB initiates a new cycle.

2 Aims of the study

The human innate immune system, faced with severe infectious diseases, is a paradigm for co-evolutional events of a competition between pathogens that are continuously optimising their invasive strategies on the one side and the defence mechanisms that have to balance their powerful force to eradicate infections but at the same time avoiding overwhelming inflammations on the other side. A major characteristicum of the innate immune system is the expression of PRRs, which bind to indispensable microbial molecules whose functions are sensitive to variations and therefore have highly conserved structures. Nevertheless, exceptions from the rules or variations within the natural limits are crucial role for the inter-individual outcome of a certain pathogen/host interaction which may range from subclinical to lethal. For the development of therapeutic or even preventive therapies it is important to understand the deviations in immune recognition which might occur on side of the host cell and on side of the pathogen.

The first part comprises a functional study of the polymorphism Asp(299)Gly of the human pattern recognition receptor TLR4 regarding LPS binding and the induction of the proinflammatory cytokine TNF and the anti-inflammatory cytokine IL-10. Furthermore, a patients study was performed to functionally associate the TLR4 polymorphism with Crohn's disease by examining the LPS-induced IL-10 release in polymorphic versus wild type patients. This section is published or submitted under the titles:

- ► IL-10 release requires stronger toll-like receptor 4-triggering than TNF- a possible explanation for the selective effects of heterozygous TLR4 polymorphism Asp(299)Gly on IL-10 release.

- ► LPS-inducible anti-inflammatory responses are not diminished in Crohn's disease patients with heterozygous TLR4 Asp(299)Gly polymorphism.

In the second part, non-classical bacterial cell wall molecules with focus on LPS from the opportunistic bacterium *Pseudomonas aeruginosa*, regarding its immune stimulatory potency, and on differentially regulated LTA expression from the extracellular and intracellular grown *Listeria monocytogenes*, are examined. These studies have been published or submitted under the following titles:

- ► Endotoxin evaluation of eleven lipopolysaccharide by whole blood assay does not always correlate with Limulus Amebocyte Lysate assay.

- ► Reduced immunostimulation by *Listeria monocytogenes* grown intracellularly is associated with altered lipo-teichoic acid expression.

3

IL-10 release requires stronger toll-like receptor 4-triggering than TNF- a possible explanation for the selective effects of heterozygous TLR4 polymorphism Asp(299)Gly on IL-10 release

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Immunobiology

3.1 Abstract

The toll-like receptor 4 Asp(299)Gly polymorphism results in an inactive receptor. Heterozygosis is associated with reduced LPS-inducible IL-10 protein and IL-10 mRNA from blood leukocytes and isolated monocytes, while numerous other mediators are not affected. We could exclude that this effect is due to differences in the kinetics of IL-10 release, in the expression of total surface TLR4 or in LPS-binding to monocytes between subjects heterozygous for the Asp(299)Gly polymorphism or homozygous carriers of the wild-type allele. Furthermore, we could show that IL-10 induction in general requires stronger LPS-triggering than TNF and is more sensitive to LPS inhibitors. The lower number of responsive,

wildtype TLR4 receptors on monocytes of heterozygotes may explain why only IL-10 release is affected.

3.2 Introduction

The Asp(299)Gly polymorphism of toll-like receptor 4 (TLR4), which mostly co-segregates with the Thr(399)Ile mutation in Europeans, was found to interrupt lipopolysaccharide (LPS)-induced TLR4 signalling in transfected THP-1 cells and to be associated with reduced responsiveness to inhaled LPS in humans [64]. While two studies about the role of the TLR4 Asp(299)Gly polymorphism in human systemic and peri-operative endotoxemia demonstrated similar responses of subjects with wild-type or heterozygous polymorphic genotype [115, 116], numerous associations with inflammatory or infectious diseases, especially inflammatory bowel disease and Gram-negative infections have been reported [117]. However, in these studies cell-based assays proving that the polymorphism carriers' ability to respond to immune stimuli is altered are mostly lacking. Erridge et al. stimulated isolated monocytes with LPS from different Gram-negative bacteria and observed no deficits of the cells from heterozygous TLR4 polymorphism carriers in releasing IL-1β [118]. In a study reported by our group, analysis of cytokine responses of blood leukocytes of 160 healthy volunteers genotyped for the Asp(299)Gly polymorphism in an ex vivo whole blood test did not result in differences in LPS-inducible release of inflammatory mediators like TNF, IL-6, IL-1β, IFNγ, G-CSF, eicosanoids or serum cytokines, except for the release of the anti-inflammatory cytokine IL-10, which was significantly reduced in the group of subjects with heterozygous TLR4 alleles [119].

IL-10 is an important anti-inflammatory cytokine mainly produced by human monocytes, and IL-10 dysfunction can result in excessive inflammation [120]. So far, there is no explanation how this selective effect of the TLR4 Asp(299)Gly polymorphism on IL-10 release is mediated. Here we show that IL-10 production is already reduced on the mRNA level, but the reduced release of IL-10 protein is not due to delayed kinetics. Furthermore, we provide evidence that IL-10 release requires stronger triggering of TLR4 than TNF release, and therefore the lower number of responsive TLR4 receptors on monocytes of heterozygous carriers of the Asp(299)Gly polymorphism may explain why only IL-10 release is affected.

3.3 Material and Methods

3.3.1 Volunteer population and TLR4 genotyping

The TLR4 Asp(299)Gly polymorphism was determined in a population of 558 volunteers recruited at the University of Konstanz, Germany, in the years 2000-2004. DNA was prepared from EDTA anticoagulated blood (Sarstedt) by the QIAamp DNA Blood Mini Kit (Qiagen). Determination of the A(896)G TLR4 SNP was performed by real-time PCR and melting point analysis according to Heesen et al. [121]. The heterozygous TLR4 polymorphism occurred with a frequency of 7.2%. Subgroups of subjects with wild-type genotype and heterozygous TLR4 polymorphism were recruited from the 558 volunteers for the investigations described below.

3.3.2 Human whole blood and monocyte incubation

Differential blood cell counts were measured routinely with a Pentra60 to rule out acute infections (ABX Technologies). Incubations of whole blood and ELISA measurements were carried out as described [119]. Monocytes were isolated by

MACS-negative selection (Miltenyi Biotec). Stimulations were performed with LPS from *Salmonella abortus equi* (*S.a.e.*), or where indicated with LPS from *Klebsiella pneumoniae* (*Kl.pn.*) (both from Sigma). In some experiments Limulus anti-LPS factor (LALF, a generous gift from F. Jordan, Charles River/Endosafe) was added. RNA from heparinized blood (Sarstedt) was isolated with the QIAamp RNA Blood Mini Kit (Qiagen) and used for reverse transcription. All experiments and measurements were carried out blindly with regard to the donors' genotypes.

3.3.3 Quantitative Real-time PCR

cDNA was quantified by Real-time PCR on a LightCycler system (Roche) with LightCycler FastStart DNA Master SYBR Green (Roche) using specific primers from Thermo Hybaid: TNF forward: 5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3′, reverse: 5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′; GAPDH forward: 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse: 5′-GAAGATGGTGATGGGATTTC-3′; IL-10 forward: 5′-CAAGTTGTCCAGCTGATCCTTCAT-3′,reverse: 5′-GGCAACCTGCCTAACATG-3′; Cyclophilin forward: 5′-CTCCTTTGAGCTGTTTGCAG-3′, reverse: 5′-GATGGCAAGCATGTGGTG-3′.

3.3.4 FACS analysis

For FACS analysis a FACS Calibur flow cytometer (Becton Dickinson) with Cell Quest software (Becton Dickinson) was used. For assessment of the monocytes' LPS-binding capacity, EDTA blood was stained with in-house produced fluorescein-5 (6)-carboxamido caproic acid N-succinimidyl ester (FCHSE)-LPS and anti-CD14 (BD Biosciences). FCHSE was used as background control. For investigation of TLR4

surface expression, $5x10^5$ peripheral blood mononuclear cells (PBMC) were prepared with CPTTM Cell Preparation Tubes (BD Biosciences) and incubated with an anti-TLR4 antibody (a kind gift from Dr. Alexander Dalpke, University of Marburg, Germany). An anti-mouse IgG-phycoerythrin (PE, DAKO) was used as label. Measurement of IgG-PE alone served as background control. Monocytes were gated according to their forward and sideward scattering properties.

3.3.5 Statistics

Statistical analysis was performed using the GraphPad Prism 4.0 program (GraphPad Software, San Diego, USA). Data are given as mean ± SEM. Significance of differences was assessed by t-test in case of two groups only or by one-way ANOVA followed by Bonferroni's post-test. IC_{50} values were determined according to a sigmoidal curve fit.

3.4 Results

The aim of this study was to investigate the selectivity of the effect of the TLR4 Asp(299)Gly polymorphism on IL-10 release. Therefore, we had to compose a newly genotyped study group (n=17 wild-type (+/+); n=10 heterozygous polymorphics (+/-)) and to reconfirm the previously observed effect of the TLR4 Asp(299)Gly polymorphism on LPS-inducible IL-10 release. Again, like in the previous study [119], stimulation of the heterozygous polymorphics' whole blood with LPS (S.a.e. 1 µg/ml) resulted in diminished IL-10 release (+\+: 0.81±0.08 ng/ml vs. +\-: 0.52±0.06 ng/ml, p=0.026), while the release of TNF was not affected (+\+: 2.96±0.38 ng/ml vs. +\-: 3.26±0.44 ng/ml, p>0.05). The same effect was also observed using purified monocytes stimulated with increasing concentrations of LPS (figure 1).

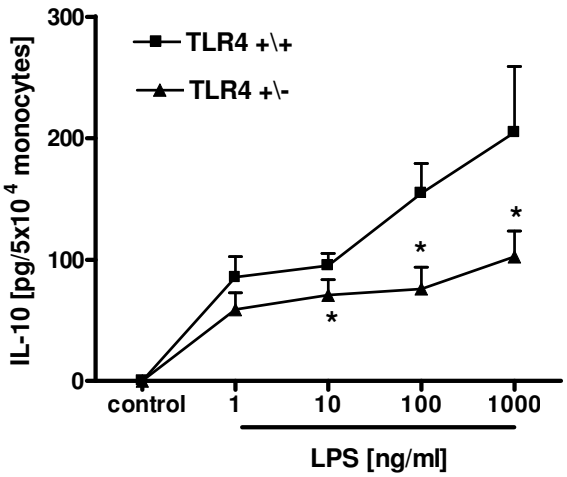

Figure 1 Heterozygous Asp(299)Gly TLR4 polymorphism is associated with reduced IL-10 but not TNF release from isolated human monocytes 5x 10^4 monocytes/well were incubated in the presence of LPS at the concentrations indicated for 20h. TNF and IL-10 were determined in the cell-free supernatants by ELISA. TLR4 +\+ indicates the wild-type (n=8) and TLR4 +\- the heterozygous polymorphic genotype (n=7). Data are means ± SEM. *$p < 0.05$ indicates significance versus the wild-type.

Analysis of mRNA expression by real-time PCR confirmed that lower LPS-inducible IL-10 release occurs already at the IL-10 mRNA level, while TNF mRNA levels were not influenced (figure 2). Since only the IL-10 release was affected by the TLR4 polymorphism, we investigated whether the reduced IL-10 levels were due to a shift in the kinetics of IL-10 release of subjects with TLR4 polymorphisms. For this purpose, we followed the release of LPS-induced IL-10 in whole blood incubations over a period of 28h. We measured IL-10 by ELISA after 5h, 10h, 15h, 20h, 25h and 30h of stimulation, but no shift in the IL-10 release curve was apparent (figure 3).

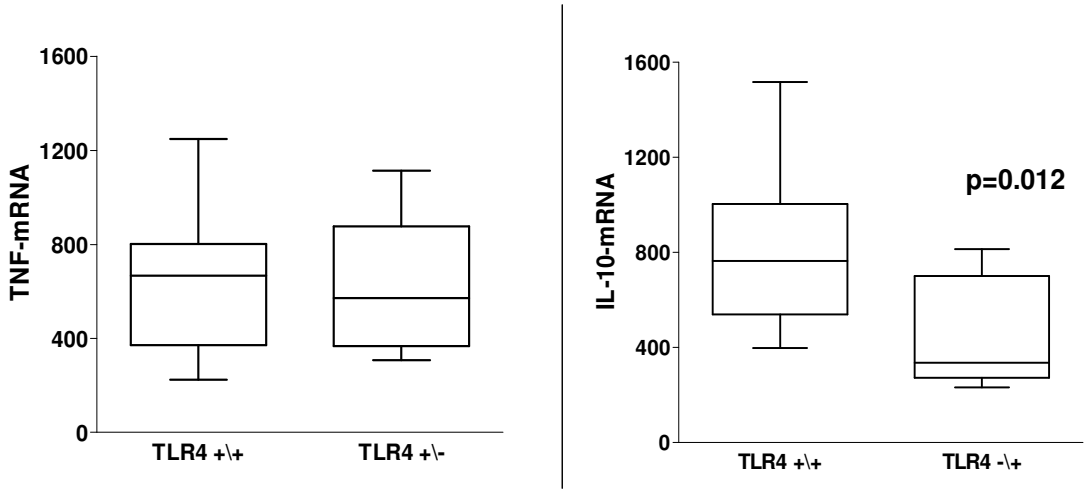

Figure 2 Heterozygous Asp(299)Gly TLR4 polymorphism is associated with reduced IL-10 but not TNF mRNA Five ml of 20% human whole blood were incubated in the presence of 1 µg/ml LPS from S.a.e. for 6h. RNA was prepared, reversely transcribed and cDNA was analyzed by real-time PCR. TNF and IL-10 data were normalized to cyclophilin cDNA. Data are presented in a box and whiskers blot as x-fold induction of mRNA. TLR4 +\+ indicates the wild-type (n=12) and TLR4 +\- the heterozygous polymorphic genotype (n=8).

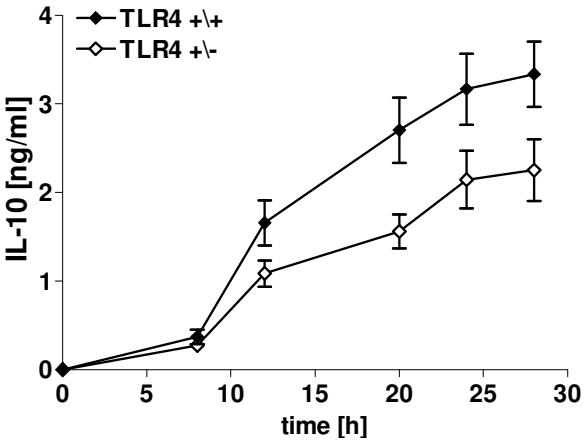

Figure 3 The kinetic of IL-10 release is not affected by the heterozygous Asp(299)Gly TLR4 polymorphism One ml of 20% human whole blood was incubated in the presence of 1 µg/ml LPS from *S.a.e.* for the time intervalls indicated. IL-10 was determined in the cell-free supernatants by ELISA. Data are means ± SEM. TLR4 +\+ indicates the wild-type (n=14) and TLR4 +\- the heterozygous polymorphic genotype (n=8).

Next we assumed that the difference in the density of total TLR4 surface expression might be responsible for the observed effects. When we compared the total TLR4 surface expression of monocytes from six wild-type and nine heterozygous TLR4 polymorphic donors by FACS analysis, we detected a higher density of TLR4 on monocytes from heterozygous polymorphic donors (median of relative fluorescence: (+\+): 11.92±1.00 vs. (+\-): 17.60±2.00; p=0.042). To confirm that the polymorphic TLR4 variant was transcribed, we investigated TLR4 mRNA by real-time PCR using specific Hybprobes designed for genotyping, which were 100% specific for the wild-type gene, but possessed one mismatch for the polymorphic TLR4 variant. The LightCycler-performed melting point analysis of the products revealed that indeed for carriers of the heterozygous polymorphisms both the wild-type (melting point 61°C) and the polymorphic mRNA variant (melting point 56°C) are transcribed in equal shares (figure 4). To investigate whether the LPS-binding capacity of monocytes from homozygous wild-type subjects is different from heterozygous subjects, we performed a FACS analysis. Monocytes from 37 homozygous wild-type subjects and monocytes from 18 heterozygous subjects were incubated with 0.35 ng/ml FCHSE-labelled LPS. We observed similar LPS-binding to monocytes of both groups (median of relative fluorescence: (+\+): 35.70±1.74 vs. (+\): 30.91±1.12).

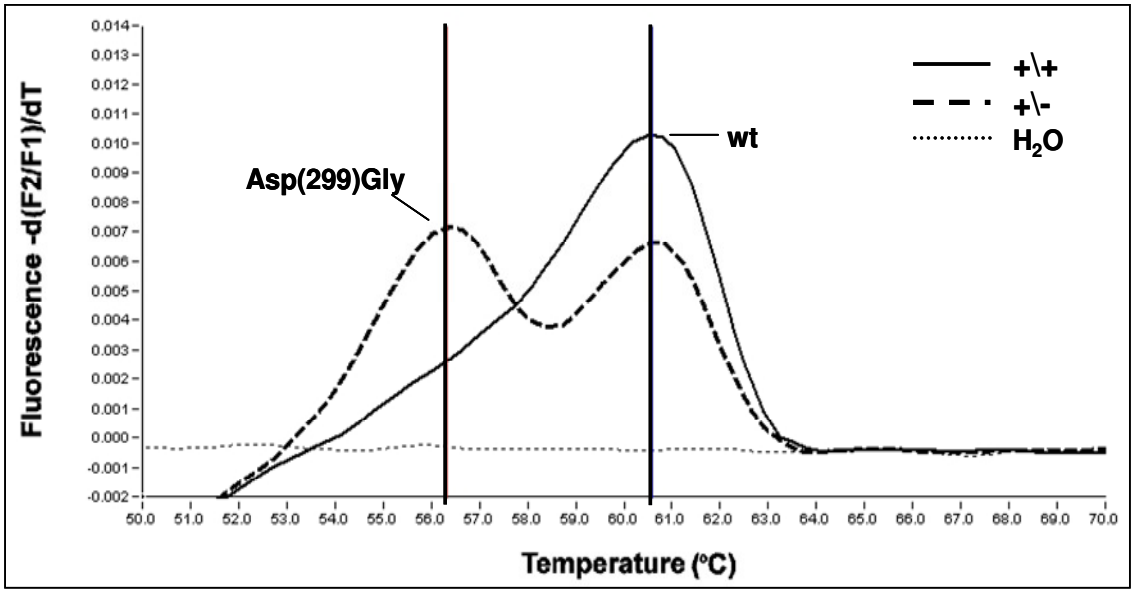

Figure 4 Carriers of the heterozygous Asp(299)Gly TLR4 polymorphism transcribe the wild-type and the polymorphic TLR4 variant RNA was prepared, reversely transcribed into cDNA and quantified by real-time PCR. The presence of the TLR4 wild-type (melting point 61°C) and/or TLR4 Asp(299)Gly polymorphic variant (melting point 56°C) was analysed by melting point analysis. TLR4 +/+ indicates the wild-type and TLR4 +\- the heterozygous polymorphic genotype.

