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Comparative characterization of bacterial immune stimuli

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Abbreviations

A	anhydro
Ab	antibody
act D	actinomycin D
ALT	alanine aminotransferase
ANOVA	analysis of variance
Bn	benzyl
Bp	base pair
CARD	caspase recruiting domain
CD	cluster of differentiation
CpG-ODN	oligodeoxynucleotide containing cytosine-guanosine dinucleotides
D	amino acid in D configuration
DAP	diaminopimelic acid, Dpm
DMSO	dimethyl sulfoxide
Dpm	diaminopimelic acid, DAP
E	glutamate
EC	effective concentration
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
GAPDH	glyceraldehyd-3-phosphate dehydrogenase
GlcNAc	N-acetylglucosamine
GpC-ODN	oligodeoxynucleotide containing guanosine-cytosine dinucleotides
i	iso
IC	inhibitory concentration
IFN	interferon
IL	interleukin
K	lysine
L	amino acid in L configuration
LAL	Limulus amebocyte lysate
LALF	LPS-specific binding protein

LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LRR	leucine-rich repeats
LTA	lipoteichoic acid
M	N-acetylmuramic acid
M(ADiQ)	muramyl dipeptide, MDP
MALDI-TOF-MS	matrix assisted laser desportion ionization-time of flight mass spectrometry
MAPK	mitogen-activated protein kinase
m-DAP	*meso*-diaminopimelic acid
MDP	muramyl dipeptide
Me	methyl
MHC-II	major histocompatibility complex-II
MHz	megahertz
MurNAc	N-acetlymuramic acid
MyD88	myeloid differentiation factor 88
NBoc	*N*-*t*-butyloxycarbonyl
NBS	nucleotide binding site
NK	natural killer cell
NMR	nuclear magnetic resonance
NOD	nucleotide-binding oligomerization domain
ODN	oligodeoxynucleotides
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PGN	peptidoglycan
PO	phosphodiester
PolyB	Polymyxin B
PPG	polypropylene glycol
PRR	pathogen recognition receptor
PTO	phosphorothioate
Q	glutamine
s	soluble

SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TA	teichoic acid
TDS	*t*-hexyldimethylsilyl
T_H1	T-helper cell 1
TIR	toll/interleukin-1 receptor domain
TLR	toll-like receptor
TMB	3,3`,5,5`-tetramethylbenzidine
TMSE	2-(trimethylsilyl)ethylester
TNF	tumour necrosis factor
Z	benzyloxycarbonyl

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1 Introduction

1.1 Innate and adaptive immunity

The mammalian immune system consists of two closely interacting systems, the innate and the adaptive immunity, which struggle against infectious diseases. The innate immune system is the first line of defence answering quickly to exposure to pathogens by production of immunostimulatory cytokines, chemokines and acute phase proteins. The major players of the innate immunity are the white blood cells with phagocytic capacity, e.g. monocytes, macrophages, dendritic cells and neutrophils. These cells recognize the invading pathogens through pathogen-associated molecular patterns (PAMPs), that are expressed by a diverse group of infectious pathogens (1). The recognition of the PAMPs through the host is mediated by different receptors, the pathogen recognition receptors (PRR) (2). Most of them are expressed on immune cells, while some soluble forms are located in the blood plasma. After recognition, the innate immune system is able to prevent the rapid proliferation and spreading of the infectious mircoorganisms and additionally activate the adaptive immunity to react directly against invading pathogens. This antigen-specific immunity answers with the production of high-affinity antibodies and the generation of cytotoxic T-cells providing long-lasting protection.

1.2 Pathogen associated molecular patterns

PAMPs are highly conserved sets of molecular structures and are a heterogeneous group of molecules, but they are not produced by the mammalian body and therefore serve as bacterial signatures and danger signal for the host. PAMPs include microbial structures, such as parts of the bacterial cell wall, e.g. lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PGN), the breakdown products of the PGN called muropeptides as well as bacterial DNA.

1.2.1 Composition of the bacterial cell wall

The bacteria can be divided into two groups: Gram-negative bacteria and Gram-positive bacteria, because of their different staining properties, resulting from different architecture of their cell wall. Gram-negative bacteria possess a cytoplasma membrane and a thin PGN-layer, to which the outer membrane is attached via lipoproteins. In the outer membrane LPS is anchored with his hydrophobic part in the phospholipid layer. Gram-positive bacteria lack an outer membrane, but have instead a thick PGN-layer, which comprises 40-50 sheets to build up a big sacculus. Through this sacculus protrude the LTA, which is anchored in the cytoplasmic membrane as well as teichoic acid (TA) bound to the PGN. In the cytoplasmic compartment the bacterial DNA is located.

1.2.2 Lipopolysaccaride

LPS consist of a lipid A anchor, a polysaccharide core and chains of repetitive carbohydrates and with this lipid A anchor LPS is attached to the outer membrane. The repeating oligosaccharides are strain-specific for diverse Gram-negative species and are highly variable. In contrast, the glycolipid anchor is highly conserved, but variations in the amount of fatty acids occur between species, mainly between four to six fatty acids (3, 4). Resulting from this, different tree-dimensional structures occur: hexaacyl asymmetrical lipid A shows a conical conformation, pentaacyl lipid A has a intermediate form and tetraacyl symmetrical lipid A has a cylindrical shape (5, 6). LPS is released from destroyed bacteria or is captured by the immune system, resulting in host-responses like production and release of cytokines and chemokines as well as complement activation (7).

1.2.3 Lipoteichoic acid

About 6 % of the cell wall of Gram-positive bacteria consist of LTA, which protrude through the murein sacculus to the surface (8). Teichoic acid which has in contrast to LTA no lipid anchor, represent also a great part of the cell wall

but do not have cytokine inducing capacity (9). LTA is anchored in the cytoplasmic membrane and consist of a lipophilic anchor, which is linked via a disaccharide to the backbone. The backbone is build up of glycerol-phosphate or ribitol-phosphate units, which are repeated up to 50 times. The residues of the backbone are substituted with different groups, e.g. N-acetylglucosamine (GlcNAc), hydroxy-groups or D-alanine, in the case of *Staphylococcus aureus* (*S. aureus*) (10). Therefore the amphiphilic glycolipid is negatively charged. The physiological function of LTA is not clear so far. It has been suggested, that binding of metal ions and regulation of the activity of autolytic enzymes could play a role (11-13). The role of LTA in regard to the immune system is manifold, e.g. induction of transcription factors and therefore production and secretion of cytokines and chemokines (14-17). But most of the LTA-knowledge is controversial, because commercially LTA preparation are heavily contaminated with endotoxin, nucleic acids or lipoproteins (18, 19). Additionally, the commonly used hot phenol extraction method lead to loss of bioactivity, especially for LTA of *S. aureus* (19). A butanol extraction method yields a highly pure (> 99% purity, < 30 pg LPS per mg LTA) and cytokine inducing material, no loss of glycerophosphate units, D-alanine or GlcNAc occur (10, 20). Additionally, chemical synthesis of LTA derivatives revealed structure-function relationship of cytokine induction (16). The lipid anchor is able to stimulate the immune system alone and is therefore an important conserved immunostimulatory principle (16). Nevertheless, increased cytokine-production can be observed, when the backbone is linked to the anchor, indicating that recognition requires additional structural components. In addition, backbone substitution is necessary for pronounced immune stimulation (16). The recognition of LTA by the immune system is mainly based on macrophages and the receptors which are involved in recognition of LTA are CD14 and partially LPS-binding protein (LBP) (15, 21, 22). Controversial results have been reported, that either toll-like receptor 4 (TLR4) or TLR2 are involved in LTA signalling. First reports have shown, that LTA signalling is TLR4 dependent (14, 23, 24), but meanwhile several groups have verified that recognition of LTA is TLR2 dependent (21, 25-27). These controversial results are probable due to LPS contaminations found in commercial LTA preparations (19). However, with highly pure LTA it has been shown, that LTA is clearly TLR2-dependent (21). Additionally, synthetic LTA has

been shown to be TLR2-dependent (16). Furthermore, TLR2 forms heterodimers with TLR1 or TLR6 (28, 29). These findings led to the hypothesis, that cross-linking of immunostimulatory molecules, which mimic more closely the situation in the bacteria, is of major importance. The physiological situation in the Gram-positive bacteria is, that the LTA protrudes through the murein sacculus and leads to the suggestion, that two LTA molecules which cross-link the receptor, are necessary for entire immune stimulation. In case of LTA, cross-linking with an LTA-antibody have shown increased cytokine release (30). Nevertheless, it is still controversially discussed, whether LTA is the main immunostimulatory principle of Gram-positive bacteria or whether PGN alone or in combination with LTA elicits the effects of Gram-positive infection (31).

1.2.4 Peptidoglycan and muropeptides

The bacterial cell walls of Gram-negative and Gram-positive bacteria contain PGN. Gram-negative bacteria possess only a small layer while Gram-positive bacteria have a 40 up to 50 layer thick sacculus (32, 33). This PGN consists of sugar chains, which are linked by amino acids. These sugar chains consist of alternating units of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). The cross-linking amino acids are alternating L- and D-isomers, additionally building interpeptide bridges (34). The amino acid sequence is species-dependent, but can be distinguished in a lys-type and a DAP-type PGN. Lys-type- and DAP-type-PGN contain lysine and *meso*-diaminopimelic acid (DAP), respectively (34). The minimal structure of muropeptides, that are PGN breakdown products, is MurNAc with one amino acid, usually L-alanine. Muropeptides are released through lytic enzymes, produced from either the host or bacteria, during bacterial growth or due to antibiotic administration (35, 36). Due to the diverse amino acids occurring in the PGN of different species, a variety of muropeptides are possible. The most prominent muropeptide is muramyl dipeptide (MDP, M(ADiQ)), which represents the minimal structure for adjuvant activity (37).

1.2.5 Bacterial DNA and CpG-oligodeoxynucleotides

Bacterial DNA has a higher frequency of unmethylated CpG dinucleotides than mammalian DNA. Therefore, the host can discriminate between own and invading bacterial DNA and can answer to this danger signal. After the discovery, that bacterial DNA is immunostimulatory, synthetic oligodeoxy-nucleotides (ODN) have been produced to investigate the immune stimulatory potency of different sequences (38, 39). One difference between natural DNA and the synthetic CpG-oligodeoxynucleotides (CpG-ODN) is the backbone modification. Instead of the phosphodiester (PO) backbone of natural DNA, the synthetic CpG-ODN usually have a phosphorothioate (PTO)-modified backbone, which protects them against nuclease degradation (40, 41). Also in length there are differences, i.e. the synthetic CpG-ODN are normally between 18 and 24 base pairs long. Additionally, mixed CpG-ODN have been synthesized, where phosphodiester and phosphorothioate sequences occur. These CpG-ODN are called D-type ODN and contain phosphorothioate G-rich sequences at their end and the CpG-motif is flanked by a palindromic phosphodiester sequence. D-type ODN have a greater ability to activate plasmacytoid dendritic cells (pDC) and to induce IFNα-production, but induce only less IL-12 (42, 43). The K-type bears only a phosphorothioate backbone with one or more CpG dinucleotides (43, 44). As control ODN GpC-ODN have been synthesized, where the CpG motif is inverted to a GpC motif. In murine cells this holds true, i.e. only CpG-ODN lead to stimulation of the immune system. However, there are species-specific differences and, additionally, nonoptimal PTO-modified ODN lead to a TLR9 dependent activation of p38 MAPK (mitogen-activated protein kinase), despite mouse CpG motifs for human cells and vice versa are used (45). CpG-ODN can activate monocytes, macrophages and dendritic cells as well as B-cells, resulting in cytokine production and secretion, upregulation of MHC-II and subsequent activation of natural killer (NK) cells (46, 47). While only pDC (48, 49) and B-cells (50) express TLR9 at a high level, myeloid dendritic cells, NK cells, T-cells and macrophages express TLR9 only at low level (49). Despite, CpG-ODN are potent inducers of T_H1-responses (51). Cytokines, which direct the immune system to cellular immunity, so called T_H1 responses include TNFα and IL-12

as well as IFNγ, IL-2 and IL-13, produced by monocytes or lymphocytes, respectively. IL-10, IL-4 and IL-5 are so called T_H2 cytokines and direct the immune system to humoral immunity.

1.3 Pattern recognition receptors

The conserved structures of the bacterial cell wall, the PAMPs are recognized by the immune system via the PRR. These PRR play an important role to alert the immune system when microbial invaders have entered the host. Some PRR are available in the blood serum like lipopolysaccharide binding protein (LBP) or soluble CD14 (sCD14), while most of them are expressed on the surface of immune cells, e.g. the scavenger receptors. Very important for the recognition of LPS, LTA and PGN are the toll-like receptors (TLR). Additionally, the NOD (nucleotide-binding oligomerization domain) proteins have been discovered recently, which play a role in intracellular recognition of muropeptides.

1.3.1 Toll-like receptors

To date, 13 mammalien toll-like receptors (TLR) have been identified, 10 in humans and 12 in mice (52-55). The TLR are type-1 transmembrane proteins with an extracellular leucin-rich repeat domain and a highly conserved intracellular toll/interleukin-1 receptor domain (TIR-domain). Some of them are located intracellular like TLR9 (56), but most of them are expressed on the cell surface. Mainly, they are expressed by antigen-presenting cells like monocytes, macrophages, dendritic cells as well as neutrophils (49). Each TLR recognizes a set of conserved molecular structures. The most renown TLR receptor is TLR4, which participates in LPS signalling (57). TLR2 forms heterodimers with TLR1 or TLR6 and can thereby discriminate between diacyl- and triacyl-lipoproteins like LTA and lipoproteins (28, 29). Additionally, TLR2 was described to recognize PGN (58). A further ligand is unmethylated bacterial DNA as well as CpG-oligodeoxynucleotides (CpG-ODN), which are recognized via TLR9 (38, 56).

1.3.2 NOD receptors

The NOD receptors are subfamily members of the CATERPILLAR family of proteins. Recently, two proteins, NOD1 (also called CARD4) and NOD2 (also called CARD15), have been shown to play a role in innate immunity (59, 60). They are build up of three structural domains. The C-terminal domain recognizes the microbial components through multiple leucine-rich repeats (LRR). The central domain is important for self-oligomerization and consists of a nucleotide binding site (NBS), while the N-terminal effector domain contains a caspase recruiting domain (CARD), one or two for NOD1 and NOD2, respectively (61, 62). NOD1 recognizes as minimal structure the dipeptide γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) (63), which is present in DAP-type PGN, while NOD2 recognizes MDP (64), which is present in PGN of Gram-negative and Gram-positive bacteria. Recently, a mutation in NOD2, one of them the frameshift mutation 3020insC, has been shown to be associated with Crohn´s disease. PBMC (peripheral blood mononuclear cells) from patients with Crohn´s disease, which carry this mutation, are not able to respond to MDP stimulation (64, 65).

2 Aims of the study

Lipopolysaccharide (LPS) mirrors all the cytokine-inducing activities of whole Gram-negative bacteria and therefore LPS is thought to be the main immunostimulatory principle of Gram-negative bacteria. Gram-positive bacteria have two major components, that build up their outer surface: lipoteichoic acid (LTA) and peptidoglycan (PGN). Both have similar immunostimulatory qualities, like similar cytokine induction and both were reported to be toll-like receptor (TLR2) dependent. However, all other TLR2 agonists possess a lipid anchor, except PGN. Muropeptides are breakdown products of PGN and are recognized by the immune system via intracellular receptors, the NOD1- (nuclear oligomerization domain) or the NOD2-receptor. Muramyl dipeptide (MDP, M(ADiQ)) is known since the seventies as minimal active principle for adjuvant activity. CpG-oligodeoxynucleotides (CpG-ODN) are produced synthetically and mimic bacterial DNA sequences. Their recognition occurs via TLR9, which is located intracellular.

- The aim of the first part of this thesis was the comparative characterization of whole *Staphylococcus aureus*, LTA and PGN, in order to identify the main immunostimulatory principle of *Staphylococcus aureus*.

- In the second part of the thesis the structural requirements of muropeptides to synergize with LPS in cytokine induction have been determined. Additionally, the synergistic effect of LPS and MDP as well as mechanistically aspects has been characterized.

- The third part investigates the synergy of MDP and other muropeptides with different CpG-oligodeoxynucleotides (CpG-ODN) as well as the mechanisms with lead to the enhanced TNFα-release induced in PBMC (peripheral blood mononuclear cells).

3 MDP and other muropeptides – direct and synergistic effects on the immune system

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invited to Journal of Endotoxin Research

3.1 Abstract

Muropeptides are synthetic fragments or breakdown products of peptidoglycan (PGN), which occur naturally in Gram-negative and Gram-positive bacteria. Muropeptides are released during bacterial growth and division, through the activity of lytic enzymes like lysozyme and amidases or antibiotic treatment. After uptake of bacteria or bacterial breakdown products by phagocytic cells, muropeptides lead to intracellular signalling, altered gene expression and activation of immune responses. One naturally released partial structure of the PGN is the well-known muramyl dipeptide (MDP), the minimal structure for adjuvant activity. This review focuses on the adjuvant activity of MDP and other muropeptides, the synergy with other bacterial immune stimuli in inducing inflammatory responses, as well as possible receptors.

3.2 Introduction

For the recognition of bacteria and killing of these microbial invaders, the pattern recognition receptors (PRR) of the immune system play a key role.

Pathogens carry conserved structures, which are recognized by these PRR, leading to the activation of the innate immune system, e.g. inducing the release of inflammatory molecules such as chemokines and cytokines. The most important preserved structure is on the one hand the lipopolysaccharide (LPS) located in the outer membrane of Gram-negative bacteria, which consists of a lipid A anchor, a inner polysaccharide core and outer chains of variable repetitive carbohydrates (66). On the other hand, lipoteichoic acid (LTA) represents to a certain extent the counterpart of Gram-positive bacteria, characterized by a polyol phosphate polymer anchored in the cytoplasmic membrane (19, 26). Gram-positive and Gram-negative bacteria contain peptidoglycan (PGN), which consists of numerous glycan-chains that are cross-linked by oligopeptides. These glycan-chains are composed of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), with the muramic acid being coupled to amino acids (32, 33). Muropeptides are breakdown products of the PGN, bearing at least the MurNAc moiety and varying amino acids. One prominent muropeptide is muramyl dipeptide (MDP), which is known since the seventies as the minimal structure for adjuvant activity (37).

The conserved structures of the bacterial cell wall, the pathogen associated molecular patterns (PAMPs), are recognised by the immune system by the PRR. Immune activation by the PRR is important for anti-bacterial defence. One prominent family of these receptors are the Toll-like receptors (TLR). Up to now, 13 mammalien TLR have been identified, comprising 10 in humans and 12 in mice (52-55). The extracellular domain of the TLR family containing leucine-rich repeats and the cytoplasmic toll/interleukin-1 (IL-1) receptor (TIR) domain show similarities to the IL-1 receptor family (67) and was also found in other cytoplasmic proteins such as MyD88 (myeloid differentiation factor 88) (68). Originally, MyD88 was thought to function as ubiquitous adapter coupling these TLR with downstream signalling kinases, but MyD88-independent pathways downstream of some TLR have been described as well (1). The best known renowned TLR receptor, TLR4, participates in LPS signalling, as shown in TLR4-deficient mice (69) as well as in C3H/HeJ and C57BL/10ScCr mice (57), which carry spontaneous mutations of the tlr4 genes. LPS receptors and adaptor molecules had been identified before, like LPS-binding protein (LBP)

and CD14 (70), but since both of them lack a membrane-spanning domain it was not clear how they would mediate cell activation (7). Additionally, some molecules on the cell surface have been discovered, like MD-2, which are linked to TLR4 (71). Furthermore, by the analysis of the three-dimensional shape of lipid A structures the question of TLR-dependence was further elucidated (6): variations in the molecular composition of lipid A and the resulting three-dimensional conformation, leading to conical-shaped lipid A or cylindrical-shaped lipid A, signal via TLR4 or TLR2, respectively (5, 66). Similarly, it was shown for LTA, that either TLR2 or TLR4 were involved in LTA signalling. Some investigators have reported, that LTA signalling is TLR4 dependent (14, 23, 24), but meanwhile several other groups have confirmed, that recognition of LTA is actually TLR2-dependent (21, 25-27). Furthermore, synthetic LTA was shown to be TLR2 dependent as well (16). A likely reason for the discrepant results is rather the quality of the used materials than conformational variants as for LPS. Commercial LTA preparations are often strongly contaminated by endotoxin or other materials (18, 19, 72).

Besides LTA, another cell wall component is predominant in Gram-positive bacteria, i.e. PGN, which also has been demonstrated to induce inflammatory mediators (31, 58, 73) and lead to activation of transcription factors (74). TLR2 dependency of PGN has been described by several investigators (75-78). However recently, Travassos et al. have demonstrated that PGN preparations from Gram-negative bacteria are contaminated with lipoproteins, while PGN preparations of Gram-positive bacteria are contaminated by LTA or similar molecules, and that these contaminants lead to the TLR2 dependent activation through PGN (27, 79). Our group has made similar observations and explained, how such small LTA contaminations - we found about 200-400 ng LTA per mg PGN in commercial preparations - can induce such a strong immune activation: coating of LTA to a solid phase leads to an increase of potency by about a factor of 1000. Additionally, findings with a novel synthetic bisamphiphilic LTA, i.e. a model of two cross-linked LTAs, which demonstrates an increased immunostimulatory capacity, support this hypothesis (Traub et al., submitted).