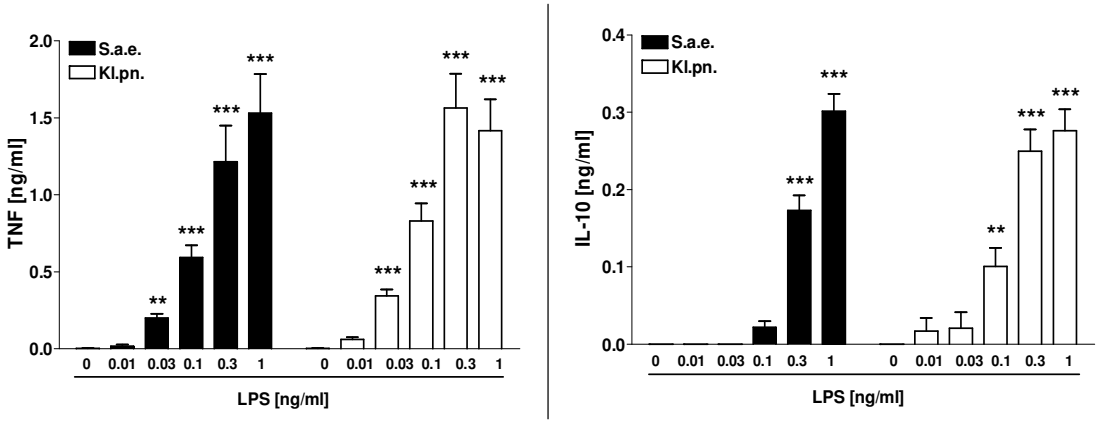

Figure 5 Induction of significant amounts of IL-10 from whole blood requires a higher LPS concentration than TNF induction One ml of 20% human whole blood from 24 healthy volunteers of the wildtype genotype was incubated in the presence of LPS at the concentrations indicated for 20h. TNF and IL-10 were determined in the cell-free supernatants by ELISA. Data are means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 and indicate significance versus the control.

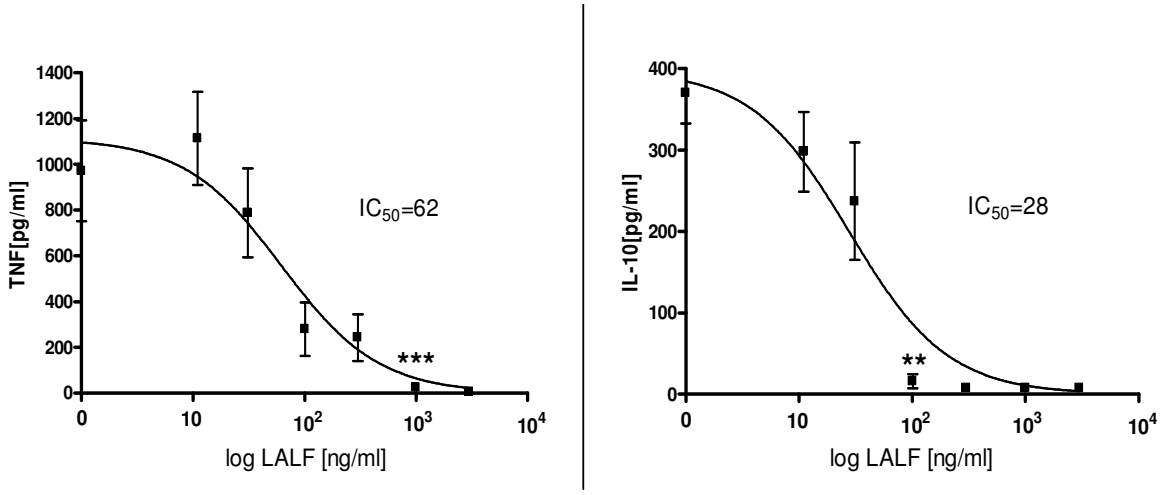

Figure 6 IL-10 induction is more sensitive to LPS inhibition than TNF One ml of 20% human whole blood from eight healthy volunteers was incubated in the presence of 1 ng/ml LPS or 1 ng/ml LPS together with 10 ng/ml-10 µg/ml LALF for 20h. TNF and IL-10 were determined in the cell-free supernatants by ELISA. Data are means ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

Since it must be assumed that the polymorphic TLR4 variant, although not being defective in LPS binding, is defective in LPS-responsiveness, we compared the sensitivity of TNF- and IL-10 release to the concentration of LPS used for stimulation. When a concentration response curve ranging from 10 pg/ml to 1 µg/ml LPS in whole blood from healthy volunteers of only the wild-type genotype was performed, we found that IL-10 release required significantly stronger LPS stimulation than TNF release (figure 5). To avoid bacterial species-specific results, these experiments were performed with LPS from two different enterobacterial strains (*S.a.e.* and *Kl.pn.*). The two LPS exhibited a similar potency to stimulate the release of TNF, IL-1β, IL-8, IL-10 and IFNγ at the highest concentration employed (shown for TNF and IL-10 in figure 3), but while 30 pg/ml of LPS from both strains resulted in a significant release of TNF, 100 pg/ml LPS (*Kl.pn.*) and 300 pg/ml LPS (*S.a.e.*) were necessary to induce

significant IL-10 release. In line with this observation, LPS (1 ng/ml)-inducible IL-10 release was significantly more susceptible to inhibition of LPS by the LPS-neutralizing factor LALF (figure 6).

3.5 Discussion

The only experimentally proven effect of the Asp(299)Gly polymorphism of TLR4 on inflammatory responses is a diminished release of the anti-inflammatory cytokine IL-10 upon in vitro stimulation of blood leukocytes from subjects with heterozygous expression of the polymorphic TLR4 variant with LPS [119]. This previous observation was now reconfirmed using a new study collective. It was shown that it also translates to isolated monocytes and that a significant reduction of IL-10 formation in case of subjects with heterozygous TLR4 polymorphism already occurs at IL-10 mRNA level, while the TNF mRNA levels remained unaffected. This, first of all, argues against an artefact of multiple testing in the previous study [119], where IL-10 was only one parameter measured among many.

The aim of this study was to investigate the underlying mechanisms responsible for the selective effect of the Asp(299)Gly polymorphism of TLR4 on cytokine release, which could not be explained by a delay in IL-10 release. In vitro transfection experiments had proven that the polymorphic variant of TLR4 is non-functional for LPS signalling [64], and it is assumed that the mutation at position 299, which is located in the extracellular LRR region of the TLR4 receptor, results in modified LPS binding. This is also supported by a recent study which provides evidence that the TLR4 mutation affects interaction with receptor agonists or co-receptors rather than intracellular signalling [122]. Therefore, it seemed likely that polymorphism carriers might suffer from impaired LPS responsiveness. However, in our study, neither the

total TLR4 surface expression nor the LPS-binding capacity of monocytes was reduced in heterozygous subjects, although it was confirmed that the polymorphic gene variant is transcribed. The latter finding suggests that some of the expressed TLR4 receptors would be aberrant and not responsive. The unaltered LPS binding capacity of monocytes could be explained by the assumption that LPS initially binds to MD-2 and is then presented to TLR4, what in case of the Asp(299)Gly variant would not result in TLR4 activation. The fact that subjects with a TLR4 polymorphism showed an increased TLR4 surface expression might even indicate that the expression of the polymorphic non-functional variant is partially compensated by a stronger expression of the wild-type TLR4.

Our results clearly indicate that IL-10 induction in general requires stronger LPS stimulation of monocytes than TNF induction and that IL-10 release is more susceptible to inhibition of LPS by a neutralizing agent. Divergent sensitivities of the TNF and IL-10 ELISA, which would influence these results, were excluded. Taken together, this means that significant IL-10 expression requires a higher density of activated receptor complexes than TNF and thus is more susceptible to a lack of functional receptors, like in the case of the TLR4 polymorphism. Although both TNF and IL-10 are released in response to TLR4 stimulation, the signal transduction pathways, which initiate gene transcription, differ. While pro-inflammatory cytokines like TNF are induced via a synergistic interplay of the NF-κB pathway and activation of the MAPK-kinases ERK1/2, JNK and p38, IL-10 induction is dependent on p38 and the transcription factor Sp1, but does not involve ERK1/2 and NF-κB [123-125], which may already explain why TNF induction is more sensitive to LPS stimulation than IL-10.

However, we investigated several key parameters known to be relevant for IL-10 induction including p38 on the basis of phosphorylated p38 by Western blot analysis, as well as the role of the MyD88 independent TRIF/IRF pathway [126], and the induction of cyclooxygenase-2 and PGE2 [127]. We observed no difference between homozygous wild-type and heterozygous polymorphic subjects in any of these experiments (unpublished data). For TNF induction it is believed that LPS binding to the TLR4 receptor complex alone is sufficient to induce TNF [128], though this has not been investigated for IL-10 so far. One might speculate that IL-10 induction requires further processes like internalization of the LPS/receptor complex and intracellular processing. Preliminary results obtained with LPS coated to surfaces support this hypothesis but were not finally conclusive.

Given the pivotal role of LPS and its receptor TLR4 in bacterial immune recognition, this study gives a first explanation of specific inflammatory alterations in heterozygous Asp(299)Gly polymorphic subjects. The resulting proinflammatory phenotype could hence be a risk factor for excessive inflammation. Consistent with this, the TLR4 Asp(299)Gly polymorphism has been convincingly linked with inflammatory bowel disease and ulcerative colitis [66], in which IL-10 reduction is known to play a decisive role [31]. Therefore, it would be of major interest to investigate LPS-inducible IL-10 levels in patients with inflammatory bowel disease carrying the Asp(299)Gly polymorphism.

4

LPS-inducible anti-inflammatory responses are not diminished in Crohn's disease patients with heterozygous Asp(299)Gly polymorphism

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Submitted to Inflammatory Bowel Disease

4.1 Abstract

Crohn's disease is an inflammatory bowel disease characterized by a relapsing or chronical inflammation of all layers of the intestinal wall. The toll-like receptor (TLR)4 Asp(299)Gly polymorphism, which is associated with reduced LPS-

inducible IL-10 release in healthy volunteers, has been linked with inflammatory bowel diseases. We have investigated by incubations of human whole blood whether in Crohn's diseases patients LPS-inducible TNF or IL-10 release is influenced by a heterozygous TLR4 polymorphisms compared to patients with a homozygous TLR4 wild type phenotype. We found that neither TNF nor IL-10 release was significantly different between both patient groups, and was furthermore comparable to cytokine release levels of healthy volunteers, indicating that probably at this stage of disease deviations in cytokine release occur only at the inflamed mucosa and cannot be detected by stimulations of leukocytes taken from the peripheral blood.

4.2 Introduction

Crohn's disease is characterized by chronic mucosal inflammation, which is discussed to be a consequence of abnormal immune responses to the autologous intestinal flora and a dysbalance of proinflammatory and regulatory cytokines. Especially IL-10, which inhibits antigen presentation as well as release of proinflammatory cytokines, turned out to play a pivotal role in the pathogenesis of Crohn's disease. It was shown that individuals that are genetically predisposed to produce less IL-10 are at higher risk of developing inflammatory bowel disease [36, 37]. Furthermore, IL-10-deficient mice develop colitis after colonisation with otherwise non pathogenic Gram-positive bacteria suggesting that under these circumstances normal immunosupressive barriers are broken [129, 130]. This has been linked to a defect of TGF-β/Smad signalling in the IL-10-deficient mice, which prevents inhibition of toll-like receptor (TLR) 2 mediated proinflammatory gene expression [131]. Since about half of the enteric indigenous flora is made up from Gram-negative bacteria, defects in TLR4 signalling

might as well be from importance. The single nucleotide polymorphisms (SNP) A(896)G of *tlr4*, which results in the exchange of the amino acid Asp by Gly at position 299, has been linked with Crohn's disease and ulcerative colitis [66, 67], where it was discussed to be associated with distinct clinical pictures [132]. Only recently, we have reported that the TLR4 Asp(299)Gly polymorphism is associated with reduced LPS-inducible IL-10 release in blood leukocytes from healthy volunteers, while the release of other proinflammatory cytokines is not affected [133]. In the present study we have investigated whether Crohn's disease patients with TLR4 Asp(299)Gly polymorphism show reduced LPS-inducible IL-10 release compared to patients with TLR4 wildtype genotype.

4.3 Material and Methods

4.3.1 TLR4 genotyping of Crohn's disease patients

The TLR4 Asp(299)Gly polymorphism was determined in a population of 63 Crohn's disease patients (sex ratio was 26 women to 37 men, median age 43, range 22-80) recruited at the University Hospital of Regensburg, Germany. The diagnosis of Crohn's disease was done on the basis of standard clinical criteria. Determination of the A(896)G *tlr4* SNP of the Crohn's disease patients was performed by SNPlex chemistry (Applied Biosystems, Foster City, CA, USA) on an automated platform with TECAN Freedom EVO and 384well TEMO liquid handling robots (TECAN, Männedorf, Switzerland) as described before [134]

4.3.2 Human whole blood incubation

Whole blood from 63 Crohn's disease patients and 30 healthy volunteers (all recruited at the University Hospital of Regensburg, Germany) was stimulated with 1

µg/ml LPS from *Salmonella abortus equi* (*S.a.e.*, Sigma). Incubations of whole blood and ELISA measurements were carried out as described [119]. All healthy volunteers were genotyped for the A(896)G *tlr4* SNP, in order to exclude carriers of the polymorphism, as previously described [133]. All experiments and measurements were carried out blindly with regard to the blood donors' genotypes.

4.3.3 Statistics

Statistical analysis was performed using the GraphPad Prism 4.0 program (GraphPad Software, San Diego, USA). Significance of differences was assessed by one-way ANOVA. Data are depicted as box and whiskers blots.

4.4 Results

To investigate whether Crohn's disease patients with TLR4 Asp(299)Gly polymorphism show reduced LPS-inducible IL-10 release in comparison to Crohn's disease patients with wild type genotype, 63 patients were genotyped and the inflammatory responses of their leukocytes were assessed by in vitro stimulation of whole blood. The genotyping revealed that 12 out of the 63 Crohn's disease patients were heterozygous carriers of the Asp(299)Gly TLR4 polymorphisms, while 51 possessed a homozygous wild type genotype. None of the patients was a carrier of a homozygous polymorphism. To compare the inflammatory capacity of leukocytes from Crohn's disease patients with and without heterozygous Asp(299)Gly TLR4 polymorphisms, LPS-inducible TNF and IL-10 release was assessed by incubations of human whole blood. A collective of 30 healthy volunteers with wild type genotype served as control group. As shown in figure 1, no significant difference in TNF or IL-10 release was detectable between Crohn's disease patients with heterozygous

Asp(299)Gly TLR4 polymorphisms or homozygous wild type genotype. Furthermore, TNF and IL-10 release of both patient groups was not different from that of blood leukocytes of healthy volunteers with homozygous TLR4 wild type genotype.

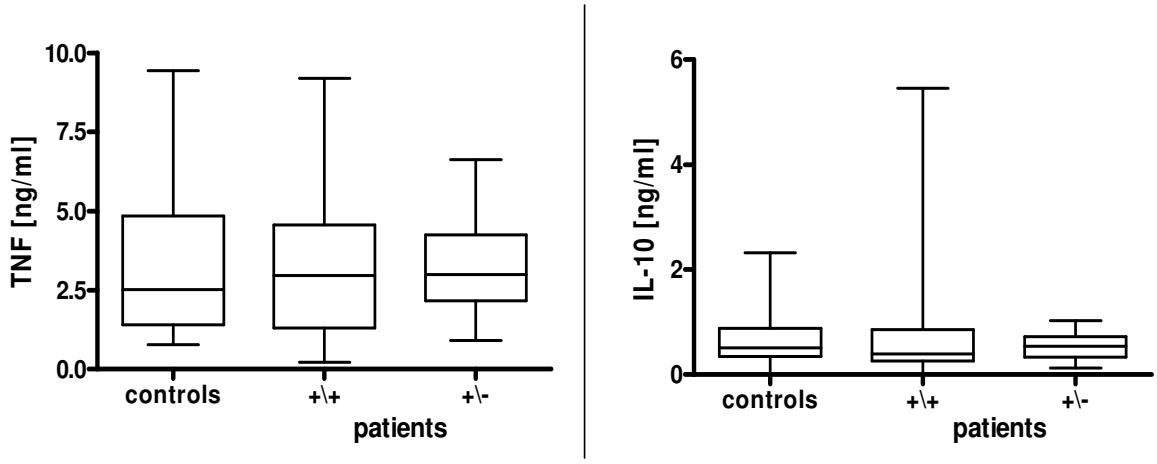

Figure 1 One ml of 20% human whole blood from 30 healthy volunteers with homozygous wild type genotype (controls), 51 Crohn's disease patients with homozygous wild type genotype (patients +/+) and 12 Crohn's disease with heterozygous polymorphic genotype (patients +/-) was incubated in the presence of 1 µg LPS for 20h. The release of TNF and IL-10 was determined in the cell-free supernatants by ELISA.

4.5 Discussion

The Asp(299)Gly polymorphism of the LPS receptor TLR4 has been linked with an increased susceptibility for inflammatory bowel disease in several studies [66, 67, 132, 135, 136] Since it was recently shown that in healthy subjects the same TLR4 polymorphism in heterozygous expression is associated with decreased LPS-inducible IL-10, while the release of other pro-inflammatory mediators remained unchanged

[133], it was tempting to investigate whether this observation also translates to Crohn' disease patients, for which reduced anti-inflammatory responses are regarded as major risk factors for disease development. We have investigated the LPS-inducible responses of 63 Crohn's disease patients of which 51 possessed a homozygous wild type genotype and 12 a heterozygous polymorphic genotype. We found that LPS-inducible TNF and IL-10 release was comparable between both groups. Furthermore, the response of blood leukocytes from Crohn's disease patients was not significantly different from that of 30 healthy volunteers with wild type genotype. The association of decreased LPS-inducible IL-10 release and the Asp(299)Gly polymorphism of TLR4 had been confirmed in two independent study groups before, one consisting of 160 healthy volunteers of which 145 were carriers of a homozygous wild type genotype and 14 of a heterozygous Asp(299)Gly polymorphism [119] and a second one comprising 27 healthy volunteers, of which 17 carried a homozygous wild type and 10 a heterozygous polymorphic genotype [133]. The decrease in LPS-inducible IL-10 already occurred on m-RNA level and was supposed to be due to a lower number of responsive TLR4 receptors on monocytes of subjects with heterozygous Asp(299)Gly polymorphism [133]. Therefore, the comparability of LPS-inducible IL-10 levels between the Crohn's disease patients with and without TLR4 polymorphism was a surprise. We would have expected that the LPS inducible IL-10 levels are diminished in patients to the same extent or even stronger as it is the case for healthy subjects with heterozygous Asp(299)Gly polymorphism. Since several patients were treated with immunosuppressive medication, we cannot exclude that LPS-induced signal transduction is biased and deviations in inducible IL-10 release, which might have been observable without medication, are obliterated now. To check for modulatory effects due to immunosuppressive medication of the patients, we had included a

group of healthy volunteers which had taken no medications and determined their LPS-inducible cytokine levels as well. Since especially TNF release, one of the most prominent proinflammatory mediators, was comparable between both patients groups and healthy controls, a dramatic influence of immune-modulatory medication can be excluded.

There is an increasing body of evidence that IL-10 is an important dampener of the ileal inflammatory processes in inflammatory bowel diseases. IL-10 concentrations for example are increased in the colon mucosa from diseased patients [137] and subjects that are genetically predisposed to produce less IL-10 are at higher risk for disease development [36, 37, 138]. We could not detect a reduced IL-10 release capacity of blood leukocytes from Crohn's disease patients with heterozygous Asp(299)Gly TLR4 polymorphism in our study. However, this was only a snap-shot taken from blood leukocytes of patients which had suffered from the disease for years and does not exclude that deviations in responses to LPS might exist which lead to significantly reduced IL-10 levels directly at the inflamed mucosal tissue of patients with Asp(299)Gly polymorphism. Therefore, it would be important to measure IL-10 concentration locally, best at the early onset of disease, to get non falsified results. If it turns out that patients with the Asp(299)Gly polymorphism are characterized by locally reduced IL-10 levels, these patients together with the group of genetically predisposed subjects might be the ideal target group that would benefit from locally administered IL-10 therapy.

4.6 Acknowledgements

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5

Endotoxin evaluation of eleven lipopolysaccharide by whole blood assay does not always correlate with Limulus Amebocyte Lysate assay

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5.1 Summary

More than 90% of all publications on endotoxin were carried out with endotoxins (lipopolysaccharide (LPS)) from enterobacteriaceae. We compared the immune stimulatory potency of eleven different LPS using human whole blood incubations. While the majority of LPS induced cytokine release equipotently, a thousand-fold more LPS from *Pseudomonas aeruginosa* or *Vibrio cholerae* was still less potent in inducing TNF, IL-1, IL-10 and IFN though it potently induced ng quantities IL-8. All LPS tested, regardless of the microorganism, showed toll-like receptor (TLR)4-dependence, except for the LPS from *P. aeruginosa* and *V. cholerae*, which were both TLR4 and TLR2 dependent. Interestingly, UV-inactivated *P. aeruginosa* bacteria, although Gram-negative, also showed TLR2- and TLR4-dependence. Re-purification

of the commercial LPS preparation by phenol re-extraction led to a complete loss of the TLR2 dependency, indicating contaminations with lipoproteins. In the Limulus Amebocyte Lysate Assay, often performed to exclude contaminations in purified water likely to originate from *P. aeruginosa*, *P. aeruginosa* LPS was only two-fold less potent than LPS from *S. abortus equi* or the assay standard LPS from E. coli. This results in an overestimation of pyrogenic burden by a factor 500 in the sample when compared with the biological activity of highly purified *P. aeruginosae* LPS in human whole blood.

5.2 Introduction

Lipopolysaccharide (LPS), which makes up about 75% of the surface of Gram-negative bacteria, is known to be their major immune stimulatory principle (for review see [139]). It is released from the bacterial surface when the bacteria multiply, or when they die and lyse, leading to the activation of immune cells, as well as epithelial, endothelial or smooth muscle cells [140]. The recognition of LPS by host cells is an important step for the induction of inflammatory processes and anti-bacterial defense mechanisms, but might also lead to multi-organ failure and shock upon excessive systemic LPS exposure [141-143]. Chemical characterization and structural analysis of LPS of numerous enterobacteriaceae have revealed common structural features. The basic structure of LPS consists of a repetitive polysaccharide (O-antigen), which forms the outer part, the core oligosaccharide and the lipid A moiety, which is embedded in the outer membrane [77-79]. The O-antigen carbohydrate chain is a polymer of repeating oligosaccharides, which differ between species and determine the serological specificity of bacteria. In contrast, the structure of the lipid A, which consists of a phosphorylated disaccharide backbone, substituted

with fatty acid, is highly conserved and exerts the endotoxic activity [80, 81, 144]. It is recognized by host immune cells via specific pattern recognition receptors, which immediately activate the host cells and stimulate cytokine release and complement activation leading to inflammatory responses [145]. The C3H/HeJ mouse has long been known to be hyporesponsive to LPS due to a spontaneous mutation of the *lps* gene [146]. Positional cloning revealed that the *lps* gene in these mice was the tlr4 gene with a point mutation [50]. At the same time some reports suggested TLR2 as LPS receptor [147, 148], but it soon became clear that the TLR2-dependent responses were induced by contaminating lipoproteins [149]. Although direct binding has not been demonstrated so far, it is believed today, that TLR4 together with MD-2 and the glycosylphosphatidylinositol anchored CD14 molecule confer sensitivity towards LPS [150]. Most of the initial studies have been done with LPS from enterobacteriaceae, since their structures were the first to be elucidated and synthesized chemically [81]. More recent studies, however, indicate that at least some LPS exist like the LPS from *Bacteroides fragilis* [151], *Leptospira interrogans* [85] or *Porphyromonas gingivalis* [86, 152] that are TLR2 dependent. During the last years, the structural and functional differences between LPS from different species became clear and the fact that the biological activity of LPS not only depends on the bacterial strain of origin but also on the cellular system used as the read-out (for review see [82, 83, 150]).

We therefore investigated the cytokine-inducing potency of eleven endotoxins from different bacterial species. As read-out system we chose the human whole blood incubation, which is likely to reflect the physiological situation, since all blood leukocytes are present in their physiological environment [153]. Further, we determined the TLR-dependence using cells from TLR2- and TLR4-defective mice.

5.3 Materials and Methods

5.3.1 Bacterial stimuli

5.3.1.1 Endotoxin

LPS from *Escherichia coli K235, Klebsiella pneumoniae, Salmonella abortus equi, Salmonella enteritidis, Salmonella typhimurium, Salmonella typhosa, Shigella flexneri, Serratia marcescens* 1A, *Vibrio cholerae* 569B (Inaba, O1), and *Pseudomonas aeruginosa* serotype 10 were purchased from Sigma, Deisenhofen, Germany. LPS from *Rhodobacter sphaeroides* was purchased from Quadratech, Epsom Surrey, England. LPS from *P. aeruginosa* and *S. abortus equi* were further purified by phenol re-extraction according to [149] to eliminate putative lipoprotein contaminations. Subsequently, dialysis of the LPS against aqua dest. was performed over night using a Spectra/Por MWCO 1 kDa membrane (Spectrum Laboratories, Inc., Ca, USA).

5.3.1.2 Bacteria

E. coli K-12 strain JM 109, a kind gift from Dr. Gerald Grütz, Charité Berlin, Germany, were grown in LB medium at 5% CO2, 37°C, and stored at –80°C in physiological saline solution containing 25% glycerol. *P. aeruginosa* S10 (purchased from ATCC, Manassas, USA) were grown in LB medium at 5% CO2, 37°C, and stored at –80°C in physiological saline solution containing 3.5% DMSO. Prior to incubation, the bacteria were washed twice with saline solution and UV-inactivated on ice for 60 min using an UV-Stratalinker 1800 (Stratagene, Jolla, CA, USA) at 9999 x 100 µJ.

5.3.2 Limulus Amoebocyte Lysate Assay

The kinetic Limulus Amoebocyte Lysate Assay (LAL, Charles River Laboratories, Sulzfeld, Germany, detection limit 0.1 EU/ml) was performed according to the manufacturer's protocol.

5.3.3 Human whole blood incubation

Human whole blood was taken from healthy volunteers using heparinized S-monovettes® (Sarstedt, Nürmbrecht, Germany) and diluted 1:5 with RPMI 1640 (Cambrex, Verviers, Belgium) containing 100 IU penicillin/100 µg streptomycin (Biochrom, Berlin, Germany) per ml in polypropylene reaction vials (Eppendorf, Hamburg, Germany). Stimulation was performed for 24h using sonified LPS or whole bacteria. After incubation at 37°C and 5% CO2 in humidified air, the vials were shaken and centrifuged for 2 min at 400g. The cell-free supernatants were stored at -80°C until cytokine measurement by ELISA.

5.3.4 Isolation and stimulation of murine bone marrow cells

TLR4-mutated ($TLR4^{d/d}$) C3H/HeJ and respective wild type C3H/HeN mice were purchased from Charles River Laboratories (Sulzfeld, Germany). TLR2 knock-out ($TLR2^{-/-}$) mice were generated by homologous recombination by Deltagen (Menlo Park, CA, USA) and kindly provided by Tularik (South San Francisco, CA, USA). The animals were maintained under controlled conditions (22°C and 55% humidity, constant day/night cycle of 12h) and were fed ad libitum with Altromin 1314 (Lage, Germany). They received human care in accordance with the NIH guidelines as well with the legal requirements in Germany. To obtain bone marrow cells, mice were put under terminal pentobarbital anesthesia (Narcoren, Merial, Halbergmoos, Germany). Cells were isolated from both femurs by rinsing with 10 ml PBS and transferred into siliconized glass tubes (Vacutainer, BD Biosciences, Heidelberg, Germany). After centrifugation, cells were resuspended in RPMI 1640 (Cambrex) containing 10% FCS (PAA Laboratories GmbH, Pasching, Austria) and 100 IU penicillin/100 µg streptomycin (Biochrom) per ml and transferred to 96-well plates (Greiner,

Frickenhausen, Germany) in a density of 5x 105 bone marrow cells/well. Cells where then stimulated with LPS or whole bacteria and incubated at 5% CO2, 37°C for 24h. After incubation, the cell-free supernatants were stored at –80°C until cytokine measurement. The TLR2 agonist lipoteichoic acid (LTA), prepared in house from Staphylococcus aureus [75] and the TLR4 agonist LPS from *S. abortus equi* served as control stimuli in all experiments to ensure the responsiveness of the TLR-defective cells.

5.3.5 ELISA

Cytokines were determined by sandwich ELISA based on commercial antibody pairs against human TNF, IFNγ and IL-8 (Endogen, Perbio Science, Bonn, Germany), human IL-1β and IL-6 and murine TNF (R&D, Wiesbaden, Germany) and human IL-10 (Pharmingen Becton-Dickinson, Heidelberg, 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). Recombinant cytokines used as standards were obtained from the National Institute for Biological Standards and Controls, Herts, UK (TNF, IL-1) and BD Biosciences, (IL-10, murine TNF, murine IL-6), PeproTech, Tebu, Frankfurt, Germany (IL-6 and IL-8) or Thomae, Biberach, Germany (IFNγ). Assays were carried out in flat bottom, ultrasorbent 96-well plates (Nunc, Wiesbaden, Germany).

5.3.6 Statistics

Statistical analysis was performed using the GraphPad Prism 3.0 program (GraphPad Software, San Diego, USA). Significance of differences was assessed by the t-test for two groups and by one-way ANOVA followed by Bonferroni's post-test for

experiments with more than two groups. In the figures *, ** and *** represent p values <0.05, <0.01 and <0.001, respectively.

5.4 Results

5.4.1 Immune-stimulatory potencies of eleven different LPS

The immune stimulatory potency of LPS from eleven different bacterial species, including LPS from the enterobacteriaceae *E. coli, S. abortus equi, S. enteritidis, S. typhimurium* and *S. typhosa*, as well as the enteropathogenic species *S. flexneri, S. marcescens* and *V. cholerae*, the opportunistic bacteria *K. pneumoniae* and *P. aeruginosa*, and the phototrophic bacterium *R. sphaeroides* were compared using human whole blood incubations.

For this purpose, concentration-response curves of the eleven LPS were performed and the release of the pro-inflammatory monokines TNF and IL-1β, the chemokine IL-8, the anti-inflammatory cytokine IL-10 and the lymphokine IFNγ was measured. The concentration-response curves of LPS from *E. coli, S. abortus equi, S.enteritidis, S. typhimurium, S. typhosa, S. flexneri, S. marcescens,* and *K. pneumoniae* were comparable for all cytokines measured and are exemplarily shown for E. coli LPS-induced TNF and IL-8 release in figure 1A and 1B, respectively. The minimal concentrations of these LPS necessary to induce significant cytokine release laid within one log-order for each cytokine and ranged from 0.01-0.1 ng/ml for the induction of IL-8, from 0.1–1 ng/ml for TNF, IL-1β and IFNγ, and were 1 ng/ml for IL-10 (table 1). In contrast, about a thousand-fold more LPS from *P. aeruginosa* and *V. cholerae*, i.e. 10-1000 ng/ml, were necessary to induce the release of significant amounts of TNF, IL-1β and IL-8, while no significant release of IL-10 and in case of *P. aeruginosa* also not of IFNγ could be induced.

Table 1 Minimal LPS concentration necessary for significant cytokine induction

LPS	cytokines				
	TNF	IL-1β	IL-8	IL-10	IFNγ
*E. coli**	0.1	0.1	0.1	1	1
*S. abortus equi **	1	0.1	0.1	1	1
*S. enteridies **	0.1	0.1	0.01	1	0.1
*S. typhimurium **	1	1	0.1	1	1
*S. typhosa **	0.1	0.1	0.1	1	1
*S. flexneri **	0.1	1	0.1	1	1
*S. marescencs **	0.1	0.1	0.01	1	1
V. cholerae #	10	100	1000	-	100
*K. pneumoniae **	0.1	1	0.1	1	0.1
P. aeruginosa #	1000	100	I00	-	-
R. sphaeroides #	-	100	100	-	-

Human whole blood from six volunteers was incubated with 0.1-1000 pg LPS/ml (bacterial species marked with *) or 0.1- 1000 ng LPS/ml (bacterial species marked with #) for 20h. Cytokines were determined in the cell-free supernatants by ELISA. The minimal LPS concentrations (ng/ml) that led to significant cytokine release compared to the unstimulated control ($p < 0.05$) was determined by Repeated Measures ANOVA followed by Bonferroni's Multiple Comparison test. Cytokine levels of unstimulated controls (pg/ml) were TNF, 92.2 ± 75.9; IL-1β, 6.9 ± 0; IL-8, 1065.6 ± 356.6; IL-10, 46.0 ± 29.1; IFNγ: 78.0 ± 49.3.

While the LPS from *P. aeruginosa* and *V. cholerae*, if applied in a thousand-fold higher concentrations, induced TNF of comparable amounts to E. coli LPS (figure 1A), they turned out to be very potent inducers of IL-8 (figure 1B). LPS from R. sphaeroides in high concentrations of 100 μg/ml induced the release of IL-1β and IL-8, but of no other cytokines measured. The main endogenous pyrogens are TNF, IL-1 and IL-6. On the one hand, TNF and IL-1 exert their pyrogenic potential through induction of IL-6, on the other hand, LPS can directly induce IL-6.

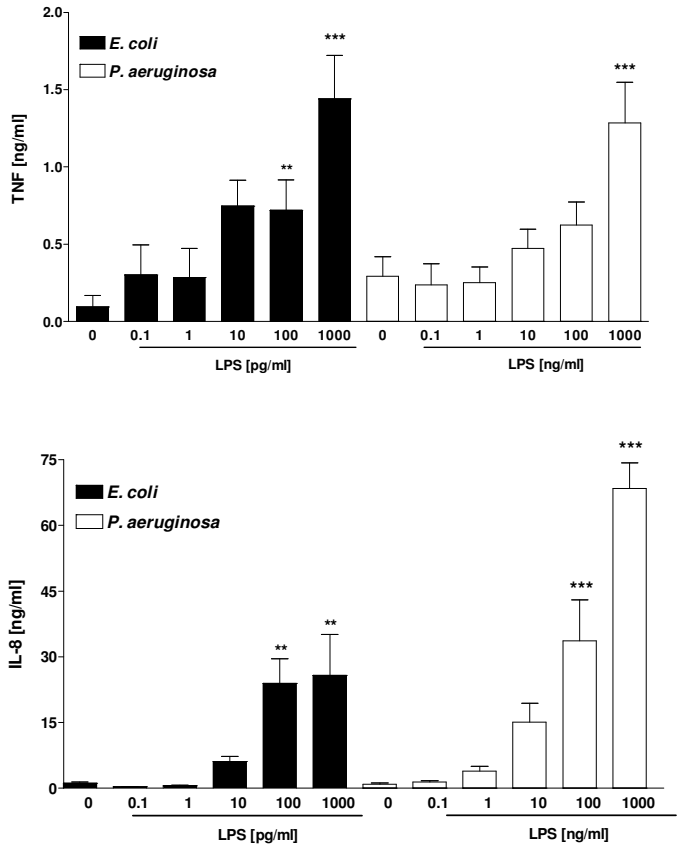

Figure 1 LPS concentration response curves One ml of 20% human whole blood was incubated with LPS from *E. coli* and *P. aeruginosa* at the concentrations indicated for 24h. TNF (A) and IL-8 (B) were determined in the cell-free supernatants by ELISA. Data are means ± SEM, n=6. ** and *** indicate significant cytokine release in comparison to the unstimulated control.

Therefore, the potency of *P. aeruginosa* LPS to induce IL-6 release has also been investigated. We found that in human whole blood, like for TNF and IL-1β thousand-fold more LPS from *P. aeruginosa* was necessary to induce IL-6 release in amounts comparable to LPS from enterobacteriaceae (IL-6 in ng/ml; LPS 10 ng/ml from S.a.e. 46 ± 5.3 versus LPS 10 µg/ml from P.a. 55 ± 6.3; n=6).

5.4.2 TLR-dependence of different LPS

In order to investigate the TLR-dependence of the different LPS, bone marrow cells from wild type and from TLR4-defective C3H/HeJ and TLR2$^{-/-}$ mice were stimulated

with the eleven LPS and the release of IL-6 was measured. All LPS showed clear TLR4 dependence, except for the LPS from *P. aeruginosa* and *V. cholerae*, which were TLR4 and TLR2 dependent. These results are exemplarily shown for five of the eleven LPS in figure 2A and 2B.