Other bacterial patterns include flagellin, which is recognized via TLR5 (80). Double-stranded RNA is as well a molecular pattern, which is recognized by the immune system. Poly(I:C) is a synthetic analogue for viral double-stranded RNA

and is dependent on TLR3 signalling (81), while the synthetic substance loxoribin is recognized by TLR7 (82). Another potent activator of the immune system is bacterial DNA. The immune cells discriminate between host and bacterial DNA on the basis of unmethylated CpG motifs that occur relatively rarely in the vertebrate genome but frequent in bacterial DNA. Synthetic oligodesoxynucleotides (ODN) containing unmethylated CpG motifs similarly activate immune cells. The recognition of bacterial DNA and CpG-ODN have been found to be impaired in TLR9 knockout mice (83). TLR9 is located intracellular, where the recognition of CpG-ODN takes place (56, 84).

Additional PRR, which are located intracellular are the cytoplasmatic NOD proteins. The NOD proteins are subfamily members of the CATERPILLAR family of proteins. The prominent members of this subfamily are NOD1 (also called CARD4) and NOD2 (also called CARD15). Both of them are located in the cytoplasmic compartment and are characterized by three structural domains: a C-terminal domain with multiple leucine-rich repeats (LRR) that recognizes components of microbes, a central nucleotide binding site (NBS), which is important for self-oligomerization of the molecule, and N-terminal effector motifs, the CARD domains, one or two for NOD1 and NOD2, respectively (59). NOD1 and NOD2 have been described as intracellular sensors for PGN degradation products. NOD1 recognizes as minimal structure the dipeptide γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP), an amino acid which is not present in eukaryotes and is therefore an effective bacterial signature (63), while NOD2 recognizes MDP (64).

In this review we summarize the actions of MDP and other muropeptides on the immune system, their strong synergistic effect with other bacterial components as well as receptors, which possibly are responsible for intracellular signalling.

3.3 MDP and muropeptides

3.3.1 The structure of muropeptides

Both, Gram-negative and Gram-positive bacteria contain peptidoglycan (PGN), Gram-negative ones possess only a small layer (*E. coli* 1 nm (32)), while Gram-positive bacteria bear a multi-layered cell wall (about 20 to 40 nm (33)) outside

the cytoplasmic membrane. Figure 1 shows the typical structure of a Gram-negative bacterium, e.g. *Escherichia c*oli. PGN consists of a glycan backbone with alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), to which four to five amino acids are linked through the lactyl group of the MurNAc residue. The amino acids occur in alternating L- and D-isomers, γ-bonded D-glutamic acid, additionally non-protein amino acids, like diaminopimelic acid (DAP), ornithine or lanthionine, which represent typical bacterial signatures. These amino acids are the cross-linking components of the glycan backbones, usually via a free amino group of a basic amino acid, such as L-lysine (lys) or *meso*-DAP, for lys-type or DAP-type PGN, respectively, and a terminal amino acid with a free carboxy group, frequently D-alanine (34). The peptide structure of a Gram-negative bacterium like *E. coli* is commonly L-Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala, where the dibasic amino acid *meso*-DAP represents the cross-linking peptide (85). In contrast, the typical structure of Gram-positive bacteria like *S. aureus* is commonly L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala, with the transpeptide bond between the lysine side chain and a poly-glycine pentapeptide (interpeptide brigde) attached to form the regular cross-links in this type of PGN (86). In contrast to the uniform structure of the glycan chain not only the lys-type or the DAP-type PGN occur, but further variations in the peptides can be found. Usually the amino acid which, is linked to the muramic acid, is L-Ala, but can be replaced by glycine or L-serine. The second position D-Glu can be replaced by amidated Glu, glycine, amidated glycine or amidated alanine. At position three, where usually *meso*-DAP or L-Lys is found, the strongest variations occur, i.e. replacement by L-ornithine, LL-DAP, hydroxy-lysine and others. Position four and five are almost always occupied by D-Ala with very little variations (34). Additionally, differences in the interpeptide bridges were found. The interpeptide bridge can consist of a single amino acid, of homo-oligopeptides, which vary between two and six amino acids and are composed of Gly or L-Ala residues, or of hetero-oligopeptides, of two to seven amino acids with different sequences (34).

While, penicillin and other β-lactam antibiotics inhibit the formation of the transpeptide bond, lytic host enzymes like lysozyme and amidase can digest the PGN of the bacterial cell wall and lead to the release of muropeptide fragments. Recently, a group of new proteins have been identified, the PGN

recognition proteins (PGRP). Four PGRPs, containing a PGN-binding domain are present in humans (PGRP-S, PGRP-L, PGRP-Iα and PGRP-Iβ) and bind PGN with high affinity (58, 60). One of these proteins, the PGRP-L, which is mainly expressed in the liver, is a N-acetylmuramoyl-L-alanine amidase, that cleaves the PGN between the sugar moiety and the peptide moiety (87). Therefore, a role of this PGN lytic enzyme in innate immune responses was suggested. However, in *Listeria monocytogenes* the major PGN hydrolase is also an N-acetylmuramoyl-L-alanyl-amidase, but it mainly releases peptidic chains and sugar moieties and not substantial amounts of muropeptides (88). Possibly, the role of this kind of amidases is rather to terminate immune reactions, than to stimulate them. Muropeptides are also released during bacterial growth and division of bacteria. In this case, mostly anhydro-muropeptides, which carry the terminal MurNAc residue in the 1,6-anhydro-form, upon breakdown of the PGN through lytic transglycosylases are formed (35). Muropeptides like the 1,6-anhydrodisaccharide tetrapeptide from *E. coli* after cleavage with amidase have been detected in human serum (36). When PGN from radiolabelled *Bacillus subtilis* cell walls was subjected to digestion by a macrophage cell line, several muropeptides were released into the medium. Analysis of the supernatant revealed the presence of a disaccharide di-, tri- and tetrapeptide (GlcNAc-MurNAc-Ala-isoGln-DAP-Ala and shortened forms) (89). One well-known muropeptide is muramyl dipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine or MDP) which is known since the seventies as the minimal structure for adjuvant activity (37). MDP corresponds to the stem peptide found in *Streptococcus pneumoniae* Ala-isoGln-Lys-Ala-Ala (90). Thus, different muropeptides occur, due to variability in PGN structure and additionally dependent on the mode of PGN digestion. Different muropeptides structures are shown in figure 2.

3.3.2 Detection and clearance of muropeptides

The body is constantly exposed to bacteria and bacterial cell wall products, which can induce acute and chronic inflammation. Measurements to proof the natural occurrence of free muropeptides in the host tissue and body fluids and to determine the average half-life have been undertaken. Muramic acid is not

synthesized by mammalian enzymes and is therefore used as a marker for bacterial cell wall breakdown products. First evidence came from the discovery of the sleep-promoting factor (factor S), which was found in cerebrospinal fluid or extracts of brain (91), as well as in human urine. Analysis revealed the presence of muropeptides (92-94). Several groups have started to quantify muropeptides in tissues and body fluids. With gas chromatography mass spectrometry or similar methods in uninfected brains and spleens of rats no muramic acid was found (95, 96), while others detected small amounts in liver, brain and kidney (100 to 150 pmol/g tissue) (97). In synovial fluids of septic (<250-1,700 ng/ml) (98) or arthritic patients (220-2000 ng/ml) (99) or in urine of patients with urinal tract infections (about 170 ng/ml) (100) muramic acid was found. In patients with pneumococcal meningitis, levels of muramic acid from 6.8 to 3,890 ng/ml were found and the authors assumed, that cell wall material of 10 ng/ml or equivalent to about 10^5 bacteria are required to induce inflammation in animal models of meningitis (96). By high pressure liquid chromatography in the intestinal content of human patients, as well as in stool samples, muramic acid has been determined and the authors suggested, that this represented a content between 20 and 87 µmol/L of MDP (101). To further elucidate the question, if bacterial breakdown products may be found after uptake from the gut, Vavricka et al. (101) hypothesized an intestinal epithelial apical di-/tripeptide transporter hPepT1, that could transport MDP. In line with these findings, orally administered MDP was detected in blood plasma of rats, but only less than 0.05% of the given dose was found (102).

Another reason for the small muropeptide content of plasma could be that muropeptides are highly water-soluble and are therefore rapidly eliminated from the circulation. Following intravenous injection of MDP in rats (1.5 mg/kg), MDP was quickly removed from the plasma. At two minutes after administration less than 35% of the injected dose was retrieved, after 2 hours MDP was nearly eliminated (102). In line with these findings, only 33% of 2 mg MDP injected in the ear vein of rabbits was detected after 30 minutes and after 4 hours no MDP was detectable in the serum anymore (103). Furthermore, also bigger muropeptides like the disaccharide pentapeptide (GlcNAc-MurNAc-Ala-isoGln-*meso*-DAP-Ala-Ala) and other structures are recovered in murine urine (104, 105).

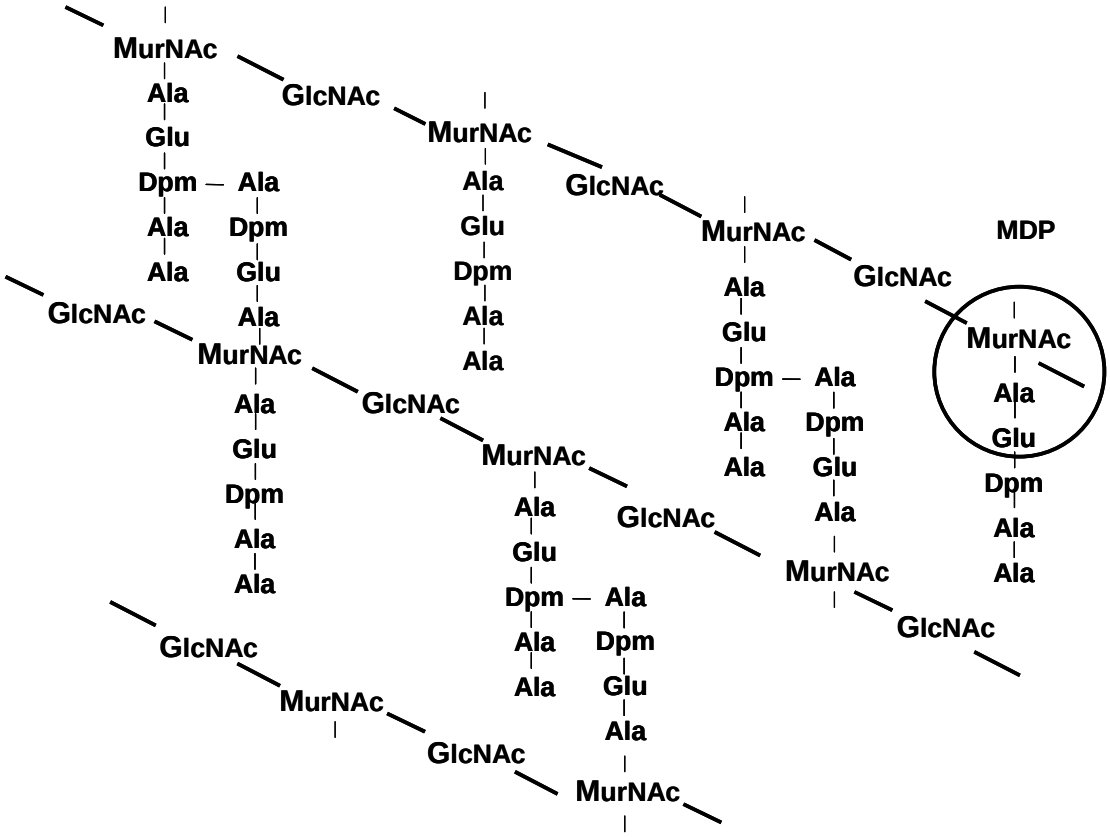


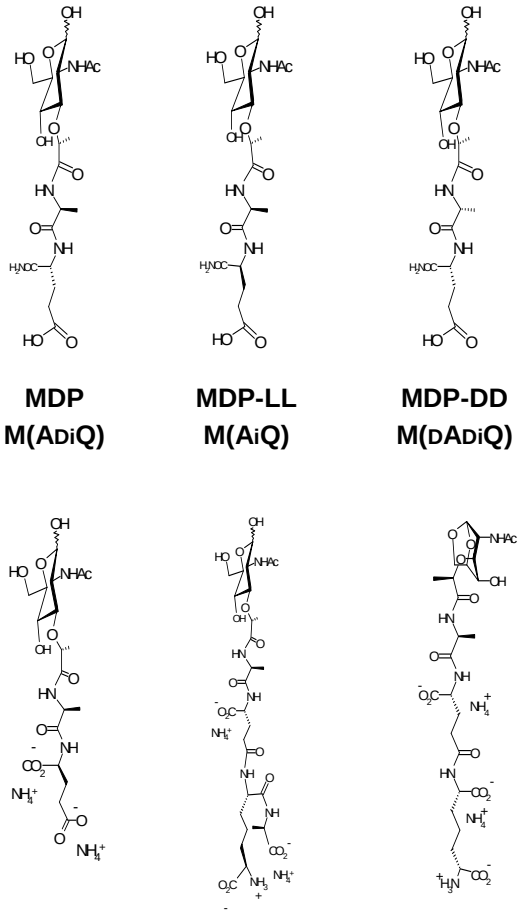
FIGURE 1: Structure of the murein sacculus of a typical Gram-negative bacterium, e.g. *Escherichia coli*. Abbreviations used in the figure: **MurNAc**: N-acetylmuramic acid, **GlcNAc**: N-acetylglucosamine, **Ala**: alanine, **Glu**: glutamate, **Dpm**: diaminopimelic acid

The proof of the presence of muramic acid in healthy animals is perhaps not possible because the detection limit (lower ng/ml concentrations) of the methods employed are still above the level of natural occurring muropeptide concentrations. From infected animals evidence is available that muropeptides are present in the body. However, in most *in vivo* and *in vitro* studies found in literature so far, very high concentrations of MDP and other muropeptides are used, i.e. typical 1 to 100 µg/ml questioning therefore their physiological relevance.

3.3.3 Effect of muropeptides on the immune system

MDP and other muropeptides target monocytes, macrophages, dendritic cells, natural killer cells, B-cells and neutrophils (106-109). In 1974, N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP) was discovered as the minimal structure responsible for adjuvant activity of the mycobacteria in

Freund´s complete adjuvant (37). Since then, a lot of efforts have been made to isolate, purify and synthesize MDP and derivatives (110, 111). MDP and other muropeptides exert many biological effects, beside adjuvant activity, such as pyrogenicity, induction of cytokines, stimulation of non-specific resistance against bacterial, viral or parasitic infections, somnogenicity, anorexia as well as immunomodulating activities like priming and synergy (112-114).



MDP **MDP-LL** **MDP-DD**
M(ADiQ) **M(AiQ)** **M(DADiQ)**

M(ADiE) **M(AγDE*m*DpmDA)** **AM(AγDE*m*Dpm)**

FIGURE 2: Different muropeptide structures. Abbreviations used in the figure: **M**: N-acetylmuramic acid, **AM**: 1,6-anhydromuramic acid, **A**: alanine; **D**: amino acid in D configuration, **L**: amino acid in L configuration, **Q**: glutamine, **E**: glutamate; **Dpm**: diaminopimelic acid, **γ**: linkage of amino acids, ***m***: meso

As immunogenic adjuvant, MDP increases the phagocytic and anti-microbial activity (115, 116) through enhancement of the expression of surface markers, which are involved in cellular adhesion processes and costimulation for antigen presentation (107, 117). In addition, MDP improves the antigen delivery as well as the enhancement of antigen processing and presentation by antigen-presenting cells, leading to increased antibody-mediated cytotoxicity (118).

Moreover, immunoadjuvant activities of MDP are enhancing the biological action of vaccine antigens through specific production of antibodies (119, 120). Furthermore, MDP as well as other muropeptides (tripeptides and disaccharide tri- and tetrapeptides) induce cellular immune defences, evidenced by skin reaction (delayed-type hypersensitivity) (37, 121). MDP augments also the effect of other immunomodulators like IFNγ (122) as well as synergizes with cytokines to stimulate the differentiation and proliferation of lymphocytes (123). Another important aspect of the adjuvant activity of MDP and other muropeptides is the direct induction of cytokines by itself, which can alter the immune response.

In some models, muropeptides are able to induce inflammatory reactions and cytokine release. However, several investigators have reported species-specific differences with regard to the direct cytokine-inducing ability of MDP and other muropeptides. Susceptibility towards MDP has been reported for *in vitro* stimulation of peritoneal macrophages from guinea pigs and rats, while no cytokine production was found in BALB/c mice as well as in other mouse strains (124, 125). In contrast, others have found cytokine-production *in vitro* by mouse peritoneal macrophages (126) as well as in bone marrow derived macrophages (127). *In vivo*, mice are rather insensitive to muropeptides when MDP was administered as an aqueous solution (LD_{50}: approx. 2200 mg/kg i.p.) (113). This could be due to rapid excretion into urine (128). Controversial results have been observed with human cells and human cell lines. In primary human monocytes, MDP is able to induce cytokine release (129, 130) and some authors suggest, that the activation of the inflammasome complex (caspase-activating complex) (131)) is responsible for this activity (132). In contrast, in human whole blood and in isolated monocytes MDP and related compounds were not able to induce cytokine release (133, 134). In the human cell line Monomac-6, MDP induced minimal expression of mRNA, but the induced mRNA was not translated into protein (135), while in the human monocytic OCT (vitamin D analogue)-differentiated THP-1 and U937 cells induction of IL-8 could be observed in response to MDP (14). A possible explanation for the controversial results could be the lack of exclusion of endotoxin contaminations or the unphysiologically high concentration of MDP.

Non-specific resistance to infections can be increased by bacterial immunstimulants via enhancing the phagocytic capacity of antigen-presenting cells. MDP stimulation decreased survival of intracellular *Salmonella typhimurium* (136), stimulated host resistance against *Klebsiella pneumoniae* (137) and *Candida albicans* infections (138) as well as killing of *Leishmania donovani* (139). MDP is therefore considered to enhance non-specific resistance of the immune system.

That MDP and derivatives belong to sleep regulatory substances and are therefore somnogenic was demonstrated by transfer experiments in which substances isolated from sleep-deprived animals induced sleep in the receiver animal (91, 92, 140). A possible explanation for the somnogenicity of muropeptides could be an indirect action by induction of endogenous pyrogens like IL-1, TNF and NO, which are probable sleep factors and have therefore a role in sleep regulation (141-143).

MDP and related muropeptides have been shown to induce some behavioural changes like hyperthermia (144), hypermetabolism, body weight loss and anorexia leading suppression of food intake during bacterial infection. However, in order to reduce food intake, very high doses of MDP (> 1 mg/kg) were necessary (145). There is some evidence that MDP may act rather via cytokines than have anorectic effects on its own (146-148). To determine the receptor-dependence of the anorectic effect, the role of CD14, TLR2 or TLR4 have been investigated. MDP reduced food intake in wild type mice of all genotypes used as well as in TLR4 deficient mice. But the anorectic effect of MDP was blunted in CD14- and TLR2-knockout mice, indicating that CD14 and TLR2 are involved in the signalling pathway of MDP-induced anorexia (149).

With DNA microarrays the response of human macrophages to MDP and therefore the induction of a macrophage activation programme, like receptors, signalling molecules and transcription factors were determined. Unfortunately, the authors only reported, that MDP induced the majority of the activation programme and no further information is available (150).

Muropeptides have a lot of diverse effects, like inducing leukocytosis and protein influx in cerebrospinal fluid or brain oedema in a rabbit model of meningitis (151). In renal cells muropeptides have been shown to induce apoptosis (152). A recent publication suggests that this is mediated via

calreticulin (153), which has also shown to be a binding protein for MDP and PGN (154).

3.4 Synergy of muropeptides with bacterial stimuli

3.4.1 Priming ability of muropeptides

Through microbial compounds like muropeptides, the susceptibility of animals to develop anaphylactic reactions and lethal toxicity in response to bacterial endotoxin can be enhanced (155). MDP and muropeptides were shown to prime animals and LPS species of low toxicity led to anaphylactic reactions and reactions to high-toxicity LPS were further augmented (125). The priming ability of MDP occurred in endotoxin-sensitive and -resistant strains, but there were variations among different mouse strains (156). Optimal MDP priming is restricted to distinct time periods (4 hours before and after administration of LPS) (156-158) and depends on the route of administration (156).

In C3H/HeJ mice structural requirements of muropeptides, to prime for induction of anaphylactic reactions by LPS have been investigated. The DAP-type muropeptide GlcNAc-MurNAc-pentapeptide induced no anaphylactic reactions, while the disaccharide-tetrapeptide and the MurNAc-tetrapeptide showed marginal reactions, but the disaccharide-tripeptide and the MurNAc-tripeptide led to death of the animals (159). MDP analogues, in which the D-isoglutamine residue of MDP was replaced by D-glutamine, D-glutamic acid or D-isoasparagine, showed lesser priming than MDP and MDP analogues with replacement in L-glutamic acid, L-glutamine or L-isoglutamine were inactive (159). Furthermore, a synthetic lactyl peptide (desmuramylpeptide) D-lactyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly (FK156) exhibited also priming activity for anaphylactic reactions (159). Thus, MDP is the minimal structure for priming effects, other structures are also effective, but apparently no fragments that are larger than the disaccharide-tetrapeptide.