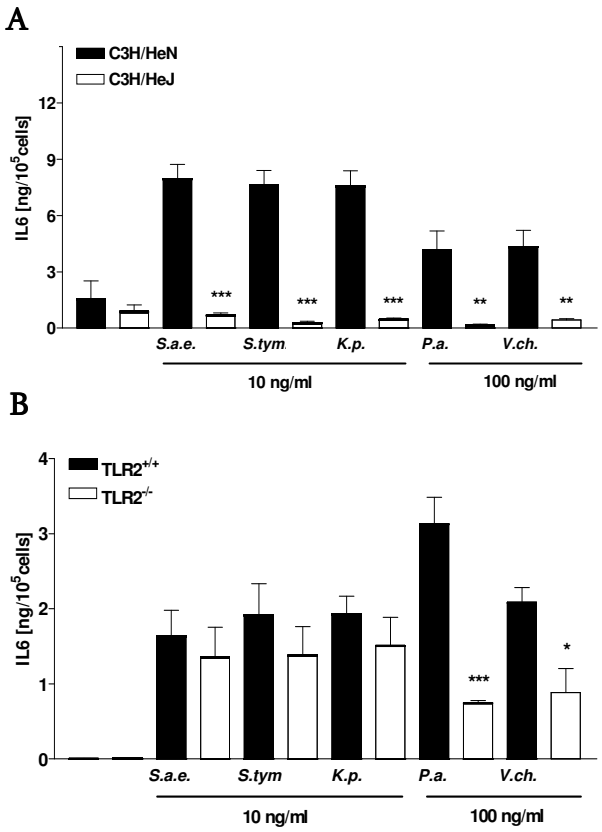

Figure 2 TLR-dependence of different LPS
5x 10^5 bone marrow cells from (A) C3H/HeN and C3H/HeJ and (B) TLR2+\+ and TLR2-\- mice were incubated with LPS from *S. abortus equi (S.a.e.), S. typhimurium (S.tym.), K. pneumoniae (K.p.), P. aeruginosa (P.a.)* and *V. cholerae (V.ch.)* in the concentrations indicated for 24h. IL-6 was determined in the cell-free supernatants by ELISA. Data are means ± SEM, n=4. *, **, *** indicate significant difference in IL-6 release in comparison to the cells from wild type mice.

5.4.3 Potency and TLR dependence of whole *P. aeruginosa* bacteria

In order to characterize the cytokine-inducing potency of whole *P. aeruginosa* bacteria, human whole blood was stimulated with different amounts of UV-inactivated *P. aeruginosa* in comparison to UV-inactivated *E. coli*. As shown in figure

3A, at least thousand-fold more *P. aeruginosa bacteria* than *E. coli* were necessary to induce comparable amounts of TNF, while the amount of maximal inducible IL-8 release was comparable (figure 3B). To investigate the TLR dependence, bone marrow cells from C3H/HeJ and TLR2-/- and their respective wild type mice were stimulated with 10^7/ml UV-inactivated P. aeruginosa or 10^5/ml UV-inactivated E. coli. Cytokine induction by *P. aeruginosa* was found to be strongly TLR4- and TLR2-dependent (IL-6 in ng/ml: C3H/HeN, 4 ± 0.7 vs. C3H/HeJ, 0.2 ± 0.03; TLR2+/+, 3 ± 0.4 vs. TLR2-/-, 0.4 ± 0.4; both n=8, p< 0.001), while E. coli induced cytokine release was only dependent on TLR4 (IL-6 in ng/ml: C3H/HeN, 2 ± 0.6 vs. C3H/HeJ, 0.8 ± 0.02, p< 0.001; TLR2+/+, 0.7 ± 0.3 vs. TLR2-/-, 0.6 ± 0.03, both n=4).

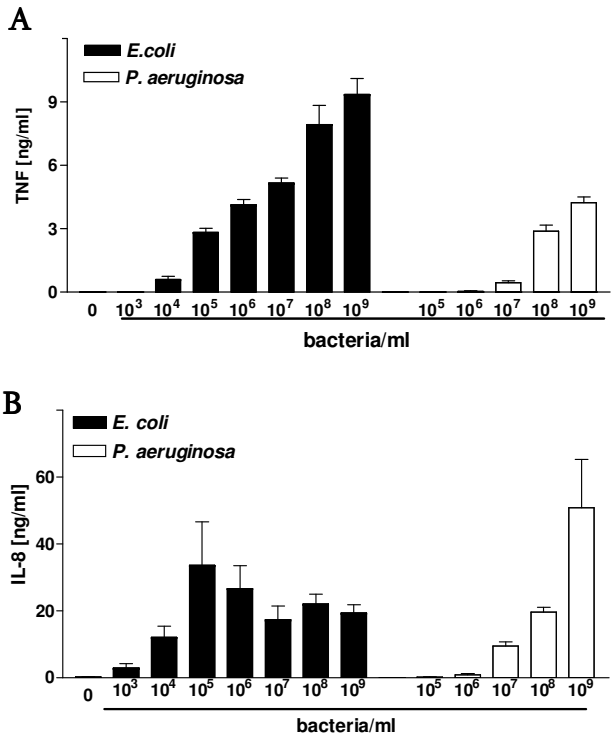

Figure 3 Comparison of cytokine induction by whole *P. aeruginosa* and *E. coli* One ml of 20% human whole blood was incubated with whole UV-inactivated *P. aeruginosa* or *E. coli* in the concentrations indicated for 24h. (A) TNF and (B) IL-8 were determined in the cell-free supernatants by ELISA. Data are means ± SEM, n=4.

5.4.4 Phenol re-extraction and pyrogenicity of *P. aeruginosa* LPS

To clarify whether the TLR2 dependence of the LPS from P. aeruginosa was due to lipoprotein contamination, phenol re-extraction of the commercial LPS preparation was performed and the cytokine-inducing potency of the re-purified LPS as well as the TLR-dependence was investigated. The phenol re-extraction led to a significant reduction of the cytokine inducing capacity of the LPS in concentrations ≥1 µg/ml, as indicated in figure 4 for TNF release. The same effect was observed for all other cytokine (data not shown). However, when commercial LPS from *S. abortus* equi was repurified, no significant loss of potency was observed (1 µg/ml LPS before vs. after repurification, TNF ng/ml: 4.4 ± 0.3 vs. 5.4 ± 0.2; n=7).

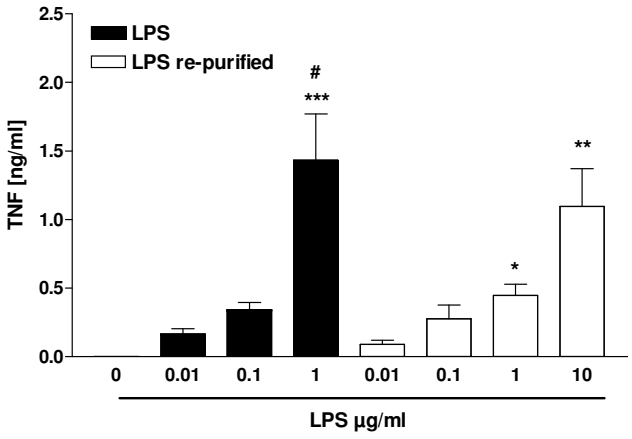

Figure 4 Comparison of cytokine induction by *P. aeruginosa* LPS before and after phenol re-extraction One ml of 20% human whole blood was incubated with LPS from *P. aeruginosa* before and after phenol re-extraction in the concentrations indicated for 24h. TNF was determined in the cell-free supernatants by ELISA. Data are means ± SEM, n=4. *, ** and *** indicate significant TNF release in comparison to the unstimulated control, # indicates significant difference in TNF release in comparison to 1 µg/ml LPS after phenol re-extraction.

The stimulation of bone marrow cells from mice defective in TLR4 or TLR2 revealed that the re-purified LPS was still TLR4-dependent (figure 5A), but had lost its TLR2-

dependent portion (figure 5B. Like in the human system, the re-purified P. aeruginosa LPS was less potent in the murine cells.

Determination of the endotoxic activity of *P. aeruginosa* and *S. abortus equi* LPS in the LAL showed that 1.7 ng *P. aeruginosa* LPS and 2 ng of the repurified *P. aeruginosa* LPS sufficed to achieve the same activity as 1 ng LPS from *S. abortus equi* or the LPS from *E. coli* O111, which served as reference material in the assay.

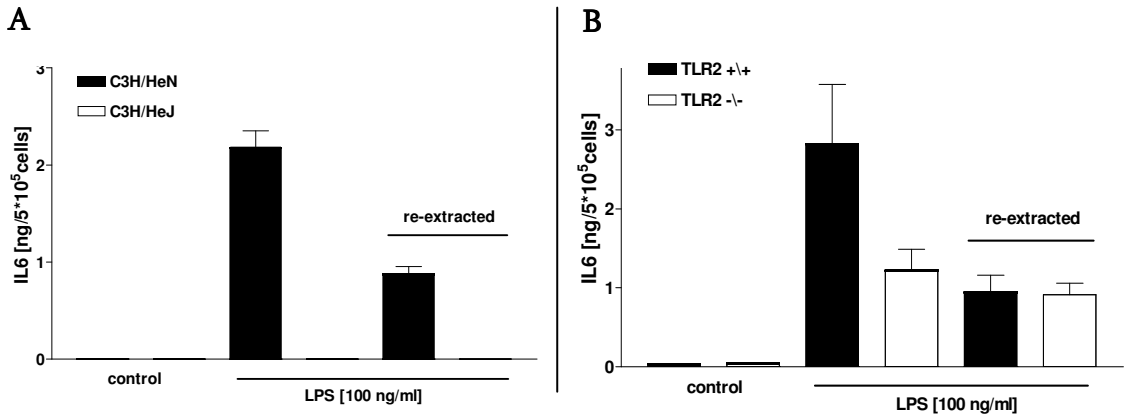

Figure 5 TLR-dependence of P. aeruginosa LPS before and after phenol re-extraction (A) 5x 105 bone marrow cells from C3H/HeN and C3H/HeJ and (B) TLR2+\+ and TLR2-\- were incubated with LPS from P. aeruginosa before and after phenol re-extraction in the concentrations indicated for 24h. IL-6 was determined in the cell-free supernatants by ELISA. Data are means ± SEM, n=4.

5.5 Discussion

Most of our knowledge on the immune stimulatory potency of endotoxins stems from studies performed with LPS from enterobacteriaceae. Only during the last years, functional differences of LPS from different bacterial species became evident, which are related to variations in LPS architecture [82, 83, 150]. It became evident that the

endotoxic activity of a given LPS also crucially depends on the cellular system used as read-out, meaning that cytokine induction in human cells can be qualitatively different from murine cells and must not be related to the activation of the Limulus coagulation cascade [83]. We have investigated the immune stimulatory potency and TLR-dependence of eleven LPS from different bacterial species. The capacity of the LPS to induce the release of a variety of pro- and anti-inflammatory cytokines from primary leukocytes was assessed in human whole blood incubations, which closely reflect the physiological situation in humans in vivo [154]. Surprisingly, we found that the immune stimulatory potential and the induced cytokine profiles of almost all LPS, although derived from different bacterial species, were remarkably similar. Except for the LPS from *P. aeruginosa* and *V. cholerae*, the minimal LPS concentration to induce significant cytokine release, laid within one log-order for each cytokine ranging from 0.01-1 ng LPS/ml. The immune stimulatory activity of LPS from *P. aeruginosa* and *V. cholerae* was about a thousand-fold weaker and even at these high concentrations not the whole spectrum of cytokines was induced. Both LPS failed to induce IL-10. It should be noted that also for the other, more potent LPS, a higher LPS concentration was necessary to obtain significant IL-10 release, compared to TNF, IL-1β or IL-8, indicating that apparently a stronger stimulation of the monocytes is necessary to trigger IL-10 release. LPS from *R. sphaeroides* in high concentrations of 100 µg/ml induced the release of IL-1 and IL-8, but of no other cytokines.

The different endotoxic potencies of LPS molecules have been attributed to their architecture, which despite a common basic structure, can vary considerably. The highest variability is found in the part distal from the bacterial surface, the O-chain of the LPS, less variability in the core structure, but even the conserved lipid A, the

structure that is recognized by innate immune receptors, shows variations in the acyl chain length, substitution patterns of the phosphates and the nature of fatty acids [83]. For full toxic activity in vivo and in vitro, a lipid A, consisting of a β-1,6-linked D-glucosamine disaccharide bearing two phosphate groups in position 1' and 4', substituted with six fatty acids 12 to 14 carbons in length, is required. Almost all deviations from this structure result in reduced endotoxic activity [77, 80].

The "optimal" lipid A structure described above is common for most enterobacteriaceae and enteropathogenic bacteria [79], i.e. in our study the LPS from *E. coli, S. abortus equi, S. enteritidis, S. typhimurium, S. typhosa, S. flexneri* and *S. marcescens,* as well as the opportunistic pathogen *K. pneumoniae,* which all exerted comparable potency in stimulating the release of various cytokines from whole blood. The LPS from *P. aeruginosa, V. cholerae* and *R. sphaeroides* also posse the di-phosphorylated β-1,6-linked D-glucosamine disaccharide, but differ in the fatty acid chain length and the type of hydroxylated and non hydroxylated fatty acid [79]. The LPS from *P. aeruginosa* contains only five fatty acids that are shorter in length (10 to 12 carbons) [150] and showed lower endotoxic activity in whole blood, which is in line with previous findings [155-157]. The O1 serotype LPS from *V. cholerae* possesses six fatty acids (12 to 16 carbons and traces of C18) [158] and was also a weaker cytokine inducing stimulus. The LPS from *R. sphaeroides* contains only five, partially unsaturated fatty acids [159], and has been shown before to act as a LPS antagonist on the level of ligand receptor interaction [84, 160, 161].

The classical hexa-acyl LPS structure from enterobacteriaceae, which is suggested to have a conical shape, seems to be the optimal structure for TLR4 activation, while the penta-acyl LPS with cylindrical shape, like the LPS from *P. gingivalis*, tends to engage TLR2 [82, 162]. In line with this, all LPS from enterobacteriaceae were TLR4-

dependent, while the LPS from *P. aeruginosa* was both TLR4- and TLR2-dependent. In case of whole, UV-inactivated P. aeruginosa, the cytokine inducing activity depended on both TLR2 and TLR4. Other reports also indicate that *P. aeruginosa* triggers both TLR2 and TLR4, as shown for the activation of airway cells [163, 164] and host defence in mice [165, 166]. Since the TLR2-dependent activity could be separated by phenol re-extraction, this appears due to contaminating lipoproteins rather than being a property of the *P. aeruginosa* LPS itself, which turned out to be TLR4-dependent only. Furthermore, a previous publication has shown, that human TLR4 also discriminates between penta-acylated and hexa-acylated LPS, like it can be isolated from cystic fibrosis patients [167]. Obviously, for weak endotoxin, the immune stimulatory effects of lipoproteins seem to be more pronounced. In this light, studies employing crude *P. aeruginosa* LPS preparations with regard to TLR dependency should be reassessed [157].

P. aeruginosa bacteria are ubiquitously found in the environment. They colonize multiple niches and use many different compounds as energy source [168, 169]. Because of their ability to grow in water at 4°C, they are a major source of contamination in purified water, especially of dialysis solutions or water prepared for injection purposes [170, 171]. They play an important role as opportunistic bacteria for patients with AIDS and for neutropenic patients, for infections of burn wounds, for lung infections in patients with nosocomial pneumonia or cystic fibrosis and acute ulcerative keratitis [172]. So far, many virulence factors of P. aeruginosa like flagella, pili, LPS, proteases, exotoxins and exoenzymes have been shown to contribute to the clinic of infection [173-175]. In the present study we demonstrate that the leukocyte activating capacity of the *P. aeruginosa* bacterium, assessed as its cytokine release pattern, as well as its TLR-dependency, is reflected by its LPS. In vivo, *P. aeruginosa*

infections are characterized by a massive influx of neutrophilic granulocytes [176, 177]. In line with this, we found that whole P. aeruginosa, like their LPS, are potent inducers of IL-8, a chemoattractant for neutrophils. We show that the similar 1000-fold weaker inducing capacity for other cytokines of the *P. aeruginosa* LPS is also seen for the whole bacterium in comparison to E. coli, which fits with previous data for E. coli and *P. aeruginosa* obtained with the cell line MonoMac6 [155]. This observed weak endotoxic activity of *P. aeruginosa* might explain the fact that although P. aeruginosa shows a widespread occurrence in the environment, nearly all clinical cases are associated with immune compromised hosts and infection is rare in healthy people [178, 179]. Others have shown that for several clinical P. aeruginosa isolates, the potency of toxicity in vivo is accompanied by a stronger cytokine release capacity in vitro [180], which supports our hypothesis. Remarkably, the endotoxic potency of the *P. aeruginosa* LPS is strongly overestimated by LAL. While by LAL, highly purified *P. aeruginosa* LPS was half as potent as reference preparations from *S. abortus equi* or *E. coli,* it was a thousand-fold less potent with regard to cytokine release from human whole blood. A 10- to 100-fold weaker pyrogenicity in rabbits compared to other LPS like the ones from *E. coli* or *S. typhimurium* has also been reported [181]. This indicates that cytokine release from human whole blood, in contrast to the LAL, reflects the potency different LPS have in vivo. Given the fact that the vast majority of LAL testing is carried out on water samples, this discrepancy for the foremost water contaminant is of critical importance. This is another example that clearly indicates that the extent of activation of the Limulus cascade cannot be directly correlated with the pyrogenic potential for humans. In this case, the endotoxic properties are overestimated by the LAL, though more often, endotoxic activities, even of synthetic Lipid A analogs, are not detected by LAL [81, 170, 182].

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6

Reduced immunostimulation by *Listeria monocytogenes* grown intracellularly is associated with altered lipoteichoic acid expression

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6.1 Abstract

We investigated whether Listeria monocytogenes, which are grown in an intracellular environment, show differences in their immune stimulatory potential compared to bacteria which replicated outside of host cells and whether this is related

to differences in lipo-teichoic acid (LTA) expression. When human blood leukocytes were stimulated with *L. monocytogenes* that had either been grown in shaking flasks at room temperature (RT) or 37°C or intracellularly in monocytic THP-1 mass cultures, we found that cytokine induction was strongest for *L. monocytogenes* grown at RT and weakest for intracellularly grown bacteria. LTA extraction revealed that surprisingly all Listeria, independently from the type of culture condition, expressed two structurally different LTA. While both LTA consisted of a glycerol phosphate backbone bound via a disaccharide to a diacyl glycerol moiety, one LTA type (LTA2) possessed a second, phosphatidyl diacyl glycerol moiety. While the immune stimulatory potential of LTA1 was comparable to that of other LTA like LTA from *S. aureus* or from *S. pneumoniae*, LTA2 was significantly weaker in inducing cytokine release and even failed to induce complement activation. In line with our observation that intracellularly grown *L. monocytogenes* possess a reduced proinflammatory potential, intracellularly grown bacteria expressed LTA1 and LTA2 in a mass relation of 1.5:1, while *L. monocytogenes* grown cell-free at room temperature expressed a relation of 16:1, indicating that a shift in expression of structural variants of LTA might be an important mechanisms to hide from the innate immune system.

6.2 Introduction

The facultative pathogen *Listeria monocytogenes* is responsible for listeriosis, a severe food-borne disease, mainly affecting immunocompromised individuals, pregnant women and their foetuses or newborns [109, 110]. Its opportunistic intracellular life cycle, and its ability to infect different cell types including monocytes, has made it an interesting model organism to study bacterial adaptation to human host cells [183].

After invasion *L. monocytogenes* is capable of escaping the phagolysosom, replicating in the cytosol, inducing rearrangement of the host cell cytoskeleton and spreading directly from cell to cell. These highly organized actions are mediated via the expression of several virulence genes. The best studied ones are encoded by the virulence regulon LIPI-1, which comprises among others the genes for listeriolysin O (hly), phospholipase C (plcA,B) and actin polymerase A (actA), and which is under direct control of the transcription factor PrfA [109, 113, 183, 184]. PrfA activity mediates the switch from the saprophyte to the pathogen and underlies complex, environment-dependent sensor mechanisms like temperature or carbon sources [109, 185-189]. In vivo *L. monocytogenes* might be exposed to the host immune system in the initial phase of infection as well as during the ongoing infection in case of host cell lysis. It is important to note, that *L. monocytogenes* which induce the initial infection have replicated in an extracellular environment, while *L. monocytogenes* which are set free from infected cells within the host have grown and replicated in an intracellular, host cell-controlled, environment. Whereas numerous publications describe the differences in gene regulation between the extracellular and the intracellular form, structural differences which might influence immune recognition have not been investigated. Major immune stimulatory compounds of Gram-positive bacteria are the cell wall components peptidoglycan, lipoproteins and LTA [74, 185]. In particular LTA is a potent inducer of cytokine release from leukocytes [103, 186] and has also been shown to induce complement activation [98]. Therefore, the aim of our study was to investigate whether *L. monocytogenes* grown in cell-free conditions differ with regard to their immunostimulatory potential from *L. monocytogenes* grown within host cells. Furthermore, we aimed to investigate whether such

differences are related to structural differences in the expression of LTA and whether they are causally related to the expression of PrfA.

6.3 Experimental Procedures

6.3.1 Bacterial strains and cultivation

The *L. monocytogenes* wild type strains ATCC 43251, DSM 12464, EGD and the EGD derived PrfA-mutant strains EGD/Δ*prfA* lacking prfA (referred to as ΔprfA in the following) and PrfA* EGD/*ΔprfA/pERL3/PrfA** constitutively expressing prfA (referred to as PrfA*; EGD wild type and mutants were a kind gift from Prof. A. Goebel (Biocenter (Microbiology), University of Würzburg, Würzburg, Germany) were grown shaking at 150 rpm in brain-heart-infusion media (BHI, BD Biosciences, Pharmingen, Heidelberg, Germany) at 37°C or RT under aerobic conditions. Bacteria were harvested during exponential growth or at the beginning of the stationary phase and washed with PBS.

6.3.2 Intracellular culture of Listeria monocytogenes

The human monocytic celline THP-1 (clone 238, T. Jungi, Berlin, Charite, Germany) was cultured in 1000 ml-bioreactors (celline classic 1000, Integra Biosciences, Bern, Switzerland) in RPMI 1640 (12-702F/U1, Lonza, Verviers, Belgium) at 37°C and 5% CO_2. For reaching the maximal amount of viable cells, the media in the cell compartment was supplemented with 20% FCS. The cells were removed for washing in RPMI 1640 and the media was changed every 4-7 days. A final cultivation step of 10 days completed the cycle. At the density of 2-3x 10^7 THP cells/ml, that is 3-4.5x 10^8 in total, *L. monocytogenes* strain ATCC 43251, harvested from the exponential growth phase, were added at a MOI of 10. After one hour, non-invasive extracellular

bacteria were removed by washing. Gentamicin (100 µg/ml) was added to prevent extracellular proliferation. After 15-18h of incubation, the cells were washed in PBS. The host cells were lysed hypotonically by resuspending in aqua dest. for 1 min. Subsequently, the bacteria were purified from cell debris and cytosolic components by discontinuous gradient centrifugation with layers of 60%, 30% and 20% of iodixanol (Optiprep, Sigma, Daisenhofen, Germany) spinning for 1h at 4°C and 19.500 rpm in a Sorvall® RC28S, SS34 rotor (DuPont, Bad Homburg, Germany). The Listeria were washed in PBS and directly transferred to further experiments..

6.3.3 LTA extraction

LTA was purified from *S. aureus* (DSM 20233), *S. pneumoniae* (R6), *L. monocytogenes* (DSM 12464, ATCC 43251, EGD, ΔprfA and PrfA*) grown under cell-free conditions at RT or 37°C or grown intracellularly in THP-1 cells by butanol/water extraction as described previously (Morath, 2001 #1382). The LTA containing fractions after hydrophobic interaction chromatography (HIC) were identified by phosphate measurement performing a phosphomolybdenum-blue assay. Endotoxin contaminations of more than 100 pg/mg LTA were routinely excluded employing the Limulus Amoebocyte Lysate Assay (Charles River Laboratories Sulzfeld, Germany).

6.3.4 GC/MS analysis of fatty acid residues

1 mg of LTA was dissolved in 0.6 ml methanol, 1 M HCl, covered with 1 ml n-heptane and incubated in glass tubes at 85°C. After 7h, the reaction mixture was shaken and the organic phase was dried. The methyl-esters of the fatty acids gained by acidic transesterification of the LTA were redissolved in n-heptane and 1.5 µl were

injected into a GC/MS (6890 Series GC-System/5973 Mass Selective Detector, Hewlett Packard, Böblingen, Germany). For normalisation, the C15 carboxylic acid methyl-ester (Merck, Darmstadt, Germany) was used as internal standard. The samples were vaporized during a gradient from 50°C to 280°C with a heating rate of 2°C/min. The peaks of the resulting chromatogram were quantified relatively and the MS scans of the peaks were matched with the NBS75K library (Hewlett Packard). The GC spectra with the MS data base matches can be viewed as supplementary data.

6.3.5 NMR spectroscopy

LTA from ATCC 43251 and DSM 12464 were analyzed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy as described for other LTA All spectra were recorded on Bruker DRX500 (500 MHz) and AVANCE 600 (600 MHz) spectrometers at 300 K using 5 mm BBI probe heads and can be viewed as supplementary data. In brief, the LTA were dissolved in D_2O with sodium 3-trimethylsilyl-3,3,2,2-tetradeuteropropanoate (TSP-d_4) added as internal chemical shift reference for ^{1}H NMR (δ_H 0.00 ppm), and acetone for ^{13}C (δ_H 30.02 ppm), respectively. For ^{31}P NMR 2% phosphoric acid was taken as external standard (δ_P 0.00 ppm). The amount of LTA in each 0.6 ml sample ranged between 4 and 11 mg. Homonuclear assignments were based on two-dimensional double-quantum-filtered correlation spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY) and rotational nuclear Overhauser effect spectroscopy (ROESY) experiments using presaturation for water suppression. TOCSY and ROESY experiments were performed in the phase-sensitive mode using mixing times of 100 ms in TOCSY and 200 ms spinlock for ROESY, respectively. ^{13}C chemical shift assignments were obtained from gradient-enhanced HSQC spectra. Data acquisition and processing were carried out using standard Bruker software. The

average number of repeating units in the poly(glycerophosphate) backbone and the percentage of substitution was determined by integration of the corresponding peak volumes in the ^{1}H NMR.

6.3.6 Whole blood incubation

Human whole blood incubations were performed as described previously [187]. The human whole blood was diluted five-fold with RPMI and stimulated over night with in house prepared LTA from *S.aureus, S. pneumoniae*, or LTA1 or LTA2 from *L. monocytogenes* or whole *L. monocytogenes* that were either alive or inactivated by UV radiation. The cell-free supernatants were stored at -80°C until cytokine measurement by ELISA.

6.3.7 Cytokine ELISA

Cytokines were measured by in-house sandwich-ELISA using commercially available antibody pairs and recombinant standards. Monoclonal antibody pairs against human TNF, IL-8 and IFNγ were purchased from Endogen (Perbio Science, Bonn, Germany), against human IL-1β and IL-6 from R&D (Wiesbaden, Germany) and against human IL-10 from BD Biosciences (Pharmingen, Heidelberg, Germany). Recombinant standards for TNF and IL-1β were kind gifts from S. Poole (NIBSC, Herts, UK), rIL-8 from PeproTech (Tebu, Frankfurt, Germany), rIFNγ from Boehringer Ingelheim (Biberach, Germany), rIL-10 and rIL-6 from BD Biosciences. The secondary biotinylated antibodies were detected by horseradish-peroxidase-conjugated streptavidin (Biosource, Camarillo, CA, USA) and TMB (3,3',5,5'-tetramethylbenzidine, 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.

6.3.8 Complement activation

L-Ficolin binding and C4 cleavage were measured as described by Lynch et al. [98]. Briefly, for both assays LTA was immobilised over night at 4°C on flat-bottom ultrasorbant 96-well plates in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Residual binding sites were blocked using Tris-buffered saline (TBS, pH 7.4), 0.1% (w/v) human serum albumine (HSA, Aventis Behring, Marburg, Germany).

For measurement of L-Ficolin binding, human serum samples from healthy volunteers (recruited at the University of Konstanz, Germany) were diluted in TBS, 10 mM $CaCl_2$, 0.05% (v/v) Triton-X100, 0.1% (w/v) HSA, pH 7.4 and incubated over night at 4°C. Then, 1 µg/ml of the human L-ficolin specific monoclonal antibody GN5 (Hycult biotechnology, Sanbio, Beutelsbach, Germany) was added and incubated for 90 min at RT. A 1:10000 dilution of the polyclonal goat-anti-rabbit peroxidase coupled detection antibody (GARPOX, DIANOVA, Hamburg, Germany) was added and incubated for 90 min at RT. TMB was used as substrate and was measured as described above.

For measurement of C4 cleavage, human serum samples from healthy volunteers (recruited at the University of Konstanz, Germany) were diluted in 20 mM Tris, 1M NaCl, 10 mM $CaCl_2$, 0.05% (v/v) Triton-X-100, 0.1% (w/v) HSA, pH 7.4 and incubated over night at 4°C. Then, human C4 purified from human serum of healthy volunteers as described previously [188] was diluted in 4 mM barbital, 145 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4, added and incubated for 90 min at 37°C. A 1:1000 dilution of an anti-C4c antibody (Quidel, San Diego, USA) which was biotin conjugated according to standard procedures was added and incubated for 90 min at

RT. Washing between the incubation steps was carried out with TBS, 0.05% Tween20, 5 mM $CaCl_2$. After incubation with horseradish-peroxidase-conjugated streptavidin for 30 min at RT, TMB was used as substrate and was measured as described above.

6.3.9 PCR

DNA was prepared from 1x 10^9 *L. monocytogenes* using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany). cDNA was reversely transcribed using the Superscript III Reverse Transcriptase (Invitrogen) from RNA prepared with the TRIzol LS Reagent (Invitrogen). Quantitative real-time (qRT) PCR was carried out for the analysis of hly, prfA and rrsA with a LightCycler rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany) using LightCycler FastStart DNA Master SYBR Green (Roche). Specificity of the product was checked by melting-point analysis and agarose gel electrophoresis. For the quantification of transcripts, a standard curve of genomic DNA was created for all products. Methodical errors were corrected via normalisation by cDNA from rrsA (16S rRNA; [189]). All primers were purchased from Thermo Hybaid, Ulm, Germany: hly (LLO; [190]): sense: 5´-CAT GGC ACC ACC AGC ATC T-3´, anti-sense: 5´-ATC CGC GTG TTT CTT TTC GA-3´; prfA (PrfA; [191]): sense: 5'-GAT ACA GAA ACA TCG GTT GGC-3'; anti-sense: 5'-GTG TAA TCT TGA TGC CAT CAG T-3'; rrsA [189]: sense: 5'-TTA GCT AGT TGG TAG GGT-3'; anti-sense: 5'-AAT CCG GAC AAC GCT TGC-3'.

6.3.10 Statistics

Statistical analysis was performed using the GraphPad Prism Software (San Diego, USA). Data are shown as means ± standard error of the mean (SEM). For the

comparison of two groups, the paired two-tailed t-test and for more than two groups the repeated measures one-way-Anova followed by Bonferroni-post tests was applied. A p-value <0.05 was considered significant.

6.4 RESULTS

6.4.1 Immunostimulatory potential of *L. monocytogenes* harvested from different growth conditions

To compare the immune stimulatory potential of *L. monocytogenes* grown in an extracellular environment to that of *L. monocytogenes* grown in an intracellular environment, Listeria were either grown aerobically cell-free in shaking flasks at RT or 37° degrees or intracellularly in THP-1 monocytic cells. If the bacteria were cultured in shaking flasks, they were harvested from the beginning stationary growth phase. For intracellular cultivation, mass cultures were established using the THP-1 monocytic cell line grown in bioreactors. The bioreactors allowed the monocytic cells to achieve a density of 3x 10^7 cells per ml with cluster formation. These conditions afforded an efficient bacterial replication with cell to cell spreading of the bacteria. Extracellular survival of the bacteria was inhibited by the use of antibiotics. Replication was stopped after 15-18h, before lysis of the host cells would start and bacteria were purified from host cell debris. By that time, bacterial numbers had increased by a factor of 10-100 (data not shown). To investigate and compare the immune stimulatory potential of the whole bacteria, the bacteria were UV-inactivated and used for in vitro stimulation of human blood leukocytes followed by subsequent measurement of cytokine release by ELISA.

As shown in figure 1, we found that the induction of IL-6 by intracellularly grown bacteria was significantly weaker than by *L. monocytogenes* grown at 37°C or RT.

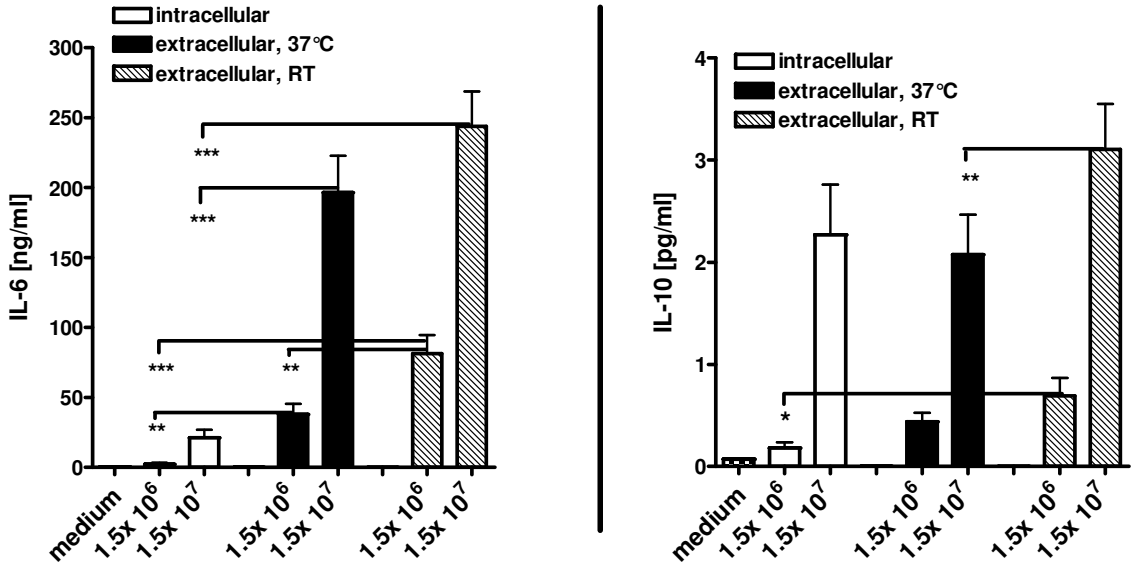

Figure 1 Intracellular cultivation of L. monozytogenes results in reduced cytokine release Whole blood was incubated for 24h with intracellular grown *L. monocytogenes* or cell-free grown *L. monocytogenes* cultivated at RT or 37°C at the concentrations indicated (all were Listeria strain ATCC 43251). Release of cytokines was measured by ELISA. Data are means ± SEM, n=8. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

This decrease in the induction of cytokines held also true for IL-1β, TNF and IL-8 (data not shown). In case of the anti-inflammatory IL-10, the differences between the culture conditions were less pronounced. Control experiments confirmed that the inflammatory potential of Listeria grown in shaking flasks was not altered if the bacteria were harvested from the exponential phase. Furthermore, by the use of extracellularly grown bacteria, it was excluded that the purification of intracellularly grown Listeria using iodixanol affects cytokine induction (data not shown).

6.4.2 LTA extraction from *L. monocytogenes* and structural analysis

Since LTA represents an important immune stimulatory component of Gram-positive bacteria, we aimed to extract LTA from *L. monocytogenes* grown in shaking flasks at

RT, 37°C or intracellular in THP-1 mass cultures in order to see, whether structural changes might be associated with the different immunostimulatory capacities of the bacteria. LTA was first prepared from Listeria grown in shaking flasks at 37°C. After butanol/water extraction of the bacteria, the extract was separated by HIC and the LTA containing fractions were determined by measurement of their phosphate content. According to the phosphate profile (figure 2A), LTA was extracted in two distinct peaks. Peak 1, comprising the fractions 44-50, corresponds to the phosphate peak that is usually observed after LTA extraction from Gram-positive bacteria like for example for LTA from *S. aureus* or *S. pneumoniae* [74, 100], whereas the second LTA peak, fractions 53-64, had not been observed for other bacterial species before. To determine the structural differences, both LTA peaks were pooled separately (named LTA1 (fractions 44-50) and LTA2 (fractions 53-64)) and subjected to NMR and MS analysis.