The priming mechanisms of MDP for subsequent LPS or streptococcal bacterial cell administration, which led to anaphylactic reactions, was suggested to be induced by complement-dependent platelet degradation, which accumulate in the liver and the lung and induce acute inflammation with severe tissue

destruction leading to anaphylactic reaction (160-162). Additionally, a recent publication has shown that pre-treatment of MDP and subsequent injection of LPS led not to endotoxin shock in Nod2-/- mice, while wild type mice react in the known way. The authors suggested an essential role of NOD2 for priming ability of MDP (163).

In several models, MDP preincubation followed by LPS stimulation with regard to cytokine release were not more effective than simultaneous application. Human monocytic OCT-differentiated THP-1 cells responded with the same amount of IL-8 release when the cells were preincubated with MDP as stimulated simultaneously with LPS plus MDP (14). In line with these findings, in human whole blood preincubation of MDP and re-stimulation with LPS led only to a 10% increase of TNF release compared to the simultaneous stimulation by LPS plus MDP (134).

3.4.2 Synergy of muropeptides with LPS

MDP and other muropeptides are strong immune amplifiers *in vivo* and *in vitro* enhancing immune reactions to other immune stimuli. Muropeptides exert priming effects, when administered before LPS challenge. Beside this, strong synergistic effects mostly with regard to cytokine release could be observed, when both substances were given at the same time-point. Examples for synergistic activation through LPS and MDP are listed in table 1. Synergy of LPS and MDP occurred in human whole blood (133, 134, 164), PBMC (peripheral blood mononuclear cells) (165), monocytes (165, 166) as well as in human monocyte derived macrophages (MDM) (167). Additionally, synergistic cytokine release by LPS and MDP took place in monocytic cell lines like Monomac-6 cells, OCT-differentiated THP-1 and U937 cells (14, 167). Similar to the human systems, synergy could be observed in models like *in vivo* in rats (168), in rat liver macrophages (169) and in the murine cell line RAW264.7 (170). Different muropeptide structures were also able to induce synergy with LPS or lipid A (63, 134, 171, 172) (a summary was shown in table 1). Experiments with actinomycin D demonstrate that MDP increased LPS-induced TNF mRNA. The effect is probably due to increased *de novo* transcription, rather than to an increase in mRNA stability (134).

stimulus concentration	muropeptide concentration	endpoint	synergy	model	literature
LPS E. coli 026:B7 10 ng/ml	MDP 1 µg/ml	TNF IL-6 IL-10	3 fold 2 fold –	human whole blood	(133) (164)
LPS S.a.e 10 pg–100 µg/ml	MDP 10 ng/ml	TNF IL-1β IL-6 IL-10	400 fold 300 fold 7 fold 3 fold	human whole blood (20%)	(134)
LPS E. coli 0111:B4 10 pg/ml	MDP 1-100 ng/ml	IL-ra IL-1β TNF	10 fold 5 fold +/–	PBMC	(165)
LPS E. coli 0111:B4 10-100 ng/ml	MDP 10 µg/ml	IL-1β mRNA HLA-DR mRNA	+ +	hu MDM	(167)
LPS S.a.e. 100 pg/ml	MDP 10 ng/ml	TNF	2 fold	isolated monocytes	(134)
LPS E. coli 1 ng/ml	MDP 10 µg/ml	TNF	2 fold	mononuclear cells (MNC)	(166)
LPS E. coli 055:B5 30 ng/ml	MDP (30 min preinc) 100 µg/ml	TNF TNF mRNA	2 fold additive	Monomac-6	(135)
LPS S.a.e. 1-100 ng/ml	MDP 1-100 ng/ml	IL-8	8 fold	OCT-diff. THP-1	(14)
LPS S.a.e. 0.1-10 ng/ml	MDP 0.1-10 ng/ml	IL-8	3 fold	OCT-diff. U937	(14)
LPS E. coli 0111:B4 25 µg/kg	MDP 0.4 mg/kg	anorexia	+	rat *in vivo*	(168)
LPS E. coli 0127:B8 5 ng/well	MDP 5 µg/well	macrophage-mediated cytolysis	2-3 fold	rat liver macrophages	(169)
LPS E. coli 10 ng-1µg/ml	MDP 40 ng-25 µg/ml	TNF	3-10 fold	RAW264.7	(170)
LPS S.a.e 100 pg/ml	MurNAc-Ala GMDP MurNAc-Ala-Gln MurNAc-Ala-Gln-Lys MDP (DD) MDP (LL) 10 ng/ml	TNF	1.3 fold 5 fold 4 fold 3 fold - -	human whole blood (20%)	(134)
Lipid A E. coli (LA-15-PP)	MDP FK 156 FK 565	IL-8	5 fold 7 fold similar (ns)	OCT-diff THP-1	(172)

1-100 ng/ml	iE-DAP 1-100 µg/ml		–		
LPS S. typhimurium 100 ng/ml	iE-DAP 1 ng/ml 1 µg/ml	IL-6	100 fold 500 fold	BMDM (NOD1–/–)	(63)
LPS E. coli 100 EU/ml 1-10000 EU/ml	TCT (tracheal cytotoxin, GlcNAc-1,6 anhydro MurNAc-Ala-Glu-meso-DAP-Ala) 0,09-9,21 µg/ml	IL-1α IL-1α mRNA NO inhibition of DNA synthesis	5.5 fold + 2 fold 80%	HTE derived from hamster tracheal tissue	(171)

TABLE 1: Synergy of LPS or lipid A with MDP and other muropeptides

3.4.3 Synergy of muropeptides with other TLR-agonists

Muropeptides exerted remarkable synergistic and priming effects for TLR4-agonists with regard to cytokine induction in various cells types. These findings led to the question, if MDP and other muropeptides also induce synergistic effects with other TLR-agonists. Examples are listed in table 2. Beside LPS, LTA as TLR2-agonist showed synergy with MDP and other muropeptides in the OCT-differentiated monocytic cell line THP-1 (14). Additionally, in the murine cell line J774.2 the same effects could be observed (173). In contrast, in human whole blood no synergy of LTA and MDP has been found (figure 3). In PBMC with 10% serum the effect was weaker than in PBMC without serum (own unpublished results). In line with this, in human monocytes with low serum (2.5%) synergy of LTA and MDP was observed (22). This led to the hypothesis, that serum components blunt the synergistic effect of LTA and MDP, which requires further studies. Other TLR2-agonists like MALP-2 (figure 3) (166, 174), PGN (135) and Pam3Cys (163, 166, 172, 174) were also able to synergize with MDP and other muropeptides. The TLR3-agonist poly(I:C) have been shown to induce synergistic production of TNF, IL-6 and IL-12p40 (163, 166). In contrast, flagellin (TLR5-agonist) as well as loxoribin (TLR7-agonist) are not able to induce synergy with MDP (166), while others reported that flagellin as well as R848 (Resiquimod) exert synergy with MDP (174). The TLR9-agonist CpG-ODN (figure 3) induce synergistic cytokine release with MDP and other muropeptides (172) (Traub et al. submitted), while others reported no synergy

between CpG-ODN and MDP (166). Non-TLR agonists like SEB and OKT-3 were no synergists to MDP (figure 3). The PKC activator PMA in contrary was able to induce synergistic effects with MDP (figure 3). MDP and other muropeptides thus revealed synergistic effects with a variety of different TLR-agonists, indicating a possible interplay of the extracellular TLR receptors and the intracellular NOD-receptors or interplay of components of their signalling pathways.

stimulus concentration	**receptor**	**muropeptide concentration**	**endpoint**	**synergy**	**model**	**literature**
LTA S. aureus 0.1-10 µg/ml	TLR2	MDP 1-100 µg/ml	IL-8	7 fold	OCT-diff THP-1	(14)
LTA S. aureus B. subtilis 0.1 µg/ml	TLR2	GMDP MDP MDP (LL) MDP (DD) GMDP 1 µg, 10 µg/ml	NO	2 fold 2 fold 1.5 fold – 3 fold	J774.2	(173)
LTA S. aureus 100 ng/ml	TLR2	MDP 10 ng/ml	TNF	–	Human whole blood (20%)	figure 3
LTA S. aureus 10 µg/ml	TLR2	MDP 50 ng/ml	TNF	-	PBMC (10% serum)	unpublished observation
LTA S. aureus 10 µg/ml	TLR2	MDP 50 ng/ml	TNF	5 fold	PBMC (without serum)	figure 3
LTA S. aureus S. pneumoniae 0.04-1 µg/ml	TLR2	MDP (30 min preinc) 100 ng/ml	TNF	5 fold 5 fold	human monocytes (2.5% serum)	(22)
MALP-2 10 ng/ml	TLR2	MDP 50 ng/ml	TNF	5 fold	PBMC (without serum)	figure 3
MALP-2 1 µg/L	TLR2	MDP 20 µg/L	TNF IL-1β	2.1 fold 3.5 fold	PBMC	(174)
MALP-2 1 µg/ml	TLR2	MDP 10 µg/ml	TNF IL-1β IL-10	2 fold 6 fold 6 fold	mononuclear cells (MNC)	(166)
PGN 100 µg/ml	TLR2	MDP (30 min preinc) 100 µg/ml	TNF TNF mRNA	2 fold additive	Monomac-6	(135)
Pam3Cys 1-100 pg/ml	TLR2	MDP FK 156 FK 565 1-100 µg/ml	IL-8	4 fold 4 fold similar (data not shown)	OCT-diff THP-1	(172)

Pam3Cys 1 µg/ml	TLR2	MDP 10 µg/ml	TNF IL-1β IL-10	2 fold 8 fold 6 fold	mononuclear cells (MNC)	(166)
Pam3Cys 10 µg/L	TLR2	MDP 20 µg/L	TNF IL-1β	1.8 fold 3.2 fold	PBMC	(174)
Pam3Cys 2 µg/ml	TLR2	MDP 1, 10 µg/ml	IL-6	2 fold	BMDM	(163)
poly(I:C) 50 µg/ml	TLR3	MDP 10 µg/ml	TNF	2 fold	mononuclear cells (MNC)	(166)
poly(I:C) 100 µg/ml	TLR3	MDP 1,10 µg/ml	IL-6 Il-12p40	3 fold 2 fold	BMDM	(163)
flagellin 10 ng/ml	TLR5	MDP 10 µg/ml	TNF	additive	mononuclear cells (MNC)	(166)
flagellin 50 µg/L	TLR5	MDP 20 µg/L	TNF IL-1β	3.4 fold 3.9 fold	PBMC	(174)
Loxoribin 5 µg/ml	TLR7	MDP 10 µg/ml	TNF	-	mononuclear cells (MNC)	(166)
R848 1 mg/L	TLR7/8	MDP 20 µg/L	TNF IL-1β	1.7 fold 2 fold	PBMC	(174)
CpG 10-1000 nM	TLR9	MDP FK156 FK 565 1-100 µg/ml	IL-8	4 fold 6 fold similar (ns)	OCT-diff THP-1	(172)
CpG 2 µM	TLR9	MDP MDP (DD) MurNAc-Ala-Gln MurNAc-Ala-Gln-Lys MurNAc-Ala-Gln-DAP Anhydro-MurNAc-Ala-Gln-DAP 50 ng/ml	TNF	2000 fold - 1200 fold 1300 fold 1300 fold 350 fold	PBMC	Traub et al., submitted
CpG 5 µg/ml	TLR9	MDP 10 µg/ml	TNF	-	Mononuclear cells (MNC)	(166)
SEB 100 ng/ml	T-cell/MHC	MDP 10 ng/ml	IL-2	–	plasma-free blood	figure 3
OKT-3 5 ng	CD3	MDP 50 ng/ml	IL-2	–	plasma-free blood	figure 3
PMA 10 ng/ml	PKC	MDP 10 ng/ml	TNF	9 fold	human whole blood (20%)	figure 3

TABLE 2: Synergy of different stimuli with MDP and other muropeptides

3.5 Receptors of muropeptides

For long, the receptor for MDP and other muropeptides were not known. Different types of receptors have been discussed, such as the 5-HT receptor,

CD14 and the TLR receptors. Recently, two intracellular proteins of the NOD family, NOD1 and NOD2, have been discovered as receptors for MDP and other muropeptides.

3.5.1 5-HT receptor

MDP has neuropharmacological activities, such as effects on sleep, analgesic properties and influence on behaviour (141, 175). Previous reports from Sevcik and Masek et al. suggest a possible interaction with the serotoninergic system (176, 177). However, recent reports from the same group showed that this is not the case if physiologically relevant concentrations in a nanomolar range are employed and no interaction of MDP with either the 5-HT_4 or the 5-HT_{1A} receptor was observed (178, 179).

3.5.2 CD14

Membrane CD14 (mCD14) is a glycosylphosphatidylinositol-anchored cell surface molecule found on cells of the myeloid linage. Since it lacks a cytoplasmatic domain, it cannot transmit activation signals into the cell. The glycosylphosphatidylinositol anchor is missing in soluble CD14 (sCD14), which is present in human serum as acute phase protein. CD14 is thought to be an adaptor molecule accepting LBP-pathogen-derived ligand complexes and passing the pathogen-derived ligand to TLR, which bear a transmembrane domain, resulting in intracellular signalling. The role of CD14 in MDP-dependent cell activation is still controversial.

MDP bound to mCD14 on human monocytes and prevented the binding of soluble PGN (180), but monomeric MDP did not bind to sCD14, while CD14 bound to MDP or GlcNAc-MDP immobilized on agarose (181). These investigators concluded, that solid-bound MDP is needed for CD14 binding. In human gingival epithelial cells CD14 and LBP, but not LBP alone, enhanced the MDP-stimulated activation (182). In line with these findings, in human monocytes the disaccharide-pentapeptide (GlcNAc-MurNAc-Ala-isoGln-*meso*-DAP-Ala-Ala) was enhanced by sCD14 preincubation, while anti-CD14 mAb

blocked the release of cytokines (183). Furthermore, in human periodontal ligament fibroblasts, which express low CD14, MDP was not inhibited by anti-CD14 mAb, while in human gingival fibroblasts, which express high CD14, MDP was inhibited by anti-CD14 mAb (184). Recently, it has been reported that CD14 is involved in mediating the anorectic effect of MDP, shown by no reduction of food intake in CD14-knockout mice (149). In addition, MDP has a priming effect on CD14 surface expression, which is upregulated 2-fold on monocytes after treatment of whole blood (133).

In contrast, MDP induced activation of a reporter gene in CD14 expressing cells was independent of CD14 (185). In line with these results, in Monomac-6 cells no reduction of TNF of the MDP synergism with LPS and PGN after preincubation with anti-CD14 mAb could be observed as well as no effect of anti-CD14 mAb on the induction of TNF (135). Furthermore, IL-8 secretion by THP-1 cells was not reduced in the presence of anti-CD14 mAb (14). These results demonstrated, that an interaction of MDP with CD14 might be possible, but has not been fully proven yet. Further experiments in CD14 knockout mice could shed some more light on this subject. Again the lack of exclusion of LPS contaminations might be a reason for some of the controversy.

3.5.3 TLR

The Toll-like receptors (TLR) have the ability to recognize pathogens and pathogen-derived products and initiate a signalling cascade, which activates the host's immune response. For this reason, several investigators have asked the question, whether the TLR play also a role in MDP or muropeptide signalling. Most studies have addressed TLR2 and TLR4.

That MDP-signalling is TLR2 dependent has been shown in human gingival epithelial cells by a reduction of IL-8 production after pre-treatment with anti-TLR2 mAb and stimulation with MDP (182). Reduced food intake in TLR4-deficient mice, but not in TLR2 knockout mice by MDP has been reported indicating that TLR2 is involved in signalling of MDP-induced anorexia (149). In contrast, in chinese hamster ovary fibroblasts transfected with CD14/TLR2 or CD14/TLR4 as well as CD14/TLR4/TLR2, no cells were activated by MDP (185, 186). In Monomac-6 cells no reduction of TNF protein and mRNA upon MDP

synergism with LPS and PGN could be observed after preincubation with anti-TLR2 or TLR4 mAb (135). Additionally, no TLR2 and TLR4 dependence of MDP could be observed in monocytic OCT-differentiated U937 cell (14). In human periodontal ligament fibroblasts and human gingival fibroblasts as well as in IFN-γ-primed oral epithelial cells anti-TLR2 and TLR4 mAb had no influence on MDP-induced IL-8 release (24, 184). Some molecules, which are recognized by TLR2, need for signalling dimerization with TLR1 or TLR6. It might be concluded that MDP as a part of PGN, which is thought to be recognized by TLR2, requires co-expression of TLR2 with TLR1 or TLR6. However, neither expression of TLR2 with TLR1 or TLR6 led to recognition of MDP (65).
Another aspect of MDP and TLR signalling could be, that MDP exerts its synergistic effect by up-regulating the mRNA expression of MyD88, an adaptor molecule of TLR. MDP indeed enhanced MyD88 expression in OCT-differentiated THP-1 cells, but no synergistic up-regulation of MyD88 mRNA could be observed with LPS plus MDP (14). Most results suggest no role of TLR in MDP signalling, however, so far not all TLR have been investigated as possible MDP receptors.

3.5.4 NOD

Recently, another family of PRR has been identified, the nucleotide-binding oligomerization domain (NOD) family of proteins, which are expressed intracellular and recognize bacteria and bacterial products, suggesting an important role in intracellular pathogen detection. The NOD proteins share homology to a class of proteins (R proteins) that are encoded by plant disease-resistance genes. Invading pathogens are recognized by plant R proteins and mediate a defence response resulting in plant disease resistance (187), implying also a function in mammals. The NOD family of proteins has several members, two of them, NOD1 and NOD2, have been initially shown to recognize LPS (188), but recent evidence has shown, that NOD1 and NOD2 actually do not detect LPS, but PGN fragments, that are usually co-extracted with LPS during purification (63, 189).

3.5.4.1 NOD1

NOD1 consists of three domains: a centrally located nucleotide oligomerization domain (NOD), C-terminal leucin rich repeats (LRR) for bacterial recognition and the N-terminal effector domain which contains one caspase recruitment domain (CARD). NOD1 is expressed in multiple tissues (190, 191). Carneiro et al. hypothesized that NOD proteins, when no stimuli are present, are negatively regulated by their LRR through folding. Signalling and self-oligomerization is possible after unfolding, when the ligand is recognized (192). NOD1 detects only PGN containing DAP at the third position, thereby distinguishing between PGN-derived compounds from Gram-negative and Gram-positive bacteria (189). The precise structure of the PGN-derived compound was identified as a DAP containing naturally occurring muropeptide, a GlcNAc-MurNAc-tripeptide (189). In addition, NOD1 detects also the DAP containing muropeptide having only one sugar moiety, a MurNAc-tripeptide, but detects not MurNAc-L-Ala-D-Glu or MurNAc-tetrapeptide, indicating, that NOD1 detects only muropeptides with a terminal *meso*-DAP. Unusually, NOD1 detects also a lactoyl-tripeptide containing DAP though only weakly in comparison to the GlcNAc-MurNAc-tripeptide, MurNAc-tripeptide or the tripeptide alone (193). The minimal motif which is detected by NOD1 is the dipeptide γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) which showed no cytokine induction in NOD1 knockout mice (193). The fact that iE-DAP lacks a sugar moiety, shows that NOD1 sensing relies on the peptidic moiety. However, the tripeptide L-Ala-γ-D-Glu-*meso*-diaminopimelic acid showed higher activity, suggesting that the L-Ala residue is required for optimal detection by NOD1 (193). Another synthetic dipeptide, i.e. γ-D-glutamic-*meso*-diaminopimelic acid (iQ-DAP), is also able to stimulate NOD1 (63). Interestingly, some Gram-positive bacteria like *B. subtilis* contain DAP instead of lysine in their PGN, but are not recognized by NOD1 because the DAP amino acid is modified by an amidation reaction. Furthermore, the UDP-MurNAc-tripeptide, which is a precursor of the PGN biosynthesis pathway, is recognized by NOD1, indicating that the addition of the UDP group to the MurNAc moiety does not change the ability of NOD1 to detect the MurNAc-tripeptide (193).