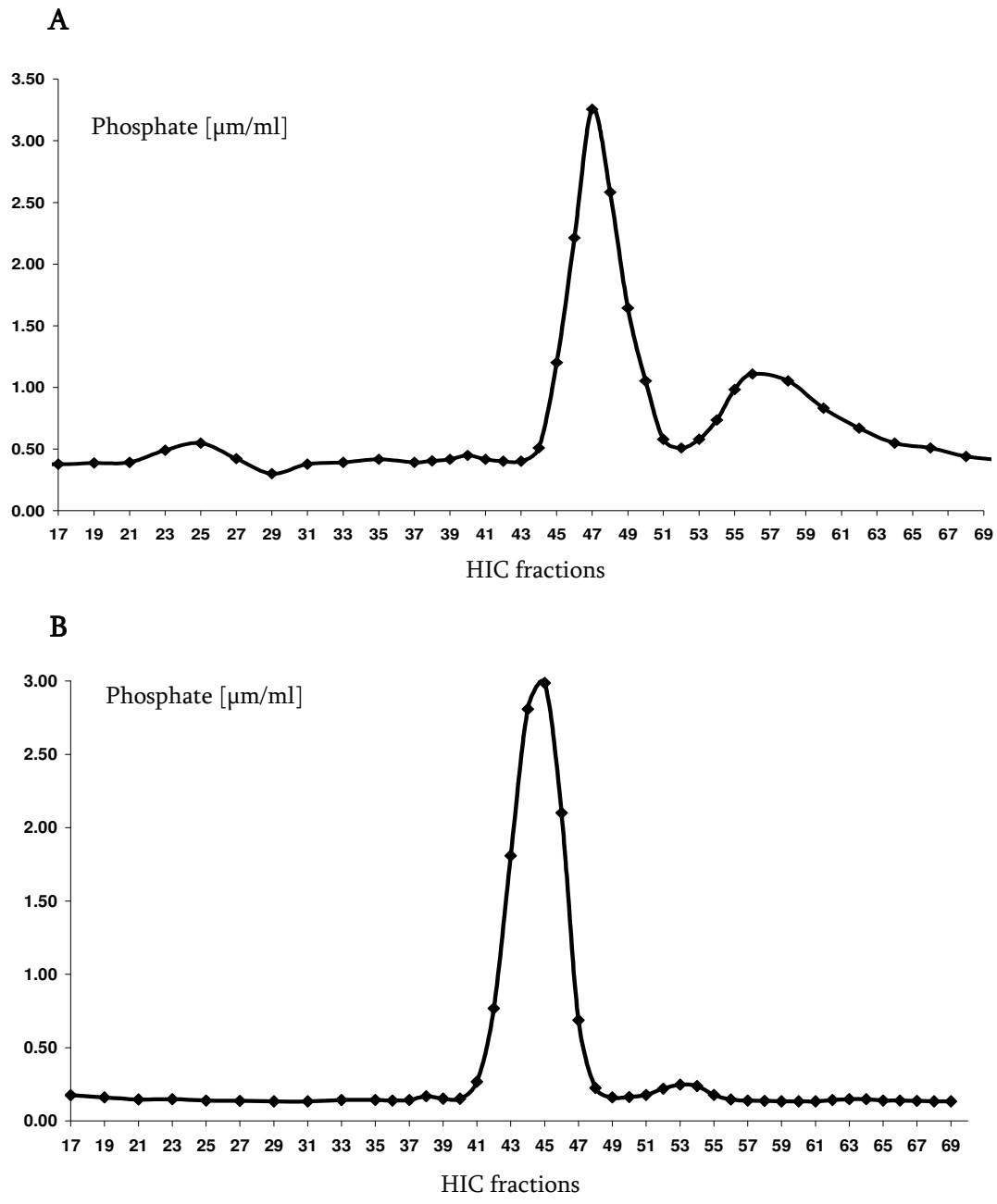

Figure 2 LTA preparation from L. monozytogenes results in two distinct LTA peaks LTA was prepared from *L. monocytogenes* (ATCC 43251) cultivated under cell-free conditions at 37°C (A) or RT (B) by butanol/water extraction. The water phase was separated via HIC and the phosphate content of the fractions was determined by phosphomolybdenum-blue assay. Both profiles are representatives for a minimum of three extractions each.

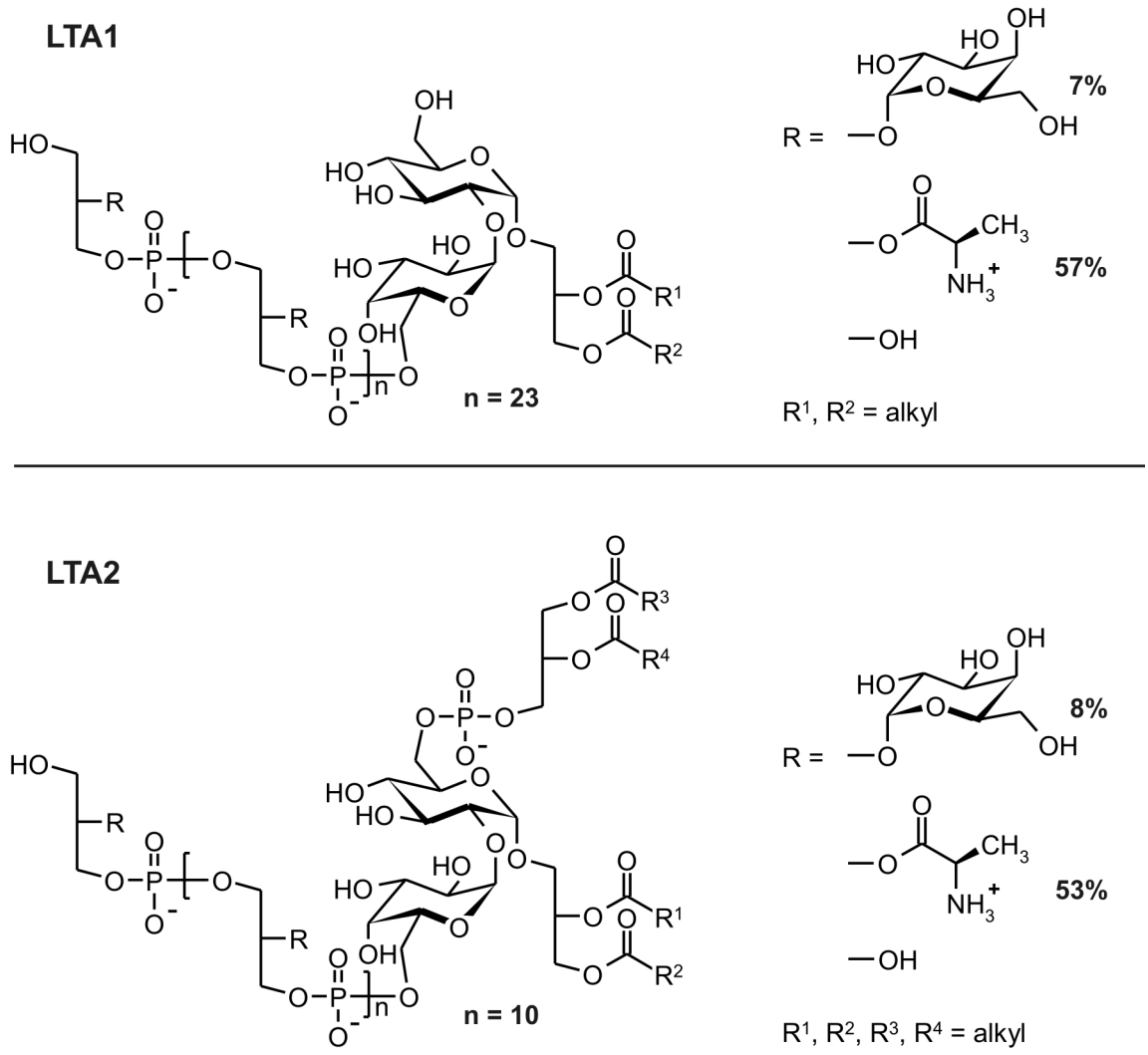

Figure 3 Structure and substitution pattern of the LTA isolated from *L. monocytogenes* strain ATCC 43251 The percentage of alanylation, galactosylation and the average (av) number of the repeating units was determined from the ^{1}H NMR spectrum.

For LTA1, a poly-glycerophosphate chain with an average number of n=23 units was calculated. About 7% were substituted with α–galactose and 57% with D-alanine. The membrane anchor was composed of a 3(1)-(2´-O-α-D-galactopyranosyl)-α–D-glucopyranosyl]-1(3),2-diacylglycerol. In the case of LTA2, approximately 10

glycerophosphate units were substituted with 8% α–galactose and 53% D-alanine. The membrane anchor showed an uncommon second diacyl glycerol moiety: 3(1)[6´-phosphatidyl-2´-O-(α-D-galactopyranosyl)-α–D-glucopyranosyl]-1(3),2-diacylglycerol. The acyl chains could be characterized as saturated and showed linear, iso- and antiso- branched methyl groups. Similar results were also obtained for LTA prepared from a second L. monocytogenes strain (DSM 12464, data not shown). To determine the differences in the structure of the acyl chains of the lipid anchors of LTA1 and LTA2 in greater detail, GC/MS analysis of the carboxylic acid-methyl esters gained by acidic transesterification of LTA1 and LTA2 was performed. In both cases, acyl chain lengths of 14, 16 and 18 were detected. The C18 acyl chains were unbranched, whereas the C16 acyl chains were partly branched with a methyl group on position 14, and the C14 chains carried a methyl group on either position 12 or 9. Taken together it turned out that in LTA1 the major portion of acyl chains (≅ 80%) consists of methylated C14 and C16 chains, while in LTA2 the amount of branched and unbranched acyl chains was comparable.

LTA1 %	carboxylic-methyl ester	LTA2 %
9.42	C18	28.87
6.99	C16	24.02
32.24	C16 ME (14)	17.09
39.51	C14 ME (12)	22.86
11.84	C14 ME (9)	7.16

Table 1 Carboxylic acids of LTA1 and LTA2
The fatty acids of LTA1 and LTA2 underwent acetous transesterification and the resulting carbocylic-methyl esters were analysed via GC/MS. The chain lengths are given and the position of natural methyl groups (ME) is indicated. For the relative quantification of the products in each sample, the signals of three experiments were first set into relation to the internal standard (C15 methyl-ester) and then the portion of every product was calculated and given as percent of total carboxylic-methyl esters.

6.4.3 Influence of different growth conditions on expression levels of LTA1 and LTA2

Influence of different growth conditions on expression levels of LTA1 and LTA2

So far, we had only examined the LTA expression of *L. monocytogenes* grown cell-free at 37°C. To investigate whether the different culture conditions have an influence on the expression of LTA1 and LTA2, LTA was prepared from *L. monocytogenes* grown cell-free at RT as well as from *L. monocytogenes* grown intracellularly in monocytic THP-1 cells. Like for Listeria grown cell-free at 37°C, both other culture conditions resulted in the extraction of the two distinct LTA fractions LTA1 and LTA2. However, although both LTA peaks were detectable, there were significant variations with regard to the overall relation of LTA1 to LTA2, as shown for LTA prepared from Listeria grown at 37°C in comparison to RT (figure 2A and 2B). Since for LTA extraction from intracellularly grown Listeria the amount of starting material was 1000-fold less compared to Listeria grown under cell-free conditions, the phosphate content was below the detection limit of the assay. Therefore, the LTA containing fractions were identified by induction of cytokine release from whole blood. Again, two distinct peaks in the same fractions were observed. The total amount of LTA1 and LTA2 was calculated by determination of the respective dry weight of the pooled fractions. As shown figure 4, the ratio of LTA1:LTA2 as determined on the weight basis was about ten times higher for Listeria grown at RT compared to Listeria grown at 37°C or intracellularly within host cells. The same results were obtained if the cell-free cultivated bacteria were taken from the exponential growth phase (data not shown).

6.4.4 Association of LTA2 expression with activation of PrfA

It is well known that invasion of host cells leads to the induction of transcription of virulence genes by L. monocytogenes. Since transcription of many virulence genes is under the control of the transcription factor PrfA, it was tempting to speculate that PrfA activation is associated or maybe even causally related to the shift in LTA1 towards LTA2 expression. Therefore, we quantified the transcription and translation of PrfA under the different culture conditions by quantitative real time PCR. Indeed, we found that in comparison to cell-free cultivation at RT, cultivation at 37°C like intracellular cultivation resulted in 780-fold induction of gene transcription (data not shown). In order to see, whether a causal association between PrfA induction and the increase in LTA2 expression exists, we isolated the LTA from the *L. monocytogenes* wild type strain EGD, its mutant strain PrfA* constitutively expressing PrfA and from the prfA-deletion mutant ΔPrfA, which completely lacks PrfA. The temperature dependency of the LTA1 to LTA2 ratio observed in the wild type strain ATCC 43251 was also present in the wild type strain EGD, excluding strain specificity. The LTA1 to LTA2 ratio of the ΔPrfA mutant at 37°C was the same as the one from EGD at 37°C, while the PrfA* mutant at RT was rather comparable to EGD at RT (figure 4). Since we would have expected an enormous increase in expression of LTA2 in case of the PrfA* mutant, these results do not point to a causal role of PrfA for LTA2 expression.

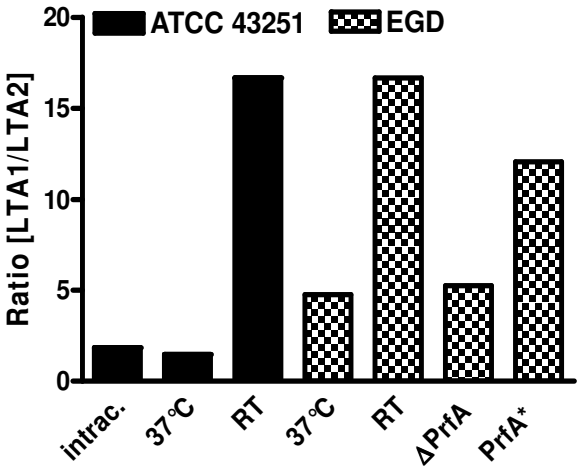

Figure 4 The LTA1 to LTA2 ratio depends on cultivation temperature
LTA was prepared from *L. monocytogenes* cultivated in THP-1 cells or under cell-free conditions at 37°C or RT by butanol/water extraction. The water phase was separated via HIC and the phosphate content of the fractions, determined by phosphor-molybdenum-blue assay, indicated the presence of LTA. The LTA1:LTA2 ratio of the pooled moieties was determined by weight and calculated by the means of at least 2 LTA extractions per culturing.

6.4.5 Immune stimulatory activity of Listeria LTA1 and LTA2

To determine the immune stimulating potential of LTA1 and LTA2 from L. monocytogenes, concentration response curves in whole blood were performed and compared to LTA prepared from *S. aureus* and *S. pneumoniae*. While cytokine release induced by LTA1 was comparable to that induced by LTA from *S. aureus* (figure 5) and *S. pneumoniae* (data not shown), LTA2 showed a shift to the right in its dose response curve by a factor 10 for the induction of the proinflammatory cytokines IL1β, IL-6, and IL-8, while its potency to induce the anti-inflammatory cytokine IL-10 was similar to LTA1. To investigate whether LTA2 possesses inhibitory properties against LTA1, both LTA were combined in different concentrations. However, neither 1 µg nor 10 µg of LTA2 added to 1 µg LTA1 had an inhibitory effect (data not shown). We have described earlier that the lektin pathway of the human complement system is activated by the LTA from *S. aureus* and *S. pneumonia* via binding to L-ficolin, which results in MASP-2 recruitment and activation of the cleavage of C4 [98, 192]. In the next step we compared the capacity of LTA1 and LTA2 from L. monozytogenes to induce complement activation to LTA from *S.*

aureus and *S. pneumoniae*. In the C4 cleavage assay LTA2 failed, even in the presence of high concentrations of human serum, to induce detectable levels of cleaved C4, while LTA1 induced C4 cleavage in a manner comparable to LTA from *S. pneumoniae* (figure 6a) and *S. aureus* (data not shown). This finding was accompanied by a 30fold reduced L-ficolin binding by LTA2 compared to LTA1 (figure 6b).

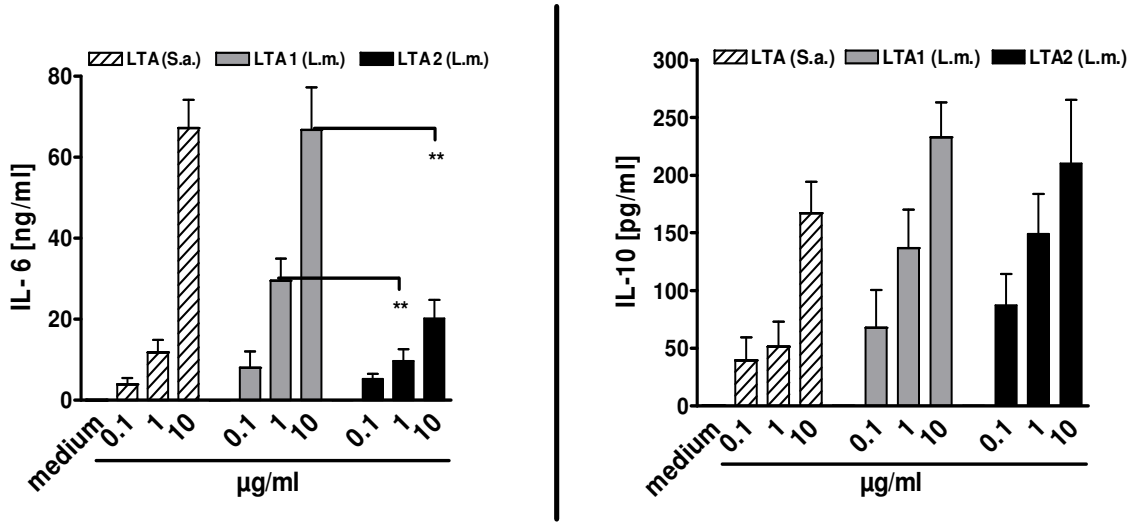

Figure 5 LTA2 possess weaker immune stimulatory potency compared to LTA1 Whole blood was incubated for 24h with LTA from *S. aureus* (ATCC 43251) or with LTA1 or LTA2 from *L. monocytogenes* (ATCC 43251), all cultivated under cell-free conditions at 37°C, at the concentrations indicated. Release of cytokines was measured by ELISA. Data are means + SEM, n=9. ** $p<0.01$

6.5 Discussion

L. monocytogenes possess the ability to infect host cells intracellularly and to quickly spread from infected host cell to other host cells. Systemic listeriosis, which has a lethality rate of 20-30% [109], mainly affects immunocompromised individuals hinting to the fact that the balance of immune system and pathogen is borderline and

competitive advantages such as immune escape might be critical. As an opportunistic intracellular pathogen *L. monocytogenes* can replicate inside and outside of host cells. In the presented work we show that the respective culture conditions seriously affect the immune stimulatory potential of Listeria. Intracellular culture of *L. monocytogenes* leads to a significantly reduced release of proinflammatory cytokines compared to cultivation under cell-free conditions in shaking flasks at RT. These changes in immunogenicity might serve to protect them from the attacks of the host's innate immune system, both inside of host cells and when they are set free from dying host cells

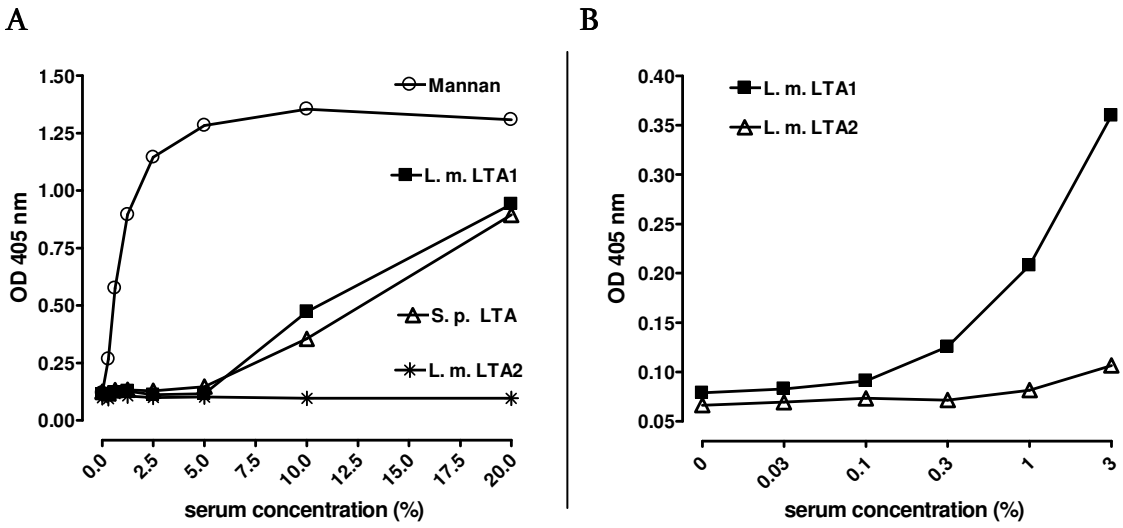

Figure 6 LTA2 fails to induce complement activation A) Plates were coated with mannan, LTA1 or LTA2 from *L. monocytogenes* (ATCC 43251) or LTA from *S. pneumoniae* (R6), all grown at cell-free conditions at 37°C, and incubated with human C4 in the presence of human serum in the concentrations indicated. The cleaving product C4c was quantified by ELISA. B) Plates were coated with LTA1 and LTA2 from *L. monocytogenes* (ATCC 43251) grown at cell-free conditions at 37°C and incubated with human serum in the concentrations indicated. L-ficolin binding was quantified by ELISA. Data from one representative experiment, carried out in duplicates, is presented as mean ± SEM.

Furthermore, we found that *L. monocytogenes* possesses the speciality of expressing two structurally different LTA. LTA in general is a basic component of the Gram-positive cell wall, and its structure is highly conserved and indispensable [193]. Thus, natural occurring LTA deficient mutants have not been detected so far. In case of Staphylococci LTA mutants, which were generated and grown under experimental conditions in the laboratory, strong impairments with regard to colonisation, virulence and cell division were observed [89, 90, 193, 194]. Furthermore, LTA is important for the homeostasis of physicochemical surface properties [92] and confers resistance to antimicrobial cationic molecules [94]. In L. monocytogenes, LTA in particular is reported as a scaffold for the virulence protein internalin B (InlB), which mediates invasion of host cells [95, 96]. LTA is also a potent stimulus for the innate immune system, inducing the expression of a variety of inflammatory molecules [74, 99] and the L-ficolin dependent pathway of complement activation [98].

In *L. monocytogenes*, the two structurally different LTA also differ with regard to their immunostimulatory potential, with LTA2 being significantly less potent in inducing proinflammatory activities than LTA1. Noteworthy, all incubations were performed on the basis of the weight, regardless of the molecular masses of the LTA. Considering the average molar mass of LTA1 (approximately 5310 g/mol) to be more than 1.5 times bigger than the one of LTA2 (approximately 3460 g/mol), the differences in cytokine induction would be even more pronounced with an equimolar manner. In line with this, culture conditions that produce Listeria with reduced immune stimulatory potential are associated with an increased expression of LTA2. It is most likely that the differences in immune stimulatory properties of the two LTA originate from the differences in their structure. LTA2 is more hydrophobic than LTA1. This was already indicated by the phosphate profile, which was determined

from the aquatic fractions after separation via hydrophobic interaction chromatography. Here, LTA2 eluted several fractions later than LTA1. The determination of the LTA's structure by NMR showed that this increased hydrophobicity of LTA2 was due to a hydrophilic backbone that was shorter in length compared to LTA1 and to the presence of a second phosphate-bound lipid anchor that was missing in LTA1. The building blocks of the sugar backbone were identical between LTA1 and LTA2 bearing the same substituents, and the same disaccharide moiety. The expression of two LTA with different structure in Listeria has also been described by Uchikawa et al. in 1986 [195], who have extracted LTA from the Listeria strains NCTC 5214, F4, 93/65 and 1383 by hot phenol extraction. However, in later years it turned out that the butanol/water extraction performed at room temperature is gentler and generates biologically active material, while phenol extraction results in partly decomposed less active or even inactive material [74, 103]. However, at least the structural analysis of our LTAs is in line with the previous report of Uchikawa et al. [195]. So far, we have prepared and investigated LTA from several different Gram-positive bacteria including *S. aureus* [75, 99], *S. pneumoniae*, [100], *B. subilits* [97] or *Lactobacilli species* [101], and observed no significant differences with regard to immune stimulation, although variations in structure existed, especially of the hydrophilic chains [196]. LTA1 from Listeria possessed comparable immune stimulatory potential like LTA from *S. aureus* or *S. pneumoniae*, but LTA2 was significant weaker in induction of proinflammatory cytokines and even failed to induce L-ficolin-dependent complement activation. Since the second diacylglycerol moiety of the lipid anchor of LTA2 is so far unique, it can be assumed that it is responsible for the reduced immune stimulatory properties. Since LTA2 does not antagonize immune stimulation by LTA1, it is most likely that sterical factors of

the three-dimensional structure or the increased hydrophobicity of LTA2 might prevent binding to immune receptors.

The increase of LTA2 expression is induced under conditions like raise of culture temperature to 37°C, which in part reflects intracellular conditions, and stronger by intracellular culture itself. This poses the question, what kind of benefit Listeria have from this shift and furthermore, how it is induced. It is cogitable, that the modifications in LTA expression might be a consequence of the switch from the saprophytic to the infectious state of Listeria. Since it is well known that intracellular infection of host cells activates a whole machinery of virulence factors, mostly under the control of the transcription factor PrfA [113] it was tempting to speculate that LTA2 expression is not only in parallel with PrfA expression but also causally related. However, the use of Listeria mutants that either constitutively express PrfA (EGD/pERL3/prfA*), or which were deleted for PrfA (EGD/ΔprfA) clearly argued against a causal relationship. The expression of PrfA of both mutants was controlled by PCR and Western blotting, in order to confirm the functionality of the mutations. The PrfA* mutant, which shows strong PrfA expression under any culture condition and therefore reflects the intracellular state of Listeria, expressed even less LTA2 in relation to LTA1 than the ΔPrfA mutant which expresses no PrfA at all. The later one rather reflects the non-virulent culture conditions and was therefore expected to express no or only small amounts of LTA2.

Taken together, these findings indicate that culture conditions that increase the virulence of Listeria also affect the structure of their LTA. However, this appears to occur independently from the transcription of the primary virulence genes, and more likely represents an adaptation to the intracellular environment - probably due to changes in the activity of enzymes that are responsible for the homeostasis of the

bacterial cell surface. Unfortunately, the pathways of LTA synthesis are not clarified sufficiently to understand the pathways responsible for implementing an alternative structure. It is hypothesised that the phosphatidyl-galactopyranosyl-glucopyranosyl-diacylglycerol is synthesized in the cytosol involving a putative *S. aureus*-YpfP-homologue [197] and transported to the periplasm where the glycerol phosphate backbone polymerizes involving a putative *S. aureus*-LtaS homologue [193]. The fast incorporation of the lipid anchor into the membrane due to the presence of four fatty acid residues in case of LTA2 instead of only two in case of LTA1 could explain the shorter backbone. It makes sense that changes of the environmental conditions, like extra- or intracellular growth or increase in temperature also affect the demands of the composition of the bacterial cell wall. Here, the presence of a second diacylglycerol anchor will drastically influence the fluidity of the cell wall. It is unclear so far, whether the increased LTA2 expression is beneficial for Listeria because it represents an adequate adaptation to the intracellular environment, which for example results in faster replication or movement of the Listeria, or whether it protects from the innate immune. Supporting the latter hypothesis, we showed that even with high concentrations of human serum, LTA2 is much less capable of inducing the L-ficolin dependent turnover of C4 and at least one important consequence will be the avoidance of the C3 mediated phagocytosis by activated macrophages, witch is essential for efficient killing of Listeria [198]. Furthermore, we surprisingly found the different culture conditions to result in changes of proinflammatory cytokine release by whole Listeria, while IL-10 release was less affected. A similar picture was obtained for LTA2, which induces less proinflammatory cytokines, but IL-10 induction was comparable to LTA1, indicating that increased LTA2 expression might render Listeria less immunstimulatory also in

terms of cytokine induction. As possible consequences one could assume the reduced levels of proinflammatory mediators together with the for LTA typical weak induction of IFNγ to impede a potent natural killer cell (NKC) activity which has been reported to be critical for resistance against *L. monocytogenes* in mice. [199, 200]. Furthermore, an increased susceptibility of mice with deficiencies in INFγ, TNF, IL-6 or IL-1β has been shown in respective knock-out models [199, 201-203]. Thus, the cytokine pattern induced by virulent Listeria and LTA2, involving the concerted suppression of these proinflammatory mediators together with the prevalence of IL-10, might account for the insufficient bactericidal activity in macrophages from the individuals at risk. In this context, a reduced activity by $T_{Helper}1$-cells is also cogitable and would underline the importance of the $CD8^+$ mediated clearing of *L. monocytogenes* but has to be investigated [204, 205]. However, if the relation of LTA1 to LTA2 expression is compared, this is similar for Listeria cultured intracellularly or cell-free at 37°C degrees, while the release or proinflammatory cytokines is less pronounced for bacteria cultured cell-free at 37°C, but significantly stronger reduced for intracellularly grown Listeria, indicating that also other factors must exist which beside increased expression of LTA2, further reduce the immune stimulatory potential of intracellularly grown Listeria.

For Listeria, this is the first report of an inducible structural modification of an essential, highly conserved cell wall component, which is associated with a benefit for immune evasion. Avoiding the strong activation of innate immune responses in case of intracellular infection of the host could be a crucial prerequisite for the establishment of a systemic infection, which can only be confined by a T-lymphocyte mediated response. This constitutes a novel adaptive immune evasion mechanism involving the inducible formation of alternative PAMPs.

6.5 Acknowledgements

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7 Summarizing Discussion

The course and outcome of an infection is determined by the nature and virulence of the pathogen, the status of the host's immune system, the host's genetic and epigenetic basis as well as the response on the treatment applied. The better we understand and control theses relations, the more adapted and successful will be the choices of prevention and therapeutic treatments.

The TLR4 polymorphism Asp(299)Gly

Of outstanding clinical relevance in this context are Gram-negative infections causing lethal septic shock or multi organ failure. The course of disease is hardly predictable and depends on the patients susceptibility and on the effectiveness of his immune reactions [24, 64, 206]. These observations might be attributed to variances of genes involved in immune defence. The LPS receptor TLR4 represents one candidate of a polymorphic gene. *In vitro* transfection experiments revealed that the Asp(299)Gly polymorphism renders the receptor non functional [64]. Our own study regarding LPS binding and LPS-induced cytokine induction in primary human blood leukocytes from individuals with the TLR4 polymorphism belongs to the few experimental studies that investigate the mechanisms which might explain the association between the TLR4 polymorphism and increased susceptibilities towards infections or chronical inflammatory disorders. Initially, in LPS inhalation experiments the Asp(299)Gly polymorphism showed a blunted bronchoconstrictive response for some subjects with the Asp(299)Gly polymorphism [64], other investigations showed a higher prevalence of the Asp(299)Gly mutation in Gram-negative sepsis and a more severe course of

disease [24, 207]. These first reports prompted several further studies, unfortunately with mostly controversial outcomes. Noteworthy, monitoring a huge collective for five years, the risk of developing carotid sclerosis was found attenuated in heterozygous individuals carrying the Asp(299)Gly polymorphism and proinflammatory serum proteins were reduced [208]. Taken together, these data suggest that the Asp(299)Gly TLR4 polymorphism might alter the initiation of inflammatory responses. Until now, only two functional studies are available, which report no influence of the TLR4 Asp(299)Gly polymorphism on inflammatory responses induced by Gram-negative stimuli [118, 119]. However, in one study from our laboratory, a decrease of LPS-induced IL-10 release from whole blood was reported [119]. In this study, more than twenty endpoints had been investigated in blood from 160 donors; therefore the physiological relevance of the statistic significance remained to be confirmed. Our follow-up study aimed to investigate the role of the Asp(299)Gly TLR4 polymorphism for LPS-inducible IL-10 release in greater detail and to clarify possible molecular mechanisms. One crucial finding was, in a new study collective, the confirmation of the decreased LPS- induced IL-10 release while TNF was not affected. IL-10 expression was found to be diminished in whole blood and in isolated monocytes already on the mRNA level. To elucidate this IL-10 specific effect, we investigated several key parameters known to be relevant for IL-10 induction including p38 [124], as well as the role of the MyD88 independent TRIF/IRF pathway [126], and the induction of cyclooxygenase-2 and PGE_2 [127]. We observed no difference between homozygous wild-type and heterozygous polymorphic subjects in any of these experiments (unpublished data). For TNF induction it is believed that LPS binding to the TLR4 receptor complex alone is sufficient to induce TNF [128], though this has not been investigated for IL-10 so far.

One might speculate that IL-10 induction requires further processes like internalization of the LPS/receptor complex and intracellular processing. Preliminary results obtained with LPS coated to surfaces support this hypothesis but were not finally conclusive. However, our results clearly indicate that IL-10 induction in general requires stronger LPS stimulation of monocytes than TNF induction and that IL-10 release is more susceptible to inhibition of LPS by a neutralizing agent. This can explain, why the polymorphism specifically affects IL-10 expression, but the mechanistic key components remain to be clarified, by which the mutation of a receptor, whose activation initiates a variety of downstream events, ends up with the remarkably exclusive impact on one cytokine only. Taken together, the influence of the mutation surprisingly implies a proinflammatory phenotype, contrasting the majority of the references mentioned above concluding an increased susceptibility towards infections and arguing with impaired proinflammatory responsiveness. In contrast, particularly the associations with severe outcomes of sepsis might be defined much better by a reduced anti-inflammatory response, which means to reconsider therapeutic strategies. Importantly, as a risk factor for excessive inflammation, the TLR4 Asp(299)Gly polymorphism has also been convincingly linked with inflammatory bowel disease and ulcerative colitis [66], in which IL-10 reduction is known to play a decisive role [31]. Therefore, it was of major interest to investigate LPS-inducible IL-10 levels in patients with Crohn's disease carrying the Asp(299)Gly polymorphism, since this disorder is discussed to be a consequence of a dysbalance of proinflammatory and regulatory cytokines [36, 37]. We designed a study in order to investigate whether Crohn's disease patients with TLR4 Asp(299)Gly polymorphism show reduced LPS-inducible IL-10 release compared to patients with TLR4 wildtype genotype. Although the polymorphism was confirmed to be associated with Crohn's

disease by our study, the phenotype of reduced LPS-induced IL-10 release was surprisingly not visible in the patients with a long-years history of this disorder. Also when the data were evaluated excluding subjects with stronger immune-modulating medication, no significant bias of the results was observed; neither was with a gender specific evaluation. We cannot exclude that the non-pathophysiological inter-individual variations of cytokine release, which we also observed in our groups of healthy individuals, account for that, but planning the study we estimated the collective to be of sufficient size. It has to be noted, that according to clinical standard criteria, the pathophysiology of Crohn's disease has a multifactorial and polygenic background. The TLR4 Asp(299)Gly polymorphism constitutes only one effector and an impact of the polymorphism possibly requires the presence of other factors like NOD2 polymorphisms (Hume IBD 08). Furthermore, this was only a snap-shot taken from blood leukocytes of patients which had suffered from the disease for years. It does not exclude that deviations in responses to LPS might exist which lead to significantly reduced IL-10 levels directly at the inflamed mucosal tissue of patients with Asp(299)Gly polymorphism. Furthermore, the impact of the polymorphism could be much more pronounced during the early development of the mucosal immune system, thus increasing the susceptibility rather than the outcome of Crohn's disease. Examining the local IL-10 concentration at the early onset of disease would be most advisable. If it turns out that patients with the Asp(299)Gly polymorphism are characterized by locally reduced IL-10 levels, these patients might be the ideal target group that would benefit from locally administered IL-10 therapy.