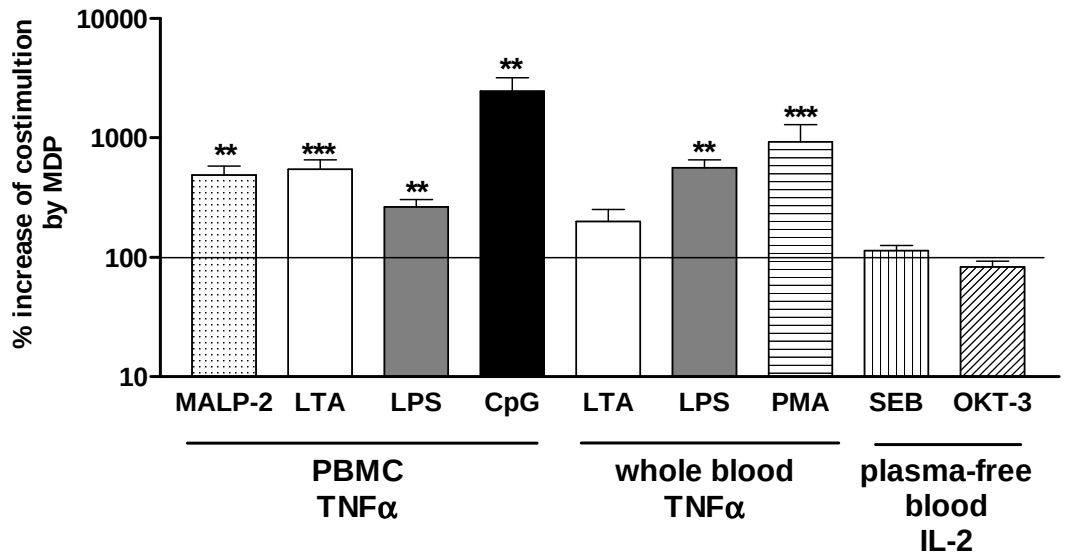


FIGURE 3: Synergistic effect of MDP with different immune stimuli. PBMC were stimulated with MALP-2 (10 ng/ml), LTA from *Staphylococcus aureus* (10 µg/ml), LPS from *Salmonella abortus equi* (10 ng/ml) or CpG-ODN 2006 (2 µM). Human whole blood was stimulated with LTA from *Staphylococcus aureus* (100 ng/ml), LPS from *Salmonella abortus equi* (100 pg/ml) or phorbol myristate acetate (PMA) (10 ng/ml). Plasma-free blood was stimulated with Staphylococcus enterotoxin B (SEB) (100 ng/ml) or OKT-3 (5 ng). All incubations were carried out in the presence or absence of 10 ng/ml MDP (human whole blood) or 50 ng/ml MDP (PBMC; plasma-free blood). After 18 hours of incubation (PBMC), 24 hours (whole blood) TNFα was determined as well as after 48 hours of incubation (plasma-free blood) IL-2 was determined in cell-free supernatants by ELISA. Data are given as percent of increase of costimulation with MDP compared to incubations in the absence of MDP and are means ∀ SEM of 4 to 8 donors. ** and *** indicates significance versus stimulus alone.

3.5.4.2 NOD2

The major structural difference between NOD1 and NOD2 is the presence of two amino-terminal CARD domains in NOD2. NOD2 is mainly expressed in monocytes, macrophages, dendritic cell, granulocytes and to lesser extent in T-lymphocytes (194). There are also reports showing that NOD2 mRNA is expressed in unstimulated epithelial cells from normal colon at low level and increased expression in macrophages and intestinal epithelial cells in Crohn´s disease patients (194, 195). Likewise, LPS and TNF induce up-regulation of NOD2 mRNA in myeloblastic and epithelial cells and induction is mediated transcriptionally. The authors suggest, that the activation of NF-κB through proinflammatory stimuli by enteropathogens or pathogenic bacteria could induce expression of NOD2 and lead to a positive feedback loop, resulting in secretion of proinflammatory cytokines and chemokines from epithelial cells

(194, 196). Recent results have shown that MDP stimulates TNF gene transcription through a NOD2-induced NF-κB activation and binding to TNF promoter (197). Furthermore, immunostaining of intestinal tissues has shown high expression of NOD2 in Paneth cells in the terminal ileum (198-200). There are several implications, that NOD2 was identified as a susceptible gene involved in Crohn´s disease, an inflammatory bowel disease, known to be influenced by both genetic and environmental factors (201-203). The most common mutation associated with Crohn´s disease is a frame-shift mutation resulting in the truncation of the terminal LRR (201). The consequence is a protein that no longer detects MDP. The reason for loss of tolerance in Crohn´s disease is not yet clear but NOD2 mutations may result in the inability of local responses in the intestinal mucosa to control bacterial infection, starting systemic responses and leading to aberrant inflammation (204). PBMC (peripheral blood mononuclear cells) from NOD2 mutants, a mutation (L1007fsinsC) also associated with Crohn´s disease, could not respond to MDP in terms of NF-κB activation and cytokine induction (65).

NOD2 is a broad sensor for Gram-negative and Gram-positive bacteria, since the MDP motif and also the corresponding naturally occurring muropeptide GlcNAc-MurNAc-L-Ala-D-isoGln is common in a lot of bacteria. MurNAc-L-Ala-D-Glu as well as muramyl tripeptides with lysine or ornithine and to lower extent amidated DAP at the third position are recognized by NOD2. PGN precursors, which are required during bacterial growth, are also recognized by NOD2. These precursors consist of UDP-MurNAc-tripeptides and one of them substituted with DAP at the third position is also recognized by NOD2. Furthermore, an intact MurNAc group which is substituted with a peptide chain is important for NOD2 signalling, because peptides lacking the MurNAc sugar moiety could not activate NOD2 (193). In addition, using MDP-analogues, where the conformation of the amino acid have been changed from L-Ala to D-Ala or D-isoGln to L-isoGln, no NF-κB activation of NOD2 transfected cells was detected any more, indicating stereoselective recognition by NOD2 (65). MDP and other muropeptides, which are recognized by NOD1 and NOD2, are probably responsible for synergistic effects with TLR agonists, indicating that TLR and NOD proteins could cooperate in immune response against bacteria (163) or that the NOD proteins play a role in cells were TLR are absent or

down-regulated. Recent reports have shown, that NOD2 can inhibit TLR2-mediated activation of NF-κB (205) and others reported a modulation of TLR pathways for the induction of cytokines by NOD2 (166, 206).

There is evidence, that NOD proteins play a role in recognition of PGN breakdown products, but the question is still open, how these molecules reach the cytosol and get in contact with NOD1 and NOD2. In most experiments, the investigators used transfection reagents or digitonin-permeabilized cells, which allow the muropeptides to enter into the cytosol (189). Phagocytosis is probably the normal way that the cell gets in contact with bacterial products, the bacteria become degraded by lysosomal proteases and muropeptides may be available for intracellular proteins. Nevertheless, how muropeptides reach the cytosol from the extracellular compartment or from the phagosome across the phagosome membrane is still unclear (192). In cells having no active phagocytosis, like intestinal epithelial cells, another pathway is possible how NOD proteins get in contact with bacterial products, i.e. through direct bacterial invasion into the cell or through a bacterial transfer apparatus. Enteroinvasive bacteria, like *Salmonella*, invasive *E. coli*, *Listeria*, *Shigella* or *Yersinia* induce for example their own uptake by epithelial cells of the intestinal mucosa (207). This could explain, how PGN compounds can enter into the cell. Girardin et al. demonstrated, that NF-κB activation through *Shigella flexneri* was inhibited by overexpression of dominant-negative NOD1 (208). Additionally, infection with enteroinvasive *E. coli* was avoided in stable transfected dominant-negative NOD1 colon epithelial cells (209). Moreover, NF-κB activation induced by *Streptococcus pneumoniae*, which invade epithelial and endothelial cells, was dependent on NOD2, as well as NOD1 and NOD2 mRNA expression was up-regulated after pneumococcal infection (210). The number of viable internalised *Salmonella typhimurium* in Caco-2 cells, that are stably transfected with NOD2, was lower than in vector-transfected cells, indicating a direct anti-bacterial activity of NOD2 (211). These findings certainly open up a possible role of NOD proteins in killing enteroinvasive bacteria. However, extracellular non-invasive bacteria, such as *Helicobacter pylori*, have been shown to be recognized by NOD1. cagPAI-positive *H. pylori* can translocate the Cag effector protein via a type IV secretory system. Although none of these Cag proteins induced

inflammation, PGN fragments secreted through the type IV secretory system, induced NOD1 dependent activation of NF-κB. Digestion and identification of the HPLC fraction of the PGN of *H. pylori* showed the GlcNAc-MurNAc-tripeptide as active component (212), as reported by Chamaillard et al. and Girardin et al. (63, 189). NOD1 deficient and NOD1 knockout mice are more susceptible to infection with cagPAI-positive *H. pylori* strains (212). Another hypothesis was, that the intestinal epithelial apical di-/tripeptide transporter hPepT1 transports MDP and activates the cell via NOD2 (101). Although, some models have shown possible mechanisms how the muropeptides enter the cytosolic compartment, the question is not yet really answered. Although no direct interaction of muropeptides with NOD proteins have been shown yet, the NOD proteins are probably the muropeptide receptors or possible downstream molecules in muropeptide signalling.

3.6 Summary

Free muropeptides are present in the body during infection. They are released due to antibiotic administration or lytic host enzymes as well as during bacterial growth and division. The diversity of PGN of the different bacterial strains leads to a lot of possible muropeptide structures. One of the prominent muropeptides is muramyl dipeptide (MDP), which represents the minimal biologically active structure, and remarkably, only little larger structures exert biological activities. Muropeptides have diverse effects on the immune system: especially they are strong immune amplifiers and induce therefore priming and synergistic effects. Direct effects on immune cells appear to be rare, i.e. they are often observed at very high concentrations only and when LPS-contaminations are not excluded. Recently, the receptors for muropeptides, NOD1 and NOD2 have been found, although possible contributions especially of CD14 and possibly TLR cannot be excluded. Remarkably, these NOD proteins are intracellular receptors suggesting especially a role in phagocytes and for intracellular pathogens. Taken together, a few very specific partial structures of PGN can be sensed intracellular amplifying immune responses. This illustrates that a combination of immune effectors orchestrates the immune responses to bacterial pathogens.

3.7 Acknowledgements

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4 Lipoteichoic acid is the major immunostimulatory component of *Staphylococcus aureus*

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Running title: LTA is a major immune stimulus

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4.1 Abstract

Lipopolysaccharide is considered the main immunostimulatory principle of Gram-negative bacteria, but it is unclear whether peptidoglycan or lipoteichoic acid, which both signal via toll-like receptor 2, represents its Gram-positive counterpart. We did not find the activity of peptidoglycan mirrored by its supposed active principle muramyl dipeptide with regard to cytokine release, synergy with lipopolysaccharide or receptor usage. Also, structurally similar peptidoglycan from Gram-negative bacteria displayed no toll-like receptor 2-dependent cytokine induction. We detected lipoteichoic acid in commercial peptidoglycan preparations. Although extractable amounts were small, lipoteichoic acid presented on a surface was more potent by three log orders than soluble lipoteichoic acid with regard to cytokine induction. The novel inhibitor polypropylene glycol blocked cytokine induction by *Staphylococcus aureus*, peptidoglycan and lipoteichoic acid with similar IC_{50}, implying a similar

mechanism of cytokine induction. Thus, cytokine induction by *Staphylococcus aureus* or peptidoglycan can be attributed to their lipoteichoic acid content.

4.2 Introduction

Lipopolysaccharide (LPS, endotoxin) has been known as the main immunostimulatory component of Gram-negative bacteria since the 1950s. However, it is still not clear what the Gram-positive counterpart to LPS is. Peptidoglycan (PGN) and lipoteichoic acid (LTA) were considered the main candidates until Travassos et al. (2004) reported, that in contrast to commercially available PGN preparations, highly purified PGN does not activate the toll-like receptor 2 (TLR2). They concluded that the TLR2-dependent stimulatory activity of peptidoglycan of Gram-positive and Gram-negative bacteria is due to LTA or lipoprotein remnants. This is an interesting hypothesis, however, some important questions remain open: Are the immunostimulatory qualities of LTA and PGN so similar? Is LTA even contained in commercial PGN preparations? Can the amount of LTA present in commercial PGN be responsible for its immunostimulatory potency?

PGN is a scaffold chains of alternating N-acetylmuramic acid and N-acetyl-glucosamine with cross-linked peptide chains, constituting about half of the Gram-positive cell wall. It induces cytokine induction via TLR2 (23), contrary to LPS, which is recognized by TLR4 and MD-2 (71), while both employ CD14 and lipopolysaccharide-binding protein (LBP) (181). Noteworthy, apart from PGN, all ligands of TLR2 have a lipid anchor.

Numerous studies have investigated the activity of the breakdown products of PGN. Among numerous synthetic muropeptides, we confirmed muramyl dipeptide (MDP) as the minimal structure which synergizes with LPS at nM concentrations (134). However, endotoxin-free preparations of MDP and other muropeptides induce no cytokine release by themselves and their adjuvant activity appears to be mediated by NOD1 and NOD2 activation, not by TLR2 (213). These discrepancies certainly suggest that the cytokine-inducing activity of PGN preparations from Gram-positive bacteria might stem from structures other than MDP.

Teichoic acids, which make up the other half of the Gram-positive cell wall, do not have cytokine inductive activity (9). LTA make up about 6% of the cytoplasmic membrane, and protrude through the murein sacculus to the surface (8). LTA consists of a lipophilic glycolipid anchor and a hydrophilic backbone with repetitive units of glycerophosphate and D-alanyl esters, free hydroxyl and glycosyl substituents. Much of the literature on the activity of LTA is unreliable, as commercially available LTA preparations were shown to be heavily contaminated with LPS, nucleic acids and lipoproteins and the classic hot phenol extraction method leads to a loss of bioactivity, especially for LTA from *S. aureus* (19, 214). An optimized preparation procedure based on ambient butanol extraction yields highly pure (> 99% purity, < 30 pg LPS per mg LTA) and potent cytokine inducing material (10, 20). Chemical synthesis of LTA enabled structure-function studies to identify the components necessary for immune recognition (16, 215). Like peptidoglycan preparations, LTA employs TLR2, CD14 and partially LBP (15, 21, 22).

In this study, we address the questions posed above by showing that the immunostimulatory qualities of LTA and PGN are indeed similar in comparison to LPS, by proving that low amounts of LTA are contained in commercial PGN, by demonstrating that presentation of immobilized LTA, as in the framework of the murein sacculus, potentiates its immunostimulatory activity, and by showing the inhibition of cytokine induction by LTA, PGN and whole Staphylococcus aureus by a novel inhibitor.

4.3 Material and Methods

4.3.1 Materials

LTA from *Staphylococcus aureus* (DSM 20233), *Bacillus subtilis* (DSM 1087), *Listeria monocytogenes* (ATCC 43251), *Streptococcus agalacticae* (DSM 6313) and *Bifidobacterium animalis* variant a and b (MB 254), both gifts from Dr. Reinscheid, University of Ulm, Germany, and *Lactobacillus plantarum* (NCIMB 8826, a gift from E. Palumbo, Université Catholique de Louvain, Belgium), and were isolated in-house by n-butanol-extraction as described (216). LTA was extracted from 48 mg peptidoglycan from *S. aureus* (Fluka, Buchs, Switzerland) by the same method. The structural identity was indicated by capillary NMR at

600 MHz (Bruker BioSpin GmbH, Rheinstetten, Germany). Other substances were PGN from *Staphylococcus aureus* ATCC 29213 (Fluka and Toxin Technology, Eubio, Vienna, Austria), *Streptococcus pyogenes* type 3 strain D58 (Toxin Technology), *Bacillus subtilis*, *Streptomyces sp*. and *Micrococcus luteus* (Sigma); LPS from *Salmonella abortus equi*, *Serratia marcesens*, *Shigella flexneri*, *Klebsiella pneumoniae* and *Escherichia coli* O26:B6 and O11:B4 (Sigma); polyethylene glycol and polypropylene glycol (Fluka), polybutylene glycol (a kind gift from H. Mach, BASF, Ludwigshafen, Germany), Limulus anti-lipopolysaccharide factor (LALF) (a generous gift from F. Jordan, Charles River/Endosafe, Charleston, USA) and muramyl dipeptide (Bachem). Luria-Bertani bacterial cultures washed in phosphate buffered saline four times were ultra violet-inactivated on ice (5 min, 1200 kJ). LTA from *S. aureus* was labeled with rhodamine by sonication with sulforhodamine Q 5-acid fluoride, dimethyl sulfoxide (DMSO) and trimethylamine, then passed through a PD-10 column. Labeling efficiency was 1-2 rhodamine per LTA. Bisamphiphilic LTA (MW 3147) was synthesized in analogy to synthetic LTA (217) and characterized by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and nuclear magnetic resonance (NMR) at 600.12 MHz and 300 K. Aqueous NH_3, PPG 1200 or glycerol, absolute ethanol and tetraethoxysilane at 50 ℃ under continuous stirring for 24 h to make PP G 1200 or glycerol surface-ended amorphous silica spheres. All non-endotoxins were tested by LAL Assay (Kinetic Chromogenic LAL, Charles River Endosafe, i.e. <0.05 endotoxin units/mg LTA or PGN).

To prepare Gram-negative PGN, *E. coli* K12 cells were extracted in boiling sodium dodecyl sulfate (SDS)/H_2O, digested with α-amylase, DNase I, lipase and pronase, then reextracted. To minimize contaminating endotoxin, samples were incubated with albumin-coated beads (Matisse-beads, a gift from Fresenius Hemocare developed as an LPS adsorber for clinical use). The remaining endotoxin content was 90 endotoxin units/mg PGN. GlcNAc content was determined by high performance liquid chromatography.

4.3.2 Methods

4.3.2.1 Incubations

Heparinized whole blood from healthy donors controlled by differential blood count was diluted 5-fold with RPMI 1640 (BioWhittaker Europe) and stimulated over night (37 ℃, 5% CO_2). For coating, 50 µl/well LTA from *S. aureus* were added to 96-well polystyrene plates (Greiner) at 37 ℃ for the times indicated. Wells were washed twice with saline before whole blood incubation. In some experiments, Alamar blue (Biosource) turnover was assessed after incubation.
Femurs from C3H/HeN and C3H/HeJ mice (Charles River), and TLR-2 knockouts on a Sv129xC57BL/6 background and wild types (kindly provided by Tularik) were flushed with ice-cold phosphate buffered saline. Bone marrow cells were plated in RPMI 1640 with 10% fetal calf serum and 100 international units/ml penicillin/streptomycin and stimulated overnight (5% CO_2 and 37 ℃).
Cytokines were measured with commercially available ELISA antibody pairs. RNA was isolated with a QIAamp© RNA Blood Mini Kit (Qiagen) including DNase digestion after 6 h human whole blood incubation. After reverse transcription, real-time PCR for TNFα and cyclophilin were performed on a LightCycler® (Roche).

4.3.2.2 Interaction of LTA and PPG

PPG 1200 or glycerol-containing spheres were incubated with rhodamine-labeled LTA under agitation. Blood erythrocytes were lysed with FACS (fluorescence activated cell sorter) lysing solution (BD Biosciences). Cells were then incubated with PPG 1200 or saline at 4 ℃ and washed twice before incubation with rhodamine-labeled LTA. In another experiment, rhodamine-labeled LTA was preincubated with PPG 1200, then washed and added to the blood cells. Fluorescence of spheres or leukocytes was measured in a FACS Calibur (BD Biosciences).

4.3.2.3 Statistics

Repeated measures analysis of variance followed by Dunnett's Multiple Comparison test and non-linear regression (sigmoidal dose response) with Kruskal Wallis test were calculated using GraphPad Prism 3.0 (GraphPad software). Data are given as means ± S.E.M.

4.4 Results and Discussion

4.4.1 PGN and LTA have similar immunostimulatory qualities

Contrary to MDP (134), PGN induces cytokine release by itself and does not synergize with LPS (figure 4A). Like LTA, PGN is a strong inducer of IL-8 but a relatively weak inducer of TNFα in whole blood in comparison to LPS (figure 4B,C). There have been other reports of similar activity of LTA and PGN, notably in dendritic cell maturation (218) and SP-D binding (219), both using highly purified LTA. Thus there appear to be more similarities between the activity of PGN and LTA than between PGN and MDP or LPS.

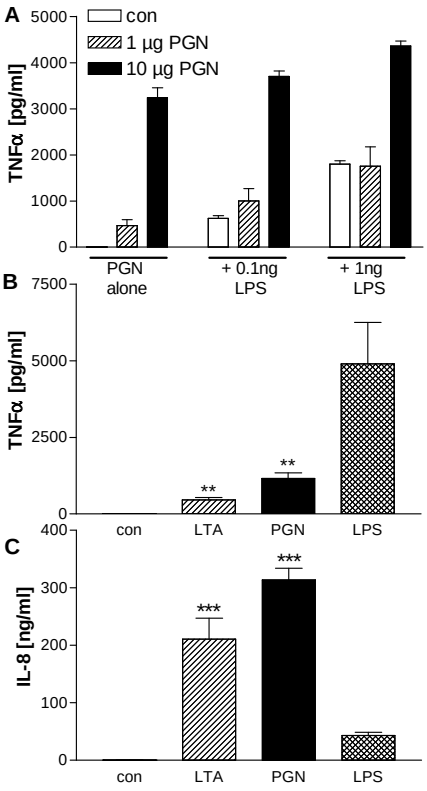

FIGURE 4:
PGN does not synergize with LPS, but induces a similar cytokine pattern to LTA. Whole blood was stimulated with (A) combinations of PGN and LPS (n=4) or (B,C) 10µg/ml LTA, PGN or LPS. Significant difference from LPS was determined by repeated measures ANOVA and Dunnet's multiple comparison test.

4.4.2 PGN from Gram-negative bacteria displays no TLR2-dependent activity

PGN from *E. coli* could not induce IL-6 release in bone marrow cells from C3H/HeJ mice with non-functional TLR4. When selectively blocking the remaining LPS contamination (< 1%) with the Limulus anti-LPS factor LALF (10 µg/ml), only little cytokine induction and no TLR2-dependency was found using bone marrow cells from control and TLR2 knockout mice (340 ± 40 vs. 250 ± 51, n=8, n.s.). As expected, commercial PGN from *S. aureus* was TLR2- and not TLR4-dependent. Thus, we confirm and complement the results of (220)) that Gram-negative PGN lacks a TLR2-dependent component, which could be LTA in case of Gram-positive PGN.

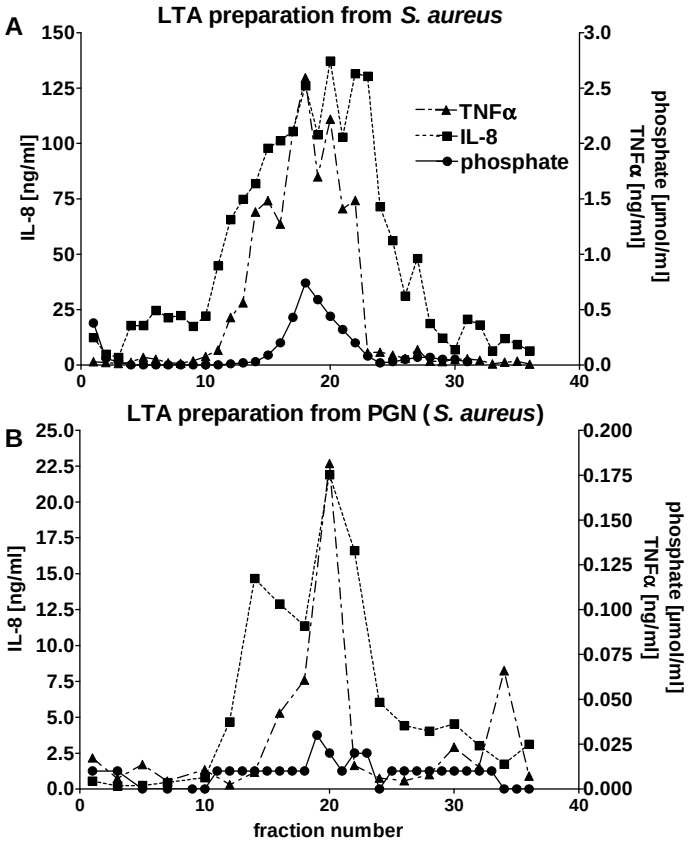

FIGURE 5:
Butanol extracts of *S. aureus* or PGN induce similar cytokine pattern and contain phosphate in the same fractions. Cytokine induction in whole blood by extracts from (A) *S. aureus* (n=2) or (B) PGN (n=4). Fractions 13 to 33 contain phosphate, the lead activity for LTA.

4.4.3 Extraction of LTA from PGN

We attempted to extract LTA from 48 mg commercial *S. aureus* PGN. The elution profile presented both cytokine inducing activity (TNFα and IL-8) and

phosphate content (lead activity for LTA) in fractions that typically contain LTA (compare figure 5A, B). The amount of LTA eluted was estimated at 10-20 µg based on cytokine induction and phosphate content. The presence of LTA in the respective fraction pool was indicated by representative NMR resonance of glycerol methine (δ_H 5.4), the anomeric proton of α-D-N-acetylglucosamine (δ_H 5.08), methyl (δ_H 0.8) and methylene (δ_H 1.3). Thus, commercial PGN does contain a small amount of LTA.

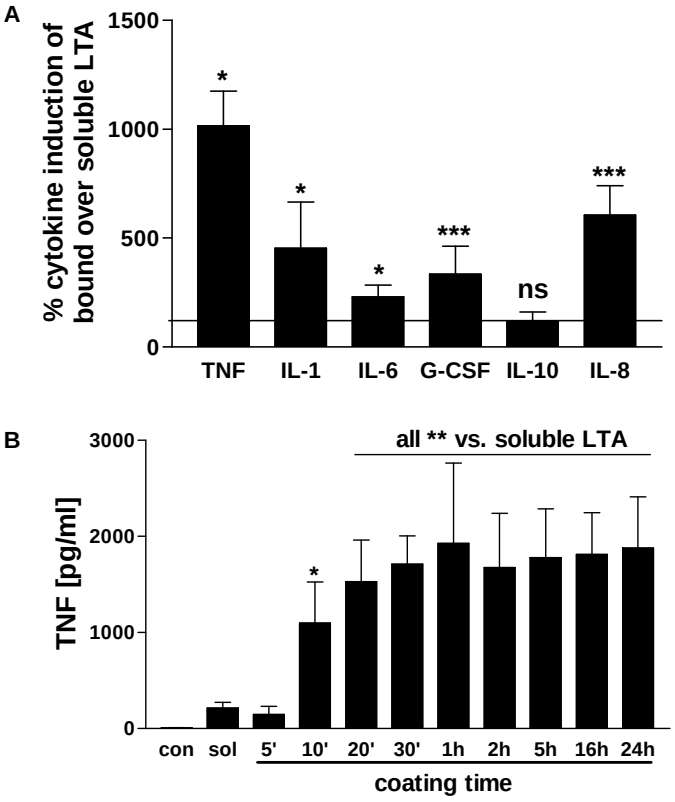

FIGURE 6:
Binding of LTA to polystyrene amplifies cytokine release. A, Ratio of cytokine response of whole blood to 2 µg/well LTA coated to polystyrene over soluble LTA (set to 100%, n=8). B, Kinetics of 2 µg/well LTA binding to polystyrene for the times indicated compared to soluble LTA (sol), n=4-12. Statistics were calculated with raw data using repeated measures ANOVA and Dunnet's multiple comparison test.

4.4.4 Strongly increased potency of LTA presented on a surface

As this small quantity of LTA would be too little to explain cytokine induction by peptidoglycan, we investigated whether LTA presented to immune cells immobilized on a surface structure might have a greater potency than soluble LTA molecules. This would resemble the physiological situation of fixation in the murein sacculus more closely. To model this situation, we preincubated LTA on 96-well plates and removed unbound molecules before incubation with blood. Measurements with rhodamine-labeled LTA showed that only about 1% (after 2 h) and 4% (after 24 h) of the 2 µg/well LTA employed remained bound to the

polystyrene surface. Further increasing the amount of LTA used resulted only in minor increases of deposition. These data were confirmed by stimulating blood with supernatant from the coated plates: Despite prior coating for 24 h, the immunostimulatory potential of the supernatant remained constant (e.g. for TNFα: soluble LTA 1890 ± 915 pg/ml, supernatant after 24 h coating 2225 ± 825 pg/ml, n=4, n.s.), confirming that only a minor percentage of the molecules could have attached to the surface during the coating process.

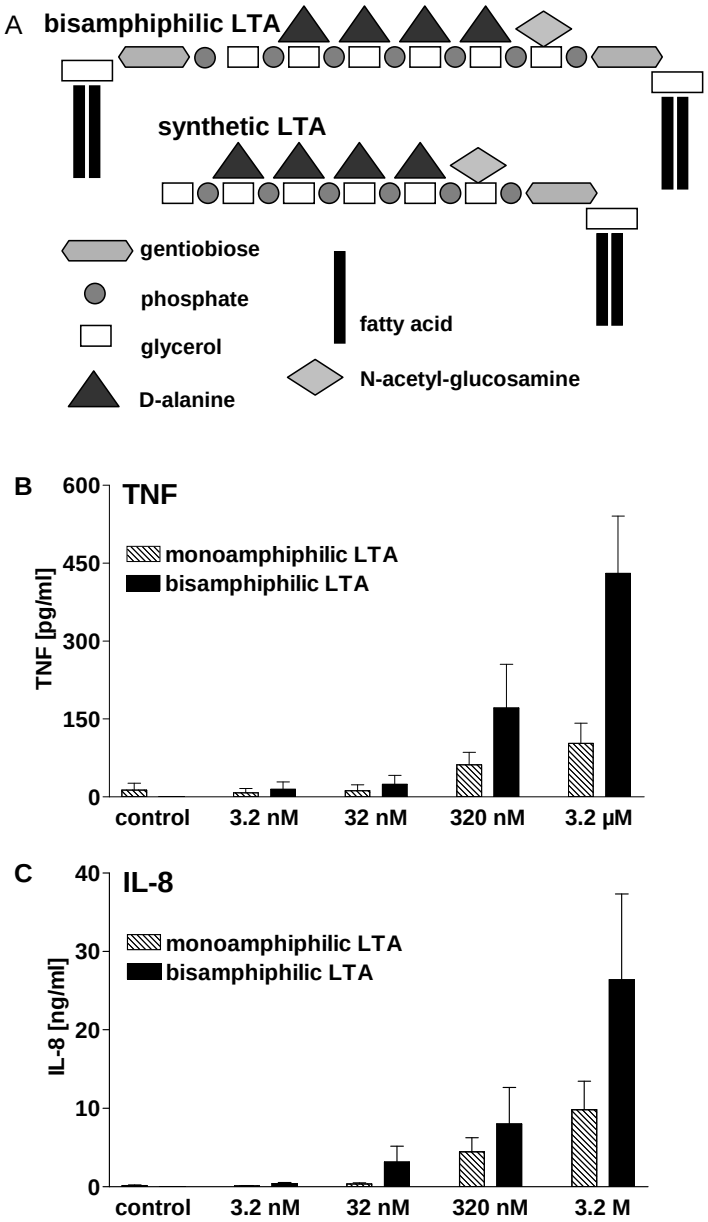

FIGURE 7:
Bisamphiphilic LTA is more potent than mono-amphiphilic LTA. (A) Structural illustration of bisamphiphilic and mono-amphiphilic LTA. (B) TNF and (C) IL-8 induction in human whole blood by equimolar quantities of bisamphiphilic and mono-amphiphilic synthetic LTA, n=4.

However, this presentation of LTA on the surface increased its cytokine-inducing activity drastically regarding the release of TNFα, IL-1β, IL-6, IL-8 and

G-CSF (figure 6A), while the release of IL-10 was not altered. This was reflected on the mRNA level measured by real-time PCR (LTA-induced TNFα mRNA at 6 h: soluble LTA: 3.6-fold ± 0.6 over control; bound LTA: 8-fold ± 0.9 over control, all normalized to cyclophilin, n=3, $p<0.05$). Cytokine levels induced by coated LTA correlated with the coating duration (figure 6B). Thus, the TNFα-inducing activity of LTA was multiplied by a factor of about 1000 (1% of the LTA was deposited, but it was 10 times more potent than soluble LTA). Going back to the 10-20 µg LTA we extracted from 48 mg PGN, a multiplication in potency by this factor can well explain the entire cytokine inductive capacity of the PGN.

Addition of soluble LTA to bound LTA (2 µg/well initial stimulus each) led to lower cytokine induction compared to bound LTA alone (for TNFα release: bound LTA alone 2.4 ± 0.5 ng/ml vs. bound + soluble LTA 1.5 ± 0.4 ng/ml, $p<0.05$, n=8), implying that soluble LTA is a partial agonist of the receptor of bound LTA with a lower efficacy.

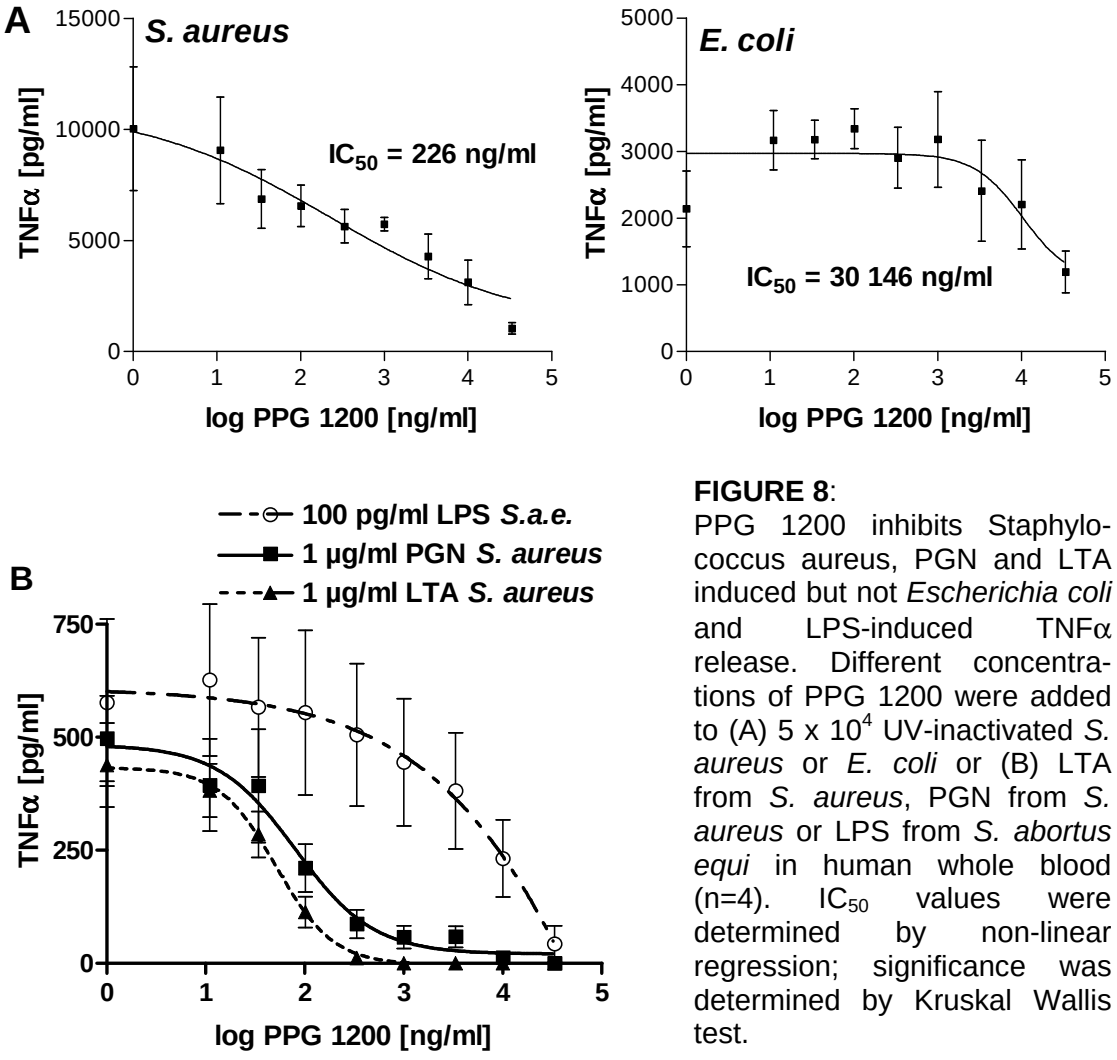

FIGURE 8:
PPG 1200 inhibits Staphylococcus aureus, PGN and LTA induced but not *Escherichia coli* and LPS-induced TNFα release. Different concentrations of PPG 1200 were added to (A) 5 x 10^4 UV-inactivated *S. aureus* or *E. coli* or (B) LTA from *S. aureus*, PGN from *S. aureus* or LPS from *S. abortus equi* in human whole blood (n=4). IC_{50} values were determined by non-linear regression; significance was determined by Kruskal Wallis test.

4.4.5 Bisamphiphilic LTA is more potent than monoamphiphilic LTA

The coat effect might be interpreted as cross-linking of individual LTA molecules. We synthesized, based on our first synthesis LTA (215), an artificial bisamphiphilic LTA (figure 7A), modeling a fixed cross-linkage of two LTA molecules. This bisamphiphilic LTA induced higher TNFα and IL-8 levels than soluble monoamphiphilic LTA at the same molarity (figure 7B,C), indicating that the increase in potency of the bound LTA may stem from cross-linkage of receptors.

Augmentation of LTA activity by cross-linking has been suggested earlier in experiments with anti-polyglycerophosphate antibodies (30). Together, these results suggest that the increased activity of bound LTA might lie in receptor clustering.

Substance **1 µg/ml LTA**	**IC_{50}** **[ng/ml]**	**p**
PPG 400	>1000	**
PPG 1200	51	***
PPG 2000	73	***
PPG 2150	72	***
PPG 3000	92	***
PPG 3250	905	***
PEG 600	>10 000	n.s.
PEG 1000	>10 000	n.s.
PEG 2000	>10 000	n.s.
PEG 3000	>3 000	n.s.
PEG 6000	>10 000	n.s.
PBG 640	>10 000	n.s.
PBG 1240	>10 000	n.s.

TABLE 3: Inhibition of LTA-induced TNFα by different glycols. 1 µg/ml LTA from *S. aureus* was incubated with different concentrations of glycols in 20% human whole blood from four different donors and TNFα release was measured by ELISA. IC_{50} values were determined by non-linear regression; significance was determined by Kruskal Wallis test.

4.4.6 Polypropylene glycol is a novel LTA-specific inhibitor

Glycols and closely related compounds, including PPG, have previously been attributed with bactericidal or bacteriostatic properties (221, 222). We investigated the inhibitory properties of polypropylene glycol (PPG) on cytokine induction by Gram-positive immune stimuli. PPG 1200 inhibited TNFα release in whole blood induced by UV-inactivated *S. aureus* while cytokine induction by *E. coli* was only affected at much higher concentrations (figure 8A). Similarly, PPG 1200 inhibited cytokine induction by either LTA or PGN from *S. aureus*, but only interfered partially with LPS-induced cytokine release at 100fold higher concentrations (figure 8B). This was also true for the release of other cytokines (for 1 μg/ml LTA, the IC_{50} of PPG 1200 for IL-1β induction was 72 ng/ml, for IL-6, 83 ng/ml and for IL-8, 161 ng/ml, n=4, all $p<0.01$). Tables 3 and 4 list the blocking efficacy of other glycol compounds such as PPG of different molecular weights, polyethylene glycol (PEG) and polybutylene glycol (PBG), as well as the respective IC_{50} values for LTA, PGN and LPS from different bacterial species. None of the glycols were cytotoxic at concentrations up to 33 μg/ml as measured by Alamar blue metabolism in blood after incubation. PPG 1200 was found to be the best inhibitory structure tested. It inhibited cytokine induction by different LTA and PGN but not by any LPS. Two of the LTA preparations were inhibited less effectively by PPG. These preparations retained some activity in TLR2 knockout mice (data not shown), indicating that this difference may be a result of minimal structural differences.

4.4.7 PPG 1200 binds LTA

To clarify whether PPG 1200, as the most effective inhibitor, interferes with ligand or receptor, we performed flow cytometry using nanobeads coated with PPG 1200 or glycerol, and rhodamine-labeled LTA. Labeled LTA retained its full ability to induce TNFα and IL-8 formation in whole blood (for 5 μg/ml stimulus: rhodamine-labeled LTA induced 470 ± 80 pg/ml TNFα and 70 ± 6.2 ng/ml IL-8, while unlabeled LTA induced 570 ± 150 pg/ml TNFα and 76 ± 6 ng/ml IL-8, n=4, both n.s.), while rhodamine alone did not induce cytokine release. The PPG-

carrying nanobeads bound more rhodamine-labeled LTA than those with glycerol (figure 9).

1 µg/ml LTA PPG 1200	**IC_{50} [ng/ml]**	**p**
S. aureus	51	***
S. agalacticae	> 3 000	*
Bifidobacterium a	118	***
Bifidobacterium b	> 1 000	*
B. subtilis	68	***
L. monocytogenes 1	474	***
L. monocytogenes 2	75	***
L. plantarum	731	**
1 µg/ml PGN PPG 1200	**IC_{50} [ng/ml]**	**p**
S. aureus (Sigma)	80	***
S. aureus (Toxin Tech.)	234	**
S. pyogenes	37	**
M. luteus	217	**
B. subtilis	407	*
10 ng/ml LPS PPG 1200	**IC_{50} [ng/ml]**	**p**
Serratia marcesens	>10 000	n.s.
Shigella flexneri	>10 000	n.s.
Klebsiella pneumoniae	>3 000	n.s.
S. abortus equi	>10 000	n.s.
E. coli (026:B6)	>10 000	n.s.
E. coli (011:B4)	>3 000	n.s.

TABLE 4: PPG 1200 specifically inhibits LTA-induced but not LPS-induced TNFα release. Different concentrations of PPG 1200 and fixed concentrations of LTA, PGN or LPS were incubated with 20% human whole blood of four different donors and TNFα release was determined by ELISA. IC_{50} values were determined by non-linear regression; significance was determined by Kruskal Wallis test.

Rhodamine-labeled LTA bound to human monocytes and could be displaced by ten-fold higher concentrations of unlabeled LTA (median fluorescence 1240 ± 100 vs. 450 ± 21, n=3). However, preincubation of leukocytes with PPG 1200 before addition of rhodamine-labeled LTA or preincubation of PPG and rhodamine-labeled LTA before addition of leukocytes did not affect the binding of rhodamine-labeled LTA to the leukocytes. Together, this indicates that LTA binds to PPG and that this complex still binds to the cells, though it cannot activate them any longer.

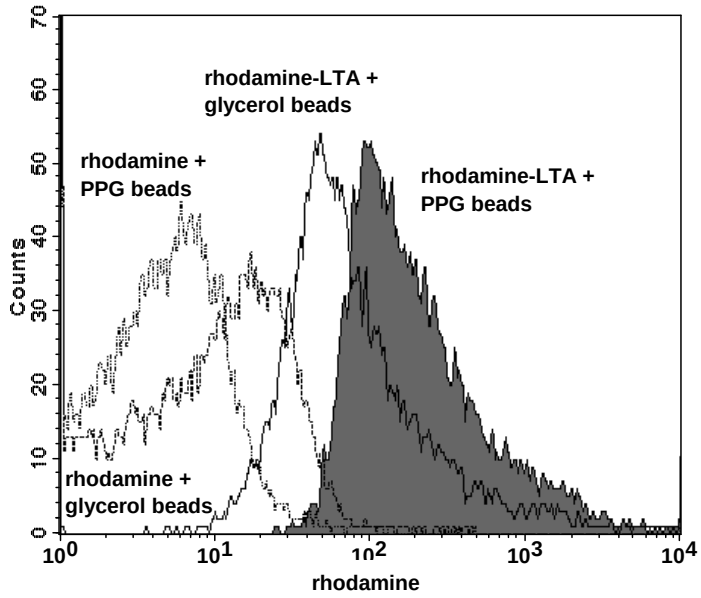

FIGURE 9:
Fluorescent LTA binds PPG-ended silica spheres. Rhodamine-labeled LTA was incubated with PPG-ended or glycerol-ended spheres, washed and measured by flow cytometry (representative of 3 independent experiments with blood from different donors).

Taken together, these studies highlight the need for stringent quality control of microbial stimuli in research on innate immunity. Minimal contaminations with extremely potent and environmentally stable structures such as LPS and here LTA are sufficient to falsify results. Contaminations with LPS can usually be excluded by LAL assay, as long as interference of the sample with LPS recovery is excluded. No such simple exclusion is yet available for LTA contamination, though the novel LTA-inhibitor PPG 1200 may prove a useful tool. Thus, much previous work on LTA and PGN as well as reports on ‘new’ ligands of TLR must be viewed critically.

Taken together, LTA appears to represent the active principle of the cytokine inducing activity of PGN and thus represents the major immunostimulatory component of Gram-positive bacteria. The identification of a specific inhibitor of LTA activity may aid in the identification of LTA-mediated actions, quality control

of bacterial preparations and, being a non-toxic compound, may have potential for clinical application in Gram-positive bacterial disease.

4.5 Acknowledgements

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5 Structural requirements of synthetic muropeptides to synergize with LPS in cytokine induction

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Running title: Structural requirements of muropeptides to synergize with LPS

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5.1 Abstract

Muropeptides contribute to the recognition of bacteria by modulating immune responses: the structural requirements for adjuvant activity were described in the seventies. During the last years, our knowledge of bacterial pattern recognition has increased dramatically and the importance of the absence of contaminations in both muropeptide preparations and other bacterial stimuli has become clear. We investigated a panel of 15 synthetic Limulus-negative muropeptides, four of them synthesized for the first time, as to their potency to synergize with lipopolysaccharide (LPS) in cytokine induction in human whole blood. No muropeptide was capable of stimulating cytokine release from human blood. However, as little as 20 nM of the muropeptides N-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, M(ADiQ)), N-acetylglucosamine-muramyl dipeptide GM(ADiQ), or C_{18}M(ADiQ) which carries a non-natural additional fatty acid, sufficed to induce an up to three log-order shift in TNFα-release in response to 100 pg/ml LPS. The release of IL-1β, IL-6, and IL-10 was also significantly enhanced though to a lesser extent. The synergistic effect was stereoselective with M(ADiQ) being the minimal active principle. Synergy was

also observed on the transcriptional level by means of real-time PCR. Smaller molecules like N-acetylmuramic acid (M), AM, carrying a naturally occurring 1,6-anhydro-bound in M or M(A), containing only the amino acid L-alanine neither synergized with LPS nor influenced the synergy of other muropeptides with LPS. In conclusion, these data show that nanomolar quantities of muropeptides dramatically potentate LPS-induced monocyte activation. This has implications for pyrogenicity testing and endotoxemia in patients.

5.2 Introduction

The adjuvant activities of degradation products of peptidoglycan, a major constituent of the Gram-negative and Gram-positive cell wall, are well known and have been intensively characterized for decades (115). In 1974, muramyl dipeptide (MDP), was found to represent the minimal active principle of peptidoglycan (37). Beside its capacity to potentate the immunogenicity of vaccines, MDP contributes to stimulation of host defence as well as resistance to cancer (223). Furthermore, MDP has been shown to prime for increased responsiveness towards LPS *in vitro* and *in vivo* (14, 127, 133, 135, 155, 156, 167, 171). This priming effect is of great importance because our understanding of pattern recognition by the innate immune system so far was primarily based on studies of effects of single components such as LPS. A major obstacle in studying interactions between different bacterial cell wall components like LPS, peptidoglycan or lipopeptides is the difficulty of purifying these components free of LPS- or lipopeptide-contaminations. Such purity is difficult to achieve with conventional isolation procedures: the microheterogeneity of these structures adds to the problem. For example, macrophages are activated by picogram/ml concentrations of LPS whereas microgram/ml concentrations of most muropeptides are required to induce cytokine release.

This problem has been overcome by chemical synthesis of defined structures. Such investigations started three decades ago in the case of muropeptides with the focus on adjuvant activity (112, 129, 224-226). At this time, pyrogen exclusion by the Limulus amebocyte lysate assay (LAL) was in its infancy. More recent work addresses the anti-tumour activity of synthesized conjugates of MDP (111) or the ability of glycoside derivatives of MDP to release IL-1β or

TNFα from murine macrophages (227). However, no pyrogen exclusion was performed in these studies. It thus appeared timely to readdress the structure-function relationship of muropeptide activity in the light of the emerging knowledge on bacterial pattern recognition. We therefore complemented a panel of commercially available muropeptide derivatives with nine structures that we synthesized ourselves. Four of these structures were synthesized for the first time. The new structures included typical peptidoglycan breakdown products of the Gram-negative and Gram-positive cell wall, like M(ADiQ) (usually ill-defined as MDP), M(A), which carries only one amino acid and AM, which consists of muramic acid (M) in an anhydroform, which are released during bacterial growth during infection or by bacteriolysis resulting from host defence mechanisms or administration of antibiotics (228, 229). Furthermore, we synthesized derivatives containing diaminopimelic acid for the first time. These substances underwent a thorough chemical analysis by NMR and MS as well as LAL testing. These highly defined muropeptide structures representing natural breakdown products of peptidoglycan of Gram-negative and Gram-positive bacteria, as well as some variants that are not found in nature, were tested as to their ability to synergize with LPS in inducing cytokine release.

5.3 Material and Methods

5.3.1 Muropeptides

Our strategy to synthesise several natural and non-natural muropeptides (Table 5, compounds 2-4 and 8-13) was partly based on the previously published synthesis of differentially protected *meso*-diaminopimelic acid (*m*-DAP) (230, 231). To this end bifunctional DAP (Table 6a) was synthesized from benzyloxycarbonyl-protected L-glutamate and a known glycine phosphonate (232, 233) via a Wittig-Horner reaction to get the 2,3-dehydro-DAP. Catalytic hydrogenation using Wilkinson's catalyst gave a 3:2 D:L-ratio of the newly created stereogenic center. Final separation by medium pressure liquid chromatography (MPLC) yielded both LL- and LD-(*meso*)-diastereomers. The assignment of the stereogenic centers of the DAP dimethylesters was accomplished by conversion of each single diastereomer to the bis-*N*-

camphanic acid amide derivative and NMR analysis (600 MHz) of the methyl ester region (234). The muramic acid moiety (Table 6b) was synthesised according to our previously published method (235, 236) from 1,3,4,6-tetra-*O*-acetyl-2-azido-2-deoxyglucopyranose (237). Saponification of the methyl ester of two and attachment of the first amino acid (L-alanine methyl ester) prevented the often observed formation of muramic acid 1',2-lactam during azide reduction (238, 239). The resulting sugar building block (Table 6c) was used for the synthesis of larger muropeptides. For 1,6-anhydromuramic acid, a new and efficient synthetic route was established: 4-*O*-benzyl-D-glucal (240) was transformed to the 1,6-anhydro-4-*O*-benzyl-2-iodo-*β*-D-glucopyranose via an *in situ* generated 4,6-di-*O*-dibutyltin ether and iodine (241, 242). Substitution of the iodine by azide and alkylation of the 3-*O*-position (236, 243) yielded the protected anhydromuramic acid (Table 6d) in seven steps with overall yield > 41%. The peptide side chains were built from commercially available, suitably protected amino acids and *m*-DAP, in solution phase using the well-known system PyBOP/NMM/CH_2Cl_2 (244) which generally provided yields > 95%. The same system was used to couple these oligopeptides to the free acids of (c) and (d) (Table 6), after alkaline cleavage of their methyl esters. Deprotection of the resulting muramylpeptides was accomplished in two to three steps: hydrogenolysis and *N*-acetylation transformed azide groups in acetamido groups and concomitantly *β*-benzyl protecting groups were removed; all ester groups were removed by alkaline saponification; acid-labile protecting groups were removed by treatment with trifluoroacetic acid in water/dioxane/methanol. The deprotection for the anhydromuropeptides had to be performed particularly carefully; acid-labile protecting groups were cleaved by treatment of the protected starting material with 50% trifluoroacetic acid/CH_2Cl_2 to prevent opening of the anhydro cycle. Afterwards, the ester groups were cleaved by aqueous alkali. Gel chromatography was used in most cases to purify the crude products from salts and impurities. Additional chromatographic methods, including ion exchange, high performance liquid chromatography, or reversed-phase chromatography were applied in some cases to obtain the pure products.

To abbreviate the different muropeptides, the following nomenclature was developed: The carbohydrate portion was abbreviated with M (*N*-acetylmuramic acid) and with AM indicating an 1,6-anhydro-form of muramic acid. GM (G = *N*-

acetylglucosamine) and $C_{18}M$ means an additional substituent of a further sugar residue or a stearic acid ester at C6-position. The peptide sequence was attached in brackets using the one letter code for amino acids and using the prefixes D, L and *m* (*meso*) for the stereochemical occurrence as well as i (iso) for structural variants. Diaminopimelic acid has not been assigned a one letter code; thus Dpm was used as abbreviation. γ and ε indicate the linkage of the amino acids. Table 5 gives the abbreviations, chemical nomenclature as well as the source (NK = synthesized by Niels Kubasch, Konstanz, Germany; Sigma, Deisenhofen, Germany; Bachem, Bubendorf, Switzerland; Calzyme, San Luis Obispo, USA) of the different compounds employed in this study. Compounds marked by an *a* are structurally identical to known fragments of peptidoglycan and can be released during bacterial growth or bacteriolysis (228, 229). All muropeptides were free of endotoxin contaminations as indicated by negative LAL test (QCL-1000, Bio Whittaker, Verviers, Belgium) in the final concentration employed in this study, i.e. all preparations contained endotoxin equivalents <0.05 endotoxin units/ml, and 0.5 endotoxin units/ml of endotoxin spikes of the sample were fully recovered showing neither interference nor synergy of LPS and muropeptides in the LAL assay.

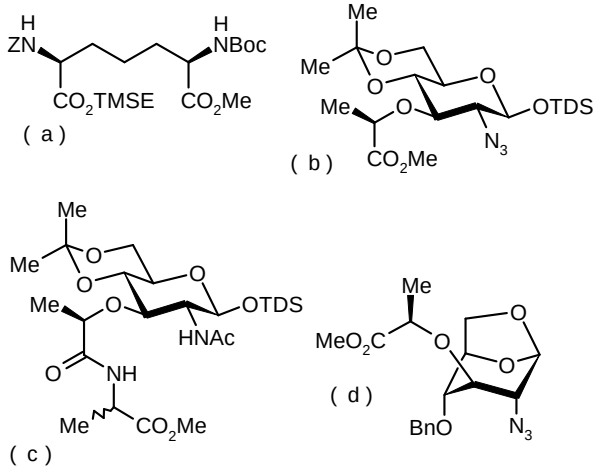
5.3.2 Whole blood incubations

Incubation of human whole blood in the presence of the bacterial stimuli was performed essentially as described (245). Heparinized blood freshly taken from healthy volunteers was diluted 5-fold with RPMI 1640 (BioWhittaker). The different stimuli were prepared as stock solutions in aqua ad injectabile (Braun, Melsungen, Germany). LPS from *Salmonella abortus equi* was purchased from Sigma and either used directly or after a further purification to eliminate putative lipopeptide contaminations by a phenol re-extraction according to Hirschfeld et al. (246). Incubations were carried out in open polypropylene reaction tubes (Eppendorf, Hamburg, Germany) at 37 °C and 5% CO_2 for 24 h. Cell-free supernatants were obtained by centrifugation at 400 x *g* for 2 min (Heraeus, Hanau, Germany) and stored at –80 °C until measurement.

No	Abbreviation	Nomenclature	Source
1	M	MurNAc	Sigma[a]
2	AM	1,6-Anhydro-MurNAc	NK[a,b]
3	M(A)	MurNAc-L-Ala	NK[a]
4	M(ADiQ)	MurNAc-L-Ala-D-isoGln	NK[a]
5	M(ADiQ)	MurNAc-L-Ala-D-isoGln	Bachem[a]
6	M(AiQ)	MurNAc-L-Ala-L-isoGln	Bachem
7	M(DADiQ)	MurNAc-D-Ala-D-isoGln	Bachem
8	M(AγDE)	MurNAc-L-Ala-γ-D-Glu	NK[a]
9	M(AγDEK)	MurNAc-L-Ala-γ-D-Glu-L-Lys	NK[a]
10	M(AγDE*m*Dpm)	MurNAc-L-Ala-γ-D-Glu-*meso*-Dpm	NK[a]
11	M(AγDE*m*DpmDA)	MurNAc-L-Ala-γ-D-Glu-*meso*-Dpm-D-Ala	NK[a]
12	AM(AγDE*m*Dpm)	1,6-Anhydro-MurNAc-L-Ala-γ-D-Glu-*meso*-Dpm	NK[a]
13	AM(AγDELLDpm)	1,6-Anhydro-MurNAc-L-Ala-γ-D-Glu-LL-Dpm	NK
14	C_{18}M(ADiQ)	6-O-stearoyl-MurNAc-L-Ala-D-isoGln	Bachem
15	M(ADiQKεC_{18})	MurNAc-L-Ala-D-isoGln-N-ε-stearoyl-L-Lys	Sigma
16	GM(ADiQ)	GlcNAc-β(1→4)MurNAc-L-Ala-D-isoGln	Calzyme[a]

[a] Represent structural compounds of peptidoglycan
[b] NK, synthesized by Niels Kubasch.

TABLE 5: Abbreviation, nomenclature and source of muropeptides



The abbreviations used are: Bn, benzyl; Me, methyl; NBoc, *N*-*t*-butyloxycarbonyl; TDS, *t*-hexyldimethylsilyl; TMSE, 2-(trimethylsilyl)ethylester; Z, benzyloxycarbonyl

TABLE 6: Synthesis of muropeptides

5.3.3 Monocytes

Peripheral blood mononuclear cells of healthy volunteers were prepared with CPT™ Cell Preparation Tubes (BD Biosciences). Negative isolation of monocytes was done with the Monocyte Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturers protocol. Cells were plated in 96-well plates at a density of 5 x 10^4 cells/well in RPMI in the presence of 10% fetal calf serum (Biochrom, Berlin, Germany) and stimulated for 24 h at 5% CO_2 and 37 °C. Supernatants were stored at –80 °C until measurement.

5.3.4 Isolated liver perfusion

C57/BL6 mice (Charles River, Sulzfeld, Germany, bred in the internal animal facility of the University of Konstanz) were pretreated (intraperitoneally) for 1 h with 2.5 µg/kg LPS, 700 µg/kg galactosamine (GalN, Roth, Karlsruhe, Germany) and 250 µg/kg M(ADiQ). After 1 h mice were put to sleep intravenously with 150 mg/kg pentobarbital (Narcoren®, Merial, Halbergmoos, Germany) and 0.8 mg/kg heparin (Sigma). The *Vena portae* and the *Vena cava inferior* of the mouse liver (C57/BL6) were cannulated and the organ was perfused blood free with a modified Krebs-Henseleit buffer with a total volume of 25 ml buffer in a closed recirculation under constant pressure conditions and samples were obtained for the times indicated in the graph. The temperature of the perfusate was kept constant at 37°C and oxygenation with pure oxygen at a pressure of 500 mbar was performed. During perfusion, the perfusate flow-through of the liver as well as the pressure were constantly measured and recorded by a special software (Lab View, National Instruments, Austin, TX). Samples for alanine aminotransferase and lactate deydrogenase measurements (247) were taken from the perfusate at different time points, as indicated in the figure 14.

5.3.5 Cytokine determination

Cytokines were measured by ELISA based on antibody pairs from Endogen, Biozol, Eching, Germany (TNFα and IL-1), R&D, Minneapolis, MN (IL-6), BD Pharmingen, Hamburg, Germany (IL-10 and interferon γ). Recombinant cytokines serving as standards were from National Institute for Biological Standards and Control (Hertfordshire, United Kingdom) (TNFα and IL-1), from Thomae (Biberach, Germany) (interferon γ) and BD Pharmingen (IL-6 and IL-10). Binding of biotinylated antibody was quantified using streptavidin-peroxidase (Dianova, Hamburg, Germany) and the substrate TMB (3,3`,5,5`-tetramethylbenzidine, Sigma).

5.3.6 Preparation of mRNA and reverse transcription

For preparation of mRNA, human whole blood incubations were stimulated with 100 pg/ml LPS and 20 nM M(ADiQ) for 2 or 4 h. After 2 h, 1 µM actinomycin D was added to some incubations and for a further 2 h. mRNA was isolated with the QiAmp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. Possible contaminating DNA was digested with the RNase-free DNase set (Qiagen). Reverse transcription for PCR was performed using a Perkin-Elmer Geneamp PCR system 9600 with 5 mM $MgCl_2$, 2 µl 10x-PCR-buffer II, 1 mM dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 2.5 µM Oligo $d(T)_{16}$ (Thermo Hybaid, Ulm, Germany), 20 units/µl RNase-inhibitor (Roche), 50 units/µl murine leukaemia virus-reverse transcriptase (Perkin Elmer Life Sciences) und 4.2 µl RNase-free water.

5.3.7 Real-time PCR

Real-time PCR was performed on a LightCycler rapid thermal cycler system (Roche). The cDNA for TNFα and GAPDH was amplified using LightCycler FastStart DNA Master SYBR Green (Roche) according to the manufacturers protocol. The $MgCl_2$ concentration was adjusted to 3 mM for TNFα and 4 mM

for GAPDH. The thermal cycling was performed (50 cycles) with an annealing temperature of 65 °C and an elongation time of 11 seconds for TNFα and 18 seconds for GAPDH. The amplification was followed by a melting program, which started at 54 °C for 45 seconds and then increased to 95 °C at 0.1 °C/second. The specific melting temperatures for TNFα and GAPDH products were 93 °C and 86.5 °C. All primers (0.5 µM) were from Thermo Hybaid: TNFα 5`-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3` (forward), 5`-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3` (reverse); GAPDH 5`-GAAGGTGAAGGTCGGAGTC-3` (forward), 5`-GAAGATGGTGATGGGATTTC-3` (reverse). The amplification products had a length of 226 bp (GAPDH) and 682 bp (TNFα), respectively.

5.3.8 Statistics

Statistical analysis was performed using the GraphPad Prism program (GraphPad Software, San Diego, CA). All data are means ± S.E.M. Significance of differences was assessed by the Wilcoxon test for two groups and by analysis of variance employing the Bonferroni test for experiments with more than two groups. Non-linear regression was calculated according to Gompertz equation for the study of Xylem cell development by Rossi et al. (248). In the figures *, ** and *** represent *p* values <0.05, <0.01 and <0.001, respectively.

5.4 Results

5.4.1 Synergistic effect of LPS and M(ADiQ)

To determine the most pronounced synergistic effect of LPS and M(ADiQ), human whole blood was incubated with 100 pg/ml LPS and increasing amounts of M(ADiQ) (2 pM to 200 nM). LAL-negative muropeptides alone induced essentially no cytokine release (< 16 pg TNFα/ml blood). A significant synergistic effect between LPS and M(ADiQ) was observed from a concentration of 20 nM of M(ADiQ), which corresponds to 10 ng/ml M(ADiQ) (figure 10). Because commercially available LPS preparations might contain

highly bioactive contaminants that can effect biological read-outs, we further purified the LPS by phenol re-extraction as published by Hirschfeld et al. (246). However, as shown in figure 11, the capacity of LPS to induce cytokine release from human whole blood was not significantly altered by the re-purification procedure. Furthermore, the synergistic effect of M(ADiQ) was not dependent on the purity of the LPS preparation.

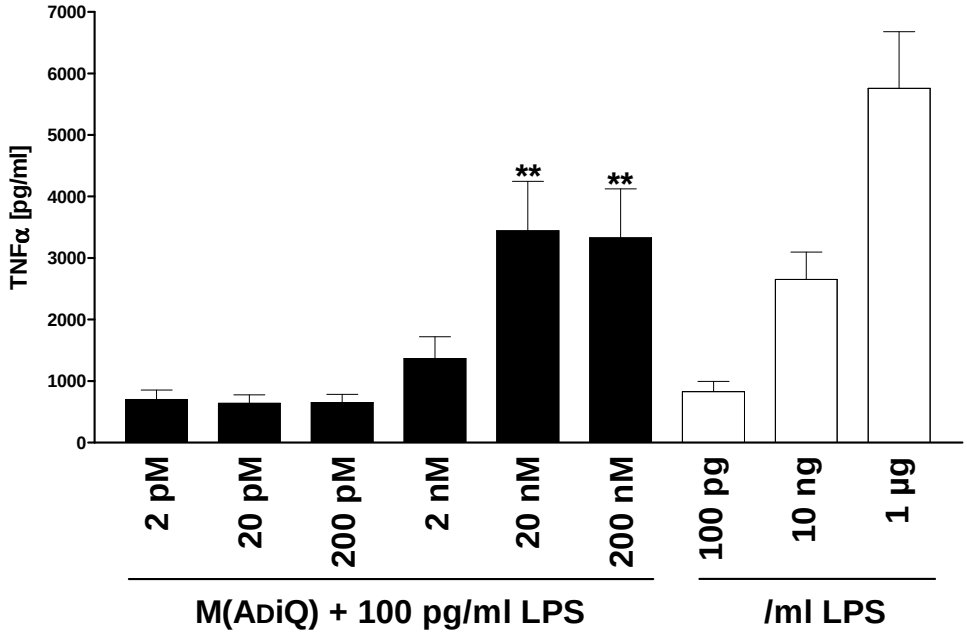


FIGURE 10: Synergistic effect of M(ADiQ) on LPS-induced TNFα release in blood. Human whole blood was stimulated with LPS in the concentrations indicated or with increasing concentrations of M(ADiQ) (2 pM to 200 nM) + 100 pg/ml LPS. After 24 h of incubation, TNFα was determined in cell-free supernatants by ELISA. TNFα levels of unstimulated controls and M(ADiQ) alone were < 3 pg/ml and < 16 pg/ml, respectively. Data are given in means ± S.E.M. of 18 donors, performed in three independent experiments. ** indicates significance compared to stimulation with 100 pg/ml LPS.

To investigate the effect of M(ADiQ) pre-incubation, human whole blood was incubated with 20 nM M(ADiQ) for 4 h and re-stimulated with 100 pg/ml LPS. The results were compared to an incubation in which LPS and M(ADiQ) were added simultaneously. Pre-incubation of M(ADiQ) led to 10% increase of TNFα release compared to simultaneous stimulation with LPS (data not shown). Since this difference was not statistically significant, stimuli were added together in all further experiments.

As indicated in figure 12a, costimulation of human whole blood with different concentrations of LPS and 20 nM M(ADiQ) led to a shift to the left of the concentration-response curve of LPS-induced TNFα release by about three log orders. Similarly, in isolated monocytes in the presence of 20 nM M(ADiQ), 100 pg/ml LPS achieved TNFα levels comparable with stimulation with 1 µg/ml LPS (figure 13).

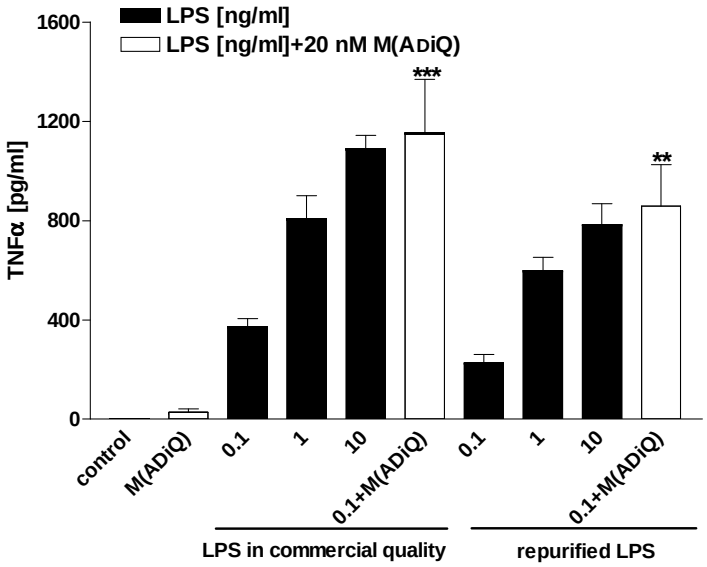


FIGURE 11: Synergistic effect of M(ADiQ) on TNFα release in blood stimulated with repurified LPS. Human whole blood was stimulated with LPS in commercial quality or re-purified LPS in the concentrations indicated in the presence or absence of 20 nM M(ADiQ). After 24 h of incubation, TNFα was determined in cell-free supernatants by ELISA. Data are means ± S.E.M. of 4 donors. ** and *** indicate significance compared to stimulation with 100 pg/ml LPS.

Furthermore, the synergistic effect of LPS and M(ADiQ), although to a lesser extent, was also observed for the release of IL-1β, IL-6 and IL-10 (figure 12b and 12c). In extension, M(ADiQ) significantly enhanced the LPS-induced liver injury in galactosamine-sensitized mice, measured *in situ* as alanine aminotransferase liberation into the perfusate of isolated livers (figure 14), indicating that *ex vivo* a similar synergistic endotoxic liver injury is observed such as *in vivo*.

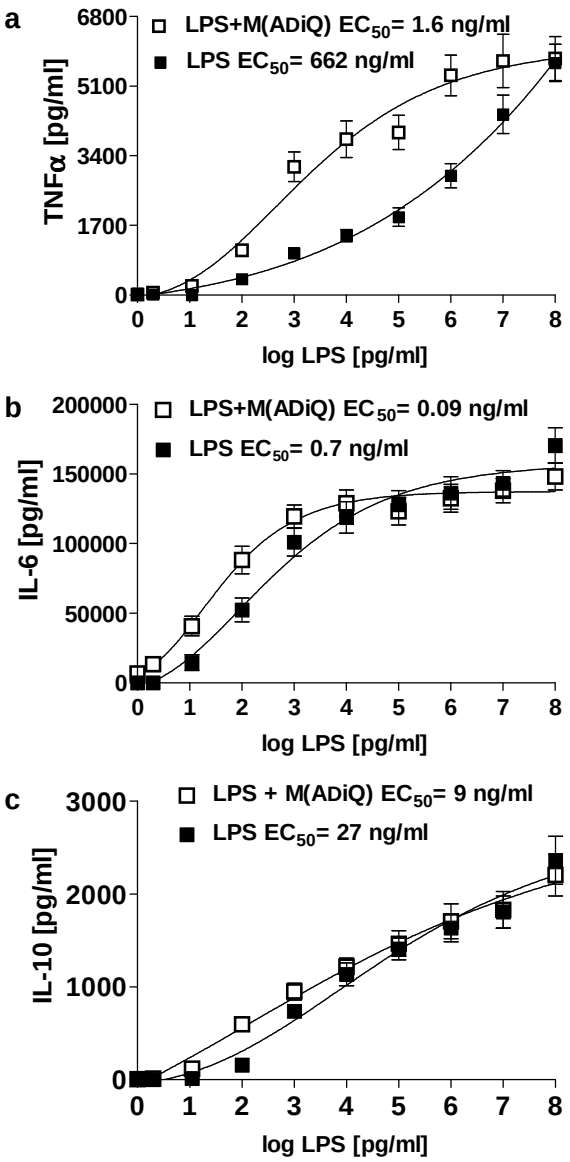


FIGURE 12:
Synergistic effect of M(ADiQ) on cytokine release in blood stimulated with different LPS concentrations. Human whole blood was stimulated in quadruplicates of 5 donors with increasing concentrations of LPS (10 pg/ml to 100 µg/ml) in the presence or absence of 20 nM M(ADiQ) . TNFα (a), IL-6 (b), and IL-10 (c) levels were determined by ELISA after 24 h of incubation in the cell-free supernatants. Non-linear regression and EC_{50} were calculated according to Gompertz. Data are means ± S.E.M.

5.4.2 Kinetics of TNFα release and mRNA formation in the presence of M(ADiQ)

TNFα release from human whole blood was determined at different time points after stimulation with 100 pg/ml LPS alone or costimulation with 20 nM M(ADiQ). A synergistic effect was evident after 4 h of incubation. Maximal TNFα release was reached after 8 h in both series of experiments, indicating that M(ADiQ) did not alter the kinetics of TNFα release (figure 15).

To investigate whether the synergistic effect of M(ADiQ) on LPS induced TNFα-release is because of enhanced transcription of TNFα mRNA, human whole blood was stimulated with 100 pg/ml LPS ± 20 nM M(ADiQ) in the presence or

absence of 1 µM actinomycin D. TNFα mRNA was determined by quantitative real-time PCR. Samples costimulated with LPS and M(ADiQ) showed an increased expression of TNFα mRNA after 4 h compared with stimulation using LPS only. The presence of 1 µM actinomycin D blunted the effect of M(ADiQ) on TNFα mRNA expression, whereas GAPDH levels remained unchanged. These results indicate that M(ADiQ) increases LPS-induced TNFα mRNA, which seems to be because of increased *de novo* transcription rather than to an increase in mRNA stability (figure 16).

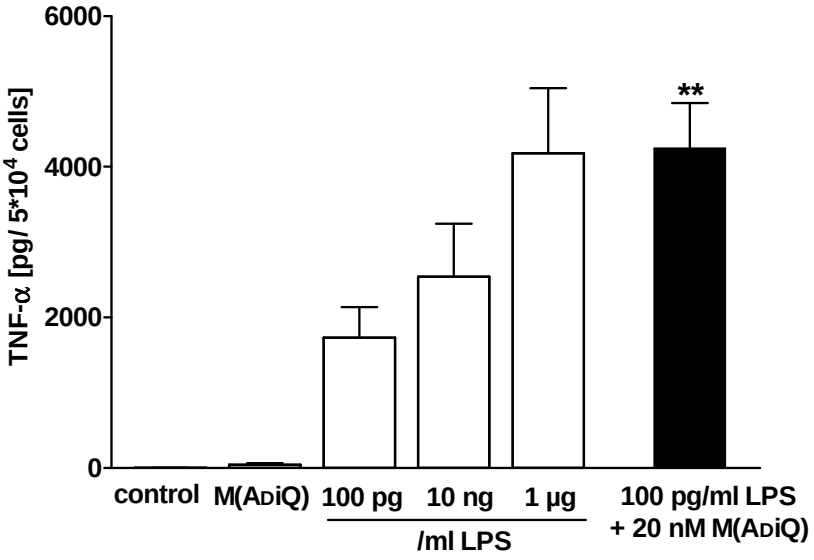


FIGURE 13: Synergistic effect of M(ADiQ) on LPS-induced TNFα release from monocytes. Isolated human monocytes were stimulated with LPS in the concentrations indicated or with 20 nM M(ADiQ) ± 100 pg/ml LPS. TNFα release was determined by ELISA after 18 h of incubation. Data are means ± SEM of 5 donors. ** indicates significance compared to stimulation with 100 pg/ml LPS.

5.4.3 Dependence of synergy on muropeptide structure

Fifteen muropeptides with different structures were investigated with regard to their synergistic activity with LPS in monocyte activation. Nine of the different muropeptide derivatives, which were not commercially available, were synthesized; the others were bought in highly pure quality (table 5). Purity and identity was confirmed by LAL, NMR and MS analysis for all muropeptides. Control experiments confirmed that 20 nM of all muropeptides induced no

significant cytokine release from human whole blood. The muramic acid (M) and the anhydroform of the muramic acid (AM) showed no synergistic effect when coincubated with LPS (data not shown). Furthermore, as depicted in figure 17, the muropeptide M(A), which carries only the amino acid L-alanine, also did not synergize with LPS, but a strong synergism was observed if this structure was extended by the addition of the amino acid isoglutamine, i.e. M(ADiQ). M(ADiQ), with the natural N-acetylglucosamine (GM(ADiQ)) showed a similar synergistic effect.

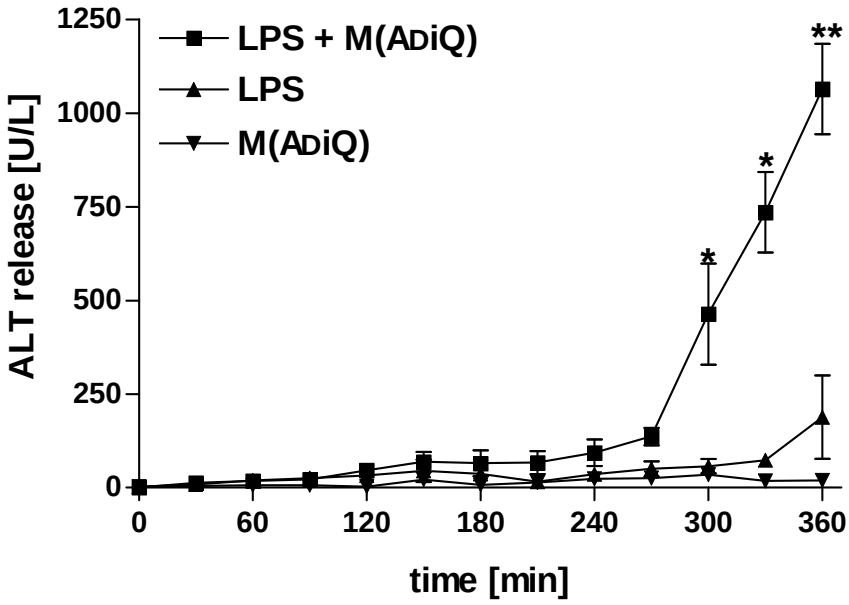


FIGURE 14: Synergistic effect of M(ADiQ) and LPS in inducing liver injury in isolated perfused mouse liver. Mice were injected (intra peritoneal) with 2.5 µg/kg LPS and 700 µg/kg galactosamine with or without 250 µg/kg M(ADiQ). One hour later, livers were excised and subjected to recirculating perfusion. The liberation of alanine aminotransferase (ALT) into the perfusate was monitored over time. LPS alone (▲) or in combination with M(ADiQ) (■), as well as M(ADiQ) alone (▼) are shown as means ± S.E.M. of 4 livers. * and ** indicate significance towards stimulation with LPS only.

The peptidoglycans of different bacteria have similar structures though the amino acids of the peptide subunits differ. Therefore, we synthesized M(ADiQ), changed the isoglutamine to a glutamate (M(AγDE)) and additionally a derivative extended by L-lysine (M(AγDEK). Both changes did not result in loss of synergistic activity compared with M(ADiQ). However, when the third amino acid was replaced by diaminopimelic acid M(AγDE*m*Dpm) the synergistic effect was

completely abolished. Synergism could also not be restored by addition of alanine (M(AγDE*m*DpmDA)). Derivatives, carrying an anhydro-muramic acid and diaminopimelic acid in either *meso* (AM(AγDE*m*Dpm) or LL-form AM(AγDELLDpm)) were also inactive.

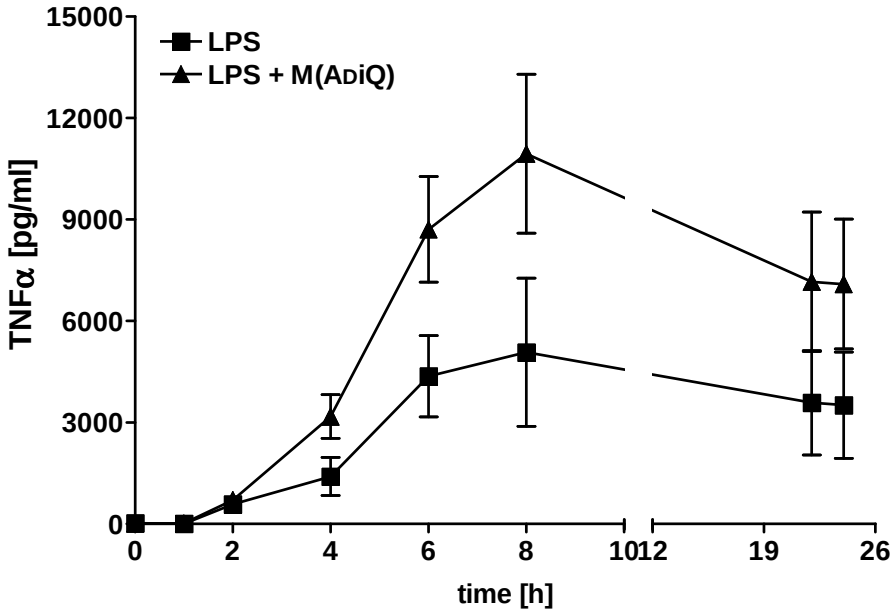


FIGURE 15: Kinetics of TNFα release in blood induced by LPS and M(ADiQ). Human whole blood was stimulated with 100 pg/ml LPS ± 20 nM M(ADiQ) for the time periods indicated. TNFα release was determined by ELISA in the cell-free supernatants. Data are means ± S.E.M. of 4 donors.

The amino acids alanine and isoglutamine in the peptide string of M(ADiQ) are naturally found in LD conformation, according to Fischers nomenclature. When the stereochemical formation was changed to the LL or DD form (M(AiQ) and M(DADiQ), respectively, no synergistic effect could be observed, indicating that the recognition is stereoselective. This series of molecules shows that M(ADiQ) and (M(AγDE) represent the minimal synergistic structure of muropeptides and that exactly two amino acids are required to reach the maximal synergistic effect.

Artificial lipophilic muropeptides have the advantage of being less rapidly eliminated *in vivo*. We found that M(ADiQ) carrying an artificial fatty acid ester bound to M (C_{18}M(ADiQ)) showed a synergistic effect comparable to M(ADiQ), whereas the addition of an artificial fatty acid ester bound to the peptide chain (M(ADiQKεC_{18})) reduced the synergistic effect (figure 17).

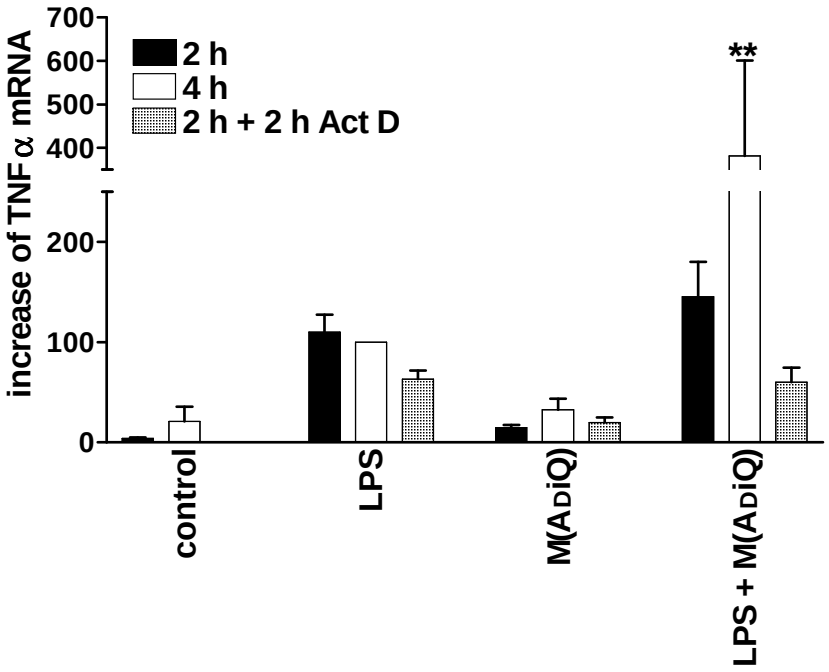


FIGURE 16: Synergy of LPS and M(ADiQ) in inducing TNFα mRNA. mRNA was isolated from human whole blood stimulated with 100 pg/ml LPS ± 20 nM M(ADiQ) in the presence or absence of 1 µM actinomycin D (Act D). Actinomycin D was given 2 h after the start of the incubation and was present last 2 h. TNFα and GAPDH levels were determined by real-time PCR. Data were normalised to GAPDH levels and are given as the percent increase above 4 h LPS stimulation. Data are means ± S.E.M. of 9 donors. ** indicates significance compared to stimulation with LPS + MDP + actinomycin D (Act D).

5.4.4 Possible antagonistic effects of small muropeptides

To investigate whether the synergistic effect of M(ADiQ) and LPS can be antagonized by smaller muropeptides, human whole blood was stimulated with 100 pg/ml LPS plus 20nM M(ADiQ) ± increasing amounts (20 nM up to 20 µM) of the smaller structures AM, M and M(A). The synergism of LPS and M(ADiQ) was not significantly reduced (maximally –25%) in any experiment indicating that the synergistic effect of M(ADiQ) is not antagonised by muropeptide structures smaller than the M(ADiQ).

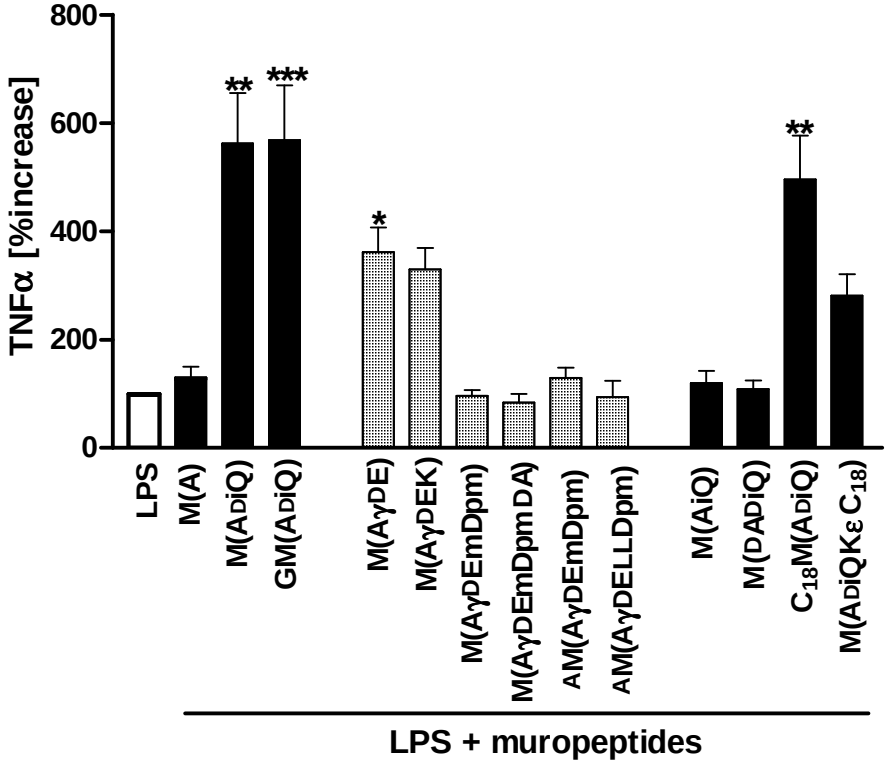


FIGURE 17: Synergistic effect of various muropeptides on LPS-induced TNFα release in blood. Human whole blood was incubated in the presence of 100 pg/ml LPS ± 20 nM of the different muropeptides indicated. TNFα was measured by ELISA after 24 h of incubation in the cell-free supernatants. TNFα levels of unstimulated controls were < 5 pg/ml. Data were normalized, 100 pg/ml LPS was set to 100% which corresponds to 700 ± 200 pg/ml TNFα. Data are given in percent and are means ± S.E.M. of 7 donors. *, ** and *** indicate significance towards stimulation with LPS only.

5.5 Discussion

The adjuvant activity of peptidoglycan has been attributed to muropeptide structures, the dipeptide M(ADiQ) (commonly but ill-defined abbreviated as MDP) being the minimal structure (37). The synergy of LPS and muropeptides to induce cytokines represents a likely but yet unproven explanation for the adjuvant activity of muropeptides. An *in vitro* synergism of M(ADiQ) with LPS in inducing cytokines was shown in various experimental settings including murine macrophages, human monocytic cell lines and whole blood (14, 127, 133, 135). M(ADiQ) alone has several times been reported to be capable of stimulating cytokine release (129, 130, 225, 227, 249, 250). However, in all these studies very high concentrations of M(ADiQ) (i.e. 1 µg/ml up to 100 µg/ml) were used and in most cases no exclusion of endotoxin contamination was carried out.

Here, we demonstrate that in human whole blood as little as 20 nM (i.e. 10 ng/ml) of M(ADiQ) and M(ADiQ)-derivatives of highly pure quality sufficed to increase the TNFα-releasing capacity of LPS about 4-fold and induced a shift to the left of the concentration-response-curve by a factor of 1000. In our hands, none of the LAL-negative preparations showed any cytokine inducing activity alone. Furthermore, this study provides a detailed biochemical analysis of the structural requirements of muropeptides to synergize with LPS in a physiological environment.

M(ADiQ), because of its low molecular weight and water solubility, is rapidly excreted from the body. After intravenous injection of M(ADiQ) (1.5 mg/kg) in rats, M(ADiQ) plasma levels immediately start to decline. Thirty minutes after injection the M(ADiQ) plasma level went from 27 µg/ml down to 4 µg/ml and was no longer detectable after 2 h (102). Microgram/ml quantities of muropeptides therefore might be relevant in case of adjuvant activities for vaccination purposes but do not seem to reflect the conditions during bacterial infection.

The very low levels of muropeptides i.e. 10 ng/ml in case of M(ADiQ), which are shown here for the first time, to suffice to synergize with low quantities of LPS, represent an observation of major importance. The fact that the synergism is also seen with highly purified LPS indicates that no contaminants like lipoproteins are responsible for this effect. Furthermore, the experiment with isolated monocytes shows that the synergistic effect is not dependent on a bystander-effect of further blood cells or serum components. The observed synergism was most pronounced for the release of TNFα, but translated also to other cytokines such as IL-1β, IL-6 and IL-10, indicating that the effect is more general. Wang et al. (133) reported a 3-fold increase of LPS (10 ng/ml)-induced TNFα from human whole blood when coincubated with 1 µg/ml M(ADiQ) and a 2-fold increase in IL-6 release, whereas IL-10 remained unchanged. The difference in IL-10 release might be because of the use of different stimulus concentrations as well as differences in the incubation time (6 hours in the study of Wang et al. *versus* 24 hours in our study). IL-10 is produced late after the initial activation of monocytes and not released in major amounts before 8 h (data not shown).

The addition of M(ADiQ) enhanced LPS-induced transcription of TNFα mRNA and expression of TNFα protein, but did not alter the kinetics of TNFα release.

This is in line with the finding of Wolfert et al. (135) who reported an increased transcription of LPS-induced TNFα mRNA in MonoMac-6 cells when coincubated with 100 µg/ml M(ADiQ). However, in their experimental setting, 100 µg/ml M(ADiQ) alone induced a 10-fold induction of TNFα mRNA, which did not result in protein expression. The lowest M(ADiQ) concentration investigated in their study was 10 ng/ml, which, however, did not induce TNFα mRNA (135). Unfortunately, this concentration was not tested for a putative synergism with LPS.

Furthermore, the results of our study indicate that the synergistic effect of LPS and M(ADiQ) also translates to mature macrophages in their organotypic environment. *In situ* perfusion of livers from mice isolated after *in vivo* treatment showed a significantly greater damage when coincubated with LPS and M(ADiQ), compared to LPS alone. Synergy with regard to lethal toxicity *in vivo* has been reported earlier (156, 159, 251). The results of our *ex vivo* experiment show that the synergy takes place at the level of injury of target organs.

Our analysis of the structure function relationship of a panel of 15 different muropeptides, containing naturally occurring structures of the Gram-negative and Gram-positive cell wall like M(A), M(ADiQ), GM(ADiQ), M(AγDE), M(AγDEK), M(AγDE*m*Dpm), M(AγDE*m*DpmDA), AM(AγDE*m*Dpm) and artificial structures like C_{18}M(ADiQ), M(ADiQKεC_{18}), M(DADiQ) and M(AiQ) revealed that M with two additional amino acids in the LD configuration (M(ADiQ) and M(AγDE)) are necessary and sufficient to exert the maximal synergistic effect with LPS in stimulating cytokine release from human whole blood. Until now, a large number of synthetic muropeptide derivatives has been developed for vaccination purposes and their biological activity has been intensively characterized (115, 224). Among these adjuvants were hydrophobic acyl derivatives, derivatives with chemical modifications at the carboxyl group, and several others. However, none of these structures is of relevance during natural bacterial infections. Kotani et al. reviewed the immunopharmacological activities of M(ADiQ) and its analogs (112). In line with our findings they reported that M(ADiQ) and M(AγDE) were most potent, while the corresponding stereoisomers (LL or DD configuration) were inactive. However, no synergistic effects with LPS in case of cytokine release were addressed in this review. The stereoselectivity of the adjuvant and toxic action of muropeptides has been

demonstrated (152, 159, 225). Our finding that the addition of a C_{18} at M does not alter the synergistic qualities, whereas comparable modification at the peptide chain blunted the effect further pronounces the importance of the amino acids and their configuration.

The introduction of Dpm into the muropeptide led to a reduction of the synergistic effect. Not much is known about the synergistic action of muropeptides containing Dpm. Takada et al. (159) showed that a muramyl tripeptide (M(AγDiQDpm) prepared from *Lactobacillus plantarum* containing Dpm, primes for anaphylactoid reactions induced by LPS in C3H/HeJ mice, but its priming activity was weak compared with M(ADiQ). Furthermore, it remains to be defined whether the nature of synergism described in these experiments in LPS non-responsive mice is translatable to the synergism described in LPS responsive cells. GAM(ADiQ*m*DpmDA) was reported to stimulate cytokine expression from human monocytes and GAM(AγDE*m*DpmDA) was shown to synergize with LPS in the activation of hamster epithelial cells (171, 250). Both substances contained Dpm. However, the structures although quite similar to our AM(AγDE*m*Dpm) or M(AγDE*m*DpmA) are not the same and furthermore, have been obtained by biochemical digestion and not by synthesis. Because microgram/ml quantities are used, neither contaminating endotoxins nor further muropeptides with a higher biological activity can be fully excluded although LAL testing was performed.

So far, no study has shown employing synthetic muropeptides larger than a dipeptide at relevant concentrations, and that such molecules have any biological activity. A proportion of 1% M(ADiQ) and less when employing microgram quantities cannot be excluded.

Taken together, our data suggest that muropeptides are natural amplifiers of biological effects. The responses to LPS differ dramatically in the absence or presence of muropeptides from Gram-negative and Gram-positive cell walls, which generally accompany LPS in natural infections. The finding that the LAL-negative preparations used here did not induce any cytokine release in human whole blood even at microgram/ml concentrations characterizes the muropeptides rather as synergists, than as independent immune stimuli. Therefore, antagonizing such effects might represent an interesting anti-inflammatory strategy leaving the LPS-based recognition of pathogens intact.

5.6 Acknowledgements

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6 Synergistic intracellular sensing by NOD2 and TLR9 enables activation of human monocytes by CpG-DNA and muropeptides

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Running title: Synergistic sensing by NOD2 and TLR9

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submitted to J Biol Chem

6.1 Abstract

During an infection with intracellular bacteria, or after phagocytosis, bacterial DNA, as well as muropeptides, are possibly sensed via the intracellular immune receptors toll-like receptor (TLR) 9 and NOD1/2. While neither synthetic CpG-oligodeoxynucleotides (ODN) nor muropeptides alone could induce significant TNF release from human peripheral blood mononuclear cells (PBMC), a

significant synergy of 100 nM MDP or various synthesized larger muropeptides with 1 µM 2006-CpG was found. The synergism was observed with different types of K- and D-type CpG-ODN and also occurred at higher concentrations with GpC-ODN, indicating that in humans the effect is not strictly sequence specific.

The synergistic effect of CpG-ODN and MDP was mediated via TLR9 and NOD2 and not dependent on TLR2 or TLR4, as confirmed by the use of bone marrow or peritoneal macrophages derived from the respective knockout mice. Furthermore, the synergism could not be observed in PBMC from human volunteers with the 3020insC mutation in NOD2. Notably, the synergism was not observed in CD14 purified monocytes alone, which lack the expression of TLR9. This indicates that the TLR9 dependent component is provided by another cell type, probably plasmacytoid dendritic cells. IFNα is likely to be one of the key mediators of this bystander effect.

6.2 Introduction

Activation of the innate immune system represents a first line of defence against invading pathogens. There is emerging evidence, that beside the interaction of immune cells with bacteria, which are present in extracellullar compartments, recognition of intracellular replicating bacteria, or bacterial particles, which are taken up by phagocytes, is also of major importance (252, 253). So far, the immune receptors toll-like receptor 9 (TLR9) and NOD proteins have been shown to be located intracellular. TLR9 is exclusively expressed in the endoplasmatic reticulum (56) and was shown to recognize unmethylated CpG DNA (48, 83), which is a characteristic bacterial DNA motif (254). TLR9 is a type-1 transmembrane protein with an extracellular leucin-rich domain and the highly conserved intracellular toll/interleukin-1 receptor domain. After ligand binding, TLR9 dimerizes and signals in a MyD88-dependent manner, leading to the activation of AP1 and NF-κB, which switches on host defense genes, for review see (1). Recent studies show, that only a limited number of cells express TLR9 at a high level, e.g. plasmacytoid dendritic cells (pDC) (48, 49, 255, 256) and B-cells (50), while myeloid dendritic cells, natural killer cells, T-cells and macrophages were found to express only low levels of TLR9 (49).

Recently, the cytoplasmatic proteins NOD1 and NOD2 were identified as intracellular pattern recognition receptors (PRR) (192, 208). Both consist of three distinct domains, a C-terminal one with a leucine-rich repeat (LRR), which is critical for ligand recognition, a central nucleotide binding site which mediates oligomerization and one or two N-terminal caspase recruiting domains (CARD), which are involved in regulation of apoptosis and activation of inflammatory responses. After ligand binding, NOD oligomerizes and CARD activates the inflammatory cascade via interaction with the downstream receptor interacting protein 2, which in turn activates NF-κB, for review see (61, 257). NOD2, whose expression is restricted to monocytes, macrophages (194) and to Paneth cells in the terminal ileum (199, 258), is a PRR for muramyl dipeptide (MDP) (64, 65), a major constituent and typical breakdown product of peptidoglycan (PGN), which is present in almost all bacterial cell walls. Mutations in the NOD2 gene have been linked to the incidence of Crohn's disease (174, 201, 202, 204) and therefore suggest a major role of NOD2 in intestinal immunity. The 3020insC mutation, a frame shift mutation, leading to the truncation of the terminal LRR, and thus to a protein which cannot detect MDP anymore, is the most common mutation associated with Crohn's disease (64, 65, 259).

NOD1 is expressed in virtually all tissues (191) and recognizes muropeptides, which contain *meso*-diaminopimelic acid (63, 189), a PGN motif which occurs in most Gram-negative bacteria, but only in a few Gram-positive bacteria such as *Listeria monocytogenes* or *Bacillus* spp. (193).

MDP is known to be a weak inducer of cytokine release on its own, even if µM quantities are used, but a potent synergist to LPS (134). The role of bacterial DNA as an immune stimulatory principle on its own is also strongly questioned, since high concentrations are necessary to achieve low levels of cytokine release. Since both represent common bacterial structures, which are released continuously during infection either by bacterial replication or after treatment with antibiotics, we aimed to investigate a putative synergism of their intracellular sensing.

6.3 Material and Methods

6.3.1 Stimuli

The different oligodeoxynucleotides (ODN) are given in table 7 and were purchased from TIB-Molbiol (Berlin, Germany), except for 2216-CpG, which was purchased from MWG Biotech (Ebersberg, Germany). The different muropeptides are given in table 8 and were purchased from Bachem (B, Weil am Rhein, Germany) or synthesized by Dr. Niels Kubasch (NK, University of Konstanz, Germany (231)). All muropeptides were negative in the LAL test (Charles River/Endosafe, Charleston, USA). Polymyxin B (PolyB) was purchased from Sigma (Deisenhofen, Germany) and the LPS-specific binding protein LALF (260, 261) was a kind gift from Dr. N. Wainwright and F. Jordan (Charles River/Endosafe).

Name	Sequence*	Length	Remarks
2006-CpG	tcg tcg ttt tgt cgt ttt gtc gtt	24	K-type
2006-GpC	tgc tgc ttt tgt gct ttt gtg ctt	24	K-type
48-ODN	tcg tcg ttt tgt cgt ttt gtc gtt tcg tcg ttt tgt cgt ttt gtc gtt	48	K-type
36-ODN	tcg tcg ttt tgt cgt ttt gtc gtt tcg tcg ttt tgt	36	K-type
12-ODN	tcg tcg ttt tgt	12	K-type
6-ODN	tcg tcg	6	K-type
polyA-ODN	aaa aaa aaa aaa aaa aaa aaa aaa	24	
2216-CpG	ggG GGA CGA TCG TCg ggg gG	20	D-type
2216-GpC	ggG GGA GCA TGC TGc ggg gG	20	D-type
K16-CpG	tcg act ctc gag cgt tct c	19	K-type
K16-GpC	tgc act ctg cag gct tct c	19	K-type

TABLE 7: Oligodeoxynucleotides
*capital letters: phosphodiester, small letters : phosphorothioate modified

6.3.2 Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) from healthy volunteers were prepared with CPT™Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, USA) according to the manufacturer's protocol. Differential blood cell counts were performed routinely to rule out acute infections (Pentra60 hematology analyzer, ABX Technologies, Montpellier, France). Cells were plated at a density of 5 x 10^5 PBMC/well in RPMI 1640 in 96-well plates (Greiner, Nürtingen, Germany). After over night stimulation, cytokines were determined from the cell-free supernatant by ELISA. Crohn's patients with and without the 3020insC mutation were identified by PCR and PBMC were prepared as previously described (206).

Abbreviation (134)	Nomenclature	Source
M(ADiQ) / MDP	MurNAc-L-Ala-D-isoGln	B
M(DADiQ)	MurNAc-D-Ala-D-isoGln	B
MAγDE)	MurNAc-L-Ala-γ-D-Glu	NK
M(AγDEK)	MurNAc-L-Ala-γ-D-Glu-L-Lys	NK
M(AγDE*m*Dpm)	MurNAc-L-Ala-γ-D-Glu-*m*Dpm	NK
AM(AγDE*m*Dpm)	1,6-Anhydro-MurNAc-L-Ala-γ-D-Glu-*m*Dpm	NK

TABLE 8: Muropeptides
M: N-acetylmuramic acid, **AM**: 1,6-anhydromuramic acid, **A**: alanine; **D:** amino acid in D configuration, **Q:** glutamine, **E:** glutamate, **K:** lysine; **Dpm:** diaminopimelic acid, **γ:** linkage of amino acids, ***m*:** meso

6.3.3 Isolation of human monocytes

Monocytes were isolated from human PBMC by negative selection with the Monocyte Isolation Kit purchased from Miltenyi Biotec (Bergisch-Gladbach, Germany) as described previously (134). Monocytes were seeded at a density of 5 x 10^4 cells/well. CD14-positive monocytes were isolated also from human PBMC by positive selection using anti-CD14 MicroBeads (Miltenyi Biotec) (262) in order to exclude pDC. 5 x 10^5 CD14 positive monocytes were seeded per

well. In both cases, cells were stimulated over night and cytokines were determined from the cell-free supernatant by ELISA.

6.3.4 Isolation of murine macrophages

Mouse peritoneal macrophages from C57/BL6 (wild type) or NOD2-/- mice (a kind gift from Dr. M. Giovannini and Dr. J.-P. Hugot, Paris, France; animals have been back-crossed to the 7^{th} generation into the C57/BL6 background) were elicited by injection of 1,5 ml of thioglycolate medium (Biorad) in the peritoneal cavity four days before peritoneal lavage with 5 ml of PBS complemented with 10 U/ml Heparin Choay from Sanofi (Gentilly, France). Cells were pooled from five to six mice and centrifuged (256g, 5 min, room temperature). Cells were suspended to 1 million cells/ml in RPMI/3% FCS and seeded in 24-well plates. After 90 min of incubation (37°C, 5% CO_2), cells were thoroughly washed with PBS to remove non-adherent cells and 500 µl of RPMI/0.2% FCS/100 U/ml penicillin/100 µg/ml streptomycin/250 ng/ml Amphotericin B were added. Cells were left untreated at least 2h before being stimulated in duplicates or triplicates. After 18h, supernatants were aliquoted and cytokines were measured by ELISA.

Bone marrow wash out cells from TLR4-mutated (C3H/HeJ, purchased from Charles River Laboratories, Sulzfeld, Germany) and TLR2-/- (a kind gift of Tularik, Inc. South San Francisco, CA, USA) and the according wild type mice were prepared. Mice were killed by injection i.v. of 150 mg/kg pentobarbital (Narcoren®, Merial, Halbergmoos, Germany) and femur and humerus were removed and rinsed with 10 ml Dulbecco´s Phosphate Buffered Saline (PAA Laboratories GmbH, Pasching, Austria). Cells were washed, resuspended in RPMI 1640 supplemented with 10% fetal calf serum, penicillin and streptomycin, plated in 96-well plates at a density of 5 x 10^5 cells/well and stimulated over night. Cytokines were determined from the cell-free supernatants by ELISA. Lipopolysaccharide (LPS) from *Salmonella abortus equi* (Sigma) and highly purified lipoteichoic acid (LTA, prepared in house from *Staphylococcus aureus* (DSM 20233) (10)) were used as control stimuli in each experiment to prove the vitality of TLR or NOD2 deficient cells and to prove the

receptor defect (TLR2$^{-/-}$/LTA and C3H/HeJ/LPS). Bone-marrow derived peritoneal macrophages from TLR9-/- mice were prepared as described (263).

6.3.5 ELISA

Cytokines were determined by ELISA based on commercial antibody pairs against human TNF (Endogen, Perbio Science, Bonn, Germany) as well as IL-1β (R&D Systems, Wiesbaden, Germany) and IL-10 (BD Biosciences Pharmingen, San Diego, USA). Recombinant cytokines used as standards were obtained from the National Institute of Biological Standards and Controls, UK (TNF, IL-1β) and BD BiosciencesPharmingen (IL-10). Murine TNF was measured with Duo Set from R&D Systems. 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).

6.3.6 FACS

Human PBMC from healthy blood donors were seeded in a density of 2.5 x 10^6 cells/well in RPMI 1640 with 10% autologous serum and 5 U/ml heparin in 24-well plates (Greiner