Non-classical bacterial immune-stimuli

Not only variations in immune receptors and signalling create an individual immune response, also the bacterial derived triggers account for the variety of pathogen-host

interactions. Even a species-specific course of infection may comprise variable features depending on the nature of the pathogen. The toxicity of Gram- negative bacteria is defined by their LPS and knowledge about structural variations of the lipid A moiety is steadily increasing [76, 79]. Investigating the immune-stimulatory potential of eleven LPS in whole blood, we found that the resulting cytokine profiles of almost all LPS, although derived from different bacterial species, were remarkably similar and seem to be a common feature of enterobacteriacea. Except for the LPS from the non-enterobacteria *P. aeruginosa* and *V. cholerae*, the minimal LPS concentration to induce significant cytokine release, laid within one log-order for each cytokine ranging from 0.01-1 ng LPS/ml. The immune stimulatory activity of LPS from *P. aeruginosa* and *V. cholerae* was about a thousand-fold weaker and even at these high concentrations not the whole spectrum of cytokines was induced. Both LPS failed to induce IL-10, a finding fitting very well to the observation in the first part of the theses that a stronger stimulation of the monocytes is necessary to trigger IL-10 release. The reason for this decreased cytokine induction clearly can be found in the structure of the lipid A. In contrast to the sterical properties of LPS from enterobacteriacea, allowing an optimal receptor-ligand binding, the LPS from *P. aeruginosa,* and *V. cholerae* differ in the fatty acid chain length and the type of hydroxylated and nonhydroxylated fatty acids [79]. The LPS from *P. aeruginosa* contains only five fatty acids that are shorter in length (10 to 12 carbons) [150]. In our study, the *P. aeruginosa* LPS showed a TLR2 dependency in line with a previous report [157]. However, we could separate the TLR2-dependent lipoprotein fraction by phenol re-extraction. Obviously, for weak endotoxins, the immune stimulatory effects of contaminating agents are much more pronounced. In this light, studies employing crude *P. aeruginosa* LPS preparations with regard to TLR dependency

should be reassessed. Because of *P. aeruginosa*'s ubiquitous occurrence and ability to grow in water at 4°C, they are a major source of contamination in purified water, especially of dialysis solutions or water prepared for injection purposes [170, 171]. They play an important role as opportunistic bacteria for patients with AIDS and for neutropenic patients, for infections of burn wounds, for lung infections in patients with nosocomial pneumonia or cystic fibrosis and acute ulcerative keratitis [172]. This observed weak endotoxic activity of *P. aeruginosa* might explain the fact that although *P. aeruginosa* shows a widespread occurrence in the environment, nearly all clinical cases are associated with immune compromised hosts and infection is rare in healthy people [178, 179]. Remarkably, while by LAL highly purified *P. aeruginosa* LPS was a thousand-fold less potent with regard to cytokine release from human whole blood, it was only half as potent as reference preparations from *S. abortus equi* or *E. coli*. A 10- to 100-fold weaker pyrogenicity in rabbits compared to other LPS like the ones from *E. coli* or *S. typhimurium* has also been reported [181]. Given the fact that the vast majority of LAL testing is carried out on water samples, this discrepancy for the foremost water contaminant is of critical importance. This is just another example that clearly indicates that the extent of activation of the Limulus cascade cannot be directly correlated with the pyrogenic potential for humans. This finding, where the endotoxic properties are overestimated by the LAL, contrasts the more frequent cases where endotoxic activities, even of synthetic Lipid A analogs, are not detected by LAL [81, 170, 182].

One kind of Gram- positive bacteria with a unique opportunistic intracellular infection cycle and immune evasion strategy is *L. monocytogenes* - the cause of food-borne listeriosis which has a fatality rate of up to 30%, particularly among infants, children and the elderly. The most frequent effects are meningitis and miscarriage or

meningitis of the foetus or newborn. Although their incidence is relatively low, their severe and sometimes fatal health consequences make them among the most serious food-borne infections [109, 110]. Despite numerous publications about the regulation of virulence and immune responses towards *L. monocytogenes*, aspects of immune recognition by PRRs as well as structural differences between saprophytic (extracellular) and pathogenic (mostly intracellular) Listeria have hardly been examined. We show that 1.) Intracellular culture of *L. monocytogenes* leads to a significantly induced release of proinflammatory cytokines compared to cultivation under cell-free conditions in shaking flasks at room temperature. 2.) Temperature alone is capable of affecting the immune stimulatory potential of Listeria. 3.) Listeria express two variants of LTA and the decrease in the immune stimulatory potential is associated with a ten-fold increase of LTA2 expression. 4.) LTA2 is a much weaker inducer of cytokines and C4 turnover compared to LTA1. These changes in immunogenicity might serve to protect *L. monocytogenes* from the attacks of the host's innate immune system, for instance if they are set free from dying host cells. Additionally, the reduction of listericidal responses inside of activated macrophages could be of great relevance.

At the moment, it is speculative whether evolution has driven the expression of LTA2 for the benefit of immune evasion. Alternatively, this could be a secondary effect of a structural modification, which in first line, meets the requirements of cell division and cell wall homeostasis in the chemically and physically different environment of human host cell compartments. Since this alternative structure is much less expressed at low temperature and so far unique for the virulent *L. monocytogenes,* we may assume that for a saprophytic bacterium such modifications of LTA structure are of disadvantage, if constitutively expressed. A promising candidate for activating the

switch in the LTA expression pattern was PrfA. It is the most prominent regulatory virulence factor in Listeria responsible for the regulation of a variety of genes involved in infection, intracellular growth and spread, also affecting cell wall components. Furthermore, analogous to LTA2 expression, its transcription is to a large degree temperature-dependent [113]. However, our findings with *prfA*-mutant Listeria strains denied any correlation. Since the syntheses pathways of listerial LTAs are fairly understood, it's difficult to identify the events leading to modifications. Noteworthy, another virulence factor reported recently, VirR, controls the modification of cell surface components and regulates the *dlt* operon which is involved in alanilation of LTA [209]. Although there is no difference in the degree of alanilation between LTA1 and LTA2, VirR would be the most obvious candidate to search further influences on LTA expression. Unfortunately, most intracellular pathogens with more or less comparable infection characteristics are not belonging to the Gram- positive bacteria; therefore, alternative study objects to investigate modulations of LTA expression are lacking. The adaptiveness uniquely displayed by the only human pathogenic Listeria strain is amazing, regarding the elaborately accomplished switch from the saprophytic to the pathogenic phenotype. Nevertheless, a single procedure modifying the indispensable cell wall molecule LTA seems to join the benefits of efficient intracellular growth and immune evasion.

This thesis considers some important factors determining the complexity of infectious disease. It shows that on host-side it is important to focus the investigations not only on genetic polymorphisms but to perform more global studies, while on the pathogen-side, small variations of PAMPs already have dramatic consequences. This, together with the increasing knowledge that is gained today about disease

susceptibilities and treatment responses, to establish an overall, fundamental understanding of the mechanisms involved in virulence and immunity.

8 Summary

The courses of infections, ranging from subclinical to lethal diseases, are determined by numerous variables of the host defence and of the nature of the pathogen. Genetic polymorphisms, concerning both regulatory promoter and coding sequences, are increasingly linked with disease susceptibility and outcome. The first part of the study was attributed to a variant of one of the human pattern recognition receptors, which constitute key elements for the initiation of an innate immune response. The heterozygous TLR4 Asp(299)Gly polymorphism has been reported to be associated with inflammatory disorders, which were assumed to be a consequence of reduced proinflammatory responses due to the polymorphism. Unfortunately, cell-based assays are lacking in all studies. Our study, which aimed to prove deviations of inflammatory responses, revealed that after LPS stimulation of blood leukocytes only the expression of the anti-inflammatory cytokine IL-10 was reduced on mRNA and protein level, resulting in a proinflammatory phenotype. We could exclude that this effect is due to differences in the kinetics of IL-10 release, in the expression of total surface TLR4 or in LPS-binding to monocytes between subjects heterozygous for the Asp(299)Gly polymorphism or homozygous carriers of the wildtype allele. Furthermore, we could show that IL-10 induction in general requires stronger LPS-triggering than TNF and is more sensitive to LPS inhibitors. The lower number of responsive, wildtype TLR4 receptors on monocytes of heterozygotes may explain why only IL-10 expression is affected.

IL-10 dysfunction can result in excessive inflammation as shown for Crohn's disease, which is characterized by chronical or relapsing mucosal inflammation. We have

investigated by incubations of human whole blood whether in Crohn's disease patients LPS-inducible TNF or IL-10 release is influenced by a heterozygous TLR4 polymorphism compared to patients with a homozygous TLR4 wildtype phenotype. Genotyping the patients and healthy controls for the *tlr4* gene, we could confirm the prevalence of the Asp(299)Gly polymorphism in the patients collective. Surprisingly, neither TNF nor IL-10 release was significantly different between both patient groups, and was furthermore comparable to cytokine release levels of healthy individuals. This indicates that probably at this stage of disease deviations in cytokine release occur only at the inflamed mucosa and cannot be detected by stimulations of leukocytes taken from the peripheral blood.

The focus of the second part of the study was the structural analysis of bacterial cell wall molecules, which are crucial for the initiation of immune defence and which also determine the extent of inflammatory reactions. Hereby we have examined non-classical cell wall molecules with endotoxic properties. We compared the immune stimulatory potency and TLR-dependency of eleven different LPS. While the majority of LPS induced cytokine release equipotentially, a thousand-fold more LPS from *Pseudomonas aeruginosa* or *Vibrio cholerae* was still less potent, though it potently induced ng quantities IL-8. All LPS tested, regardless of the microorganism, showed toll-like receptor (TLR)4-dependence, except for the LPS from *P. aeruginosa* and *V. cholerae,* which were both TLR4 and TLR2 dependent. However, re-purification of the commercial LPS preparation by phenol re-extraction led to a complete loss of the TLR2 dependency, indicating contaminations with lipoproteins. The Limulus Amebocyte Lysate Assay, often performed to exclude contaminations in purified water likely to originate from *P. aeruginosa,* resulted in an overestimation of pyrogenic burden of *P. aeruginosa* LPS by a factor 500 in the sample when compared

with the biological activity of highly purified *P. aeruginosa* LPS in human whole blood.

Another aspect was *Listeria monocytogenes*, a food-borne, opportunistic intracellular pathogen. When human blood leukocytes were stimulated with *L. monocytogenes* that had either been grown in shaking flasks at room temperature (RT) or 37°C or intracellular in monocytic mass cultures, cytokine induction was strongest for *L. monocytogenes* grown at RT and weakest for intracellular grown bacteria. LTA extraction revealed that surprisingly all Listeria, independently from the type of culture condition, expressed two structurally different LTA. While the immune stimulatory potential of LTA1 was comparable to that of other LTA, LTA2 was significantly weaker in inducing cytokine release and even failed to induce complement activation. In line with our observation that intracellular grown *L. monocytogenes* possess a reduced proinflammatory potential, intracellular grown bacteria expressed LTA1 and LTA2 in a mass relation of 1.5:1, while Listeria grown cell-free at room temperature express a relation of 16:1, indicating that a shift in expression of structural variants of LTA might be an important mechanism to hide from the innate immune system.

This thesis contributes to understand the consequences of molecular variations for host-pathogen interaction and immune defence. Especially the variations in LTA structure during intracellular adaptation of Listeria offer new targets for bactericidal therapies.

9 Zusammenfassung

Der Verlauf einer Infektion, mit einer Bandbreite von subklinisch bis hin zu letal, wird durch unzählige Variablen der Wirtszell-Verteidigung und durch den Charakter des Krankheitserregers bestimmt. Genetische Polymorphismen, sowohl regulatorische Promotor- als auch kodierende Abschnitte betreffend, werden immer mehr mit Krankheits-Anfälligkeit und –Entwicklung assoziiert. Der erste Teil der Studie beschäftigte sich mit einer Variante eines der humanen Mustererkennungs-Rezeptoren, welche als Schlüsselelemente für die Initiierung einer angeborenen Immunantwort gelten. Der heterozygote TLR4 Asp(299)Gly Polymorphismus ist mit entzündlichen Funktionsstörungen in Verbindung gebracht worden, von denen man annahm, sie seien eine Konsequenz verminderter Entzündungsreaktionen. Allerdings wurde keine Studie durch zellbasierende Experimente unterstützt. Unsere Studie, die Abweichungen in der Immunantwort nachweisen wollte, zeigte, dass nach LPS-Stimulation von Blutleukozyten ausschließlich die Expression des anti-entzündlichen Zytokins IL-10 reduziert war, und zwar auf mRNA- und Proteinbasis. Dieser Umstand weist klar einen pro-inflammatorischen Phänotyp des Polymorphismus auf. Wir konnten ausschließen, dass der Effekt Unterschieden in der Kinetik der IL-10-Ausschüttung, der Gesamtexpression von TLR4 oder der LPS-Bindekapazität von Monozyten zwischen heterozygot Polymorphen und homozygoten Trägern des Wildtyp-Allels zugrunde liegt. Außerdem konnten wir zeigen, dass IL-10-Induktion generell einer stärkeren LPS-Stimulation bedarf als TNF und sensibler gegenüber LPS-Inhibitoren reagiert. Die geringere Anzahl an responsiven Wildtyp-Rezeptoren

auf Monozyten Heterozygoter könnte eine Erklärung darstellen, warum ausschließlich IL-10 betroffen ist.

IL-10 Dysfunktionen können zu exzessiver Entzündung führen, wie es für Morbus Crohn bekannt ist. Morbus Crohn ist durch chronische oder rezidive Entzündungen der Mukosa charakterisiert. Wir untersuchten mittels Vollblutinkubationen, ob in Morbus Crohn-Patienten die LPS-induzierte TNF- oder IL-10- Freisetzung durch einen heterozygoten TLR4 Polymorphismus im Vergleich mit Patienten des homozygoten Wildtyp-Phenotyps beeinflusst wird. Die Genotypisierung des *tlr4*-Gens der Patienten sowie gesunder Kontrollen konnte die Prävalenz des Polymorphismus im Patientenkollektiv bestätigen. Erstaunlicherweise war aber weder die TNF-, noch die IL-10- Ausschüttung signifikant unterschiedlich zwischen beiden Patientengruppen. Zusätzlich war sie vergleichbar der Zytokinmengen gesunder Probanden. Dieser Umstand weist wahrscheinlich darauf hin, dass in diesem Krankheitsstadium Abweichungen in der Zytokinausschüttung nur in der entzündeten Mukosa auftreten und nicht durch Stimulation von peripheren Blutleukozyten gemessen werden können.

Im Fokus des zweiten Abschnitts der Studie standen Untersuchungen „exotischer" bakterieller Zellwandmoleküle mit endotoxischen Eigenschaften, die die Initiierung und das Ausmaß einer Immunantwort bedeutend beeinflussen können. Wir verglichen die immunstimulatorische Potenz und TLR-Abhängigkeit von elf verschiedenen LPS. Während die Mehrheit gleichermaßen Zytokine induzierte, war selbst die tausendfache Menge an *Pseudomonas aeruginosa* oder *Vibrio cholerae* LPS weniger potent, wenn auch größere Quantitäten an IL-8 induziert wurden. Alle LPS zeigten sich, unabhängig von der mikrobiellen Herkunft, TLR4-abhängig mit Ausnahme der LPS von *P. aeruginosa* und *V. cholerae*, welche sowohl TLR4- als auch

TLR2- Abhängigkeit aufzeigten. Die Aufreinigung der kommerziellen LPS-Präparationen mittels Phenol-Extraktion führte jedoch zu einem vollständigen Verlust der TLR2-Abhängigkeit, was Verunreinigungen mit Lipoproteinen indiziert. Der Limulus Amöbozyten Assay wird häufig zur Kontrolle gereinigten Wassers angewendet um Verunreinigungen, meist durch *P. aeruginosa* hervorgerufen, auszuschließen, Allerdings resultierte er in diesem Fall in einer Überschätzung der pyrogenen Belastung durch *P. aeruginosa* LPS um den Faktor 500, verglichen mit der biologischen Aktivität in Vollblut.

Ein weiterer Blickpunkt war *Listeria monozytogenes*, ein durch Nahrungsmittel übertragener, opportunistisch-intrazellulärer Krankheitserreger. Wenn humane Blutleukozyten mit *L. monozytogenes*, die entweder im Schüttelkolben bei Raumtemperatur (RT) oder 37°C oder intrazellulär in einer Monozyten-Massenkultur vermehrt worden waren, stimuliert wurden, war die Zytokininduktion für bei RT gewachsene *L. monozytogenes* am stärksten und am schwächsten für intrazellulär gewachsene. Die Isolation von LTA enthüllte erstaunlicherweise, dass *L. monozytogenes,* unabhängig der Anzuchtbedingungen, zwei strukturell verschiedene LTA exprimierten. Während die immunstimulatorische Wirkung von LTA1 vergleichbar mit anderen LTA war, war die Zytokinausschüttung durch LTA2 deutlich geringer und eine Komplementaktivierung blieb ganz aus. Parallel dazu exprimierten intrazellulär gewachsene Listerien LTA1 und LTA2 in einem Massenverhältnis von 1,5:1, während extrazellulär bei RT kultivierte Listerien eine Relation von 16:1 aufwiesen. Diese Verschiebung in der Expression struktureller LTA-Varianten könnte einen wichtigen Mechanismus darstellen, Reaktionen des angeborenen Immunsystems zu vermeiden.

Diese These liefert einen Beitrag zum Verständnis möglicher Folgen für die Wirt-Pathogen-Interaktion und die Immunverteidigung, die mit Variationen auf molekularer Ebene einhergehen können. Vor allem bieten die Modifikationen der LTA-Struktur während der intrazellulären Anpassung von *L. monozytogenes* neue Grundlagen zur Entwicklung bakterizider Therapien.

10 Declaration of author's contributions

- **O. Dehus**, S. Bunk, S. v. Aulock and C. Hermann: IL-10 release requires stronger toll-like receptor 4-triggering than TNF- a possible explanation for the selective effects of heterozygous TLR4 polymorphism Asp(299)Gly on IL-10 release. Immunobiology 2008; *in press*

The experiments were mainly performed by O. Dehus with help from S. Bunk and S. v. Aulock under the supervision of C. Hermann. The study was designed by C. Hermann and O. Dehus. The manuskript was written by O. Dehus.

- **O. Dehus**, G. Rogler, Jochen Hampe, Stefan Schreiber and C. Hermann: LPS-inducible anti-inflammatory responses are not diminished in Crohn's disease patients with heterozygous Asp(299)Gly polymorphism; *submitted to IBD*

The experiments were performed by O. Dehus under the supervision of C. Hermann. Jochen Hampe and Stefan Schreiber carried out the genotyping. The study was designed by C. Hermann, O. Dehus and G. Rogler. The manuscript was written by O. Dehus.

- **O. Dehus**, T. Hartung and C. Hermann: Endotoxin evaluation of eleven lipopolysaccharide by whole blood assay does not always correlate with Limulus Amebocyte Lysate assay. J Endotox Res 2006; 12(3):171-80

The experiments were performed by O. Dehus under the supervision of C. Hermann. The study was designed by C. Hermann and T. Hartung. The manuscript was written by C. Hermann.

- **O. Dehus**, M. Pfitzenmaier, S. Maier, N. Fischer, G.Stübs, W. Schwaeble, S. Morath, T. Hartung, A. Geyer and C. Hermann: Reduced immunostimulation by *Listeria monozytogenes* grown intracellularly is associated with altered lipo-teichoic acid expression; *submitted to JBC*

The experiments were mainly performed by O. Dehus and M. Pfitzenmaier, with minor contributions by S. Maier and C. Draing under the supervision of C. Hermann and A. Geyer. The study was designed by C. Hermann, O. Dehus and A. Geyer. Further contributions were made of G. Stübs, S. Morath, N. Fischer and T. Hartung. The manuskript was written by O. Dehus.

Konstanz, 25th of April 2008

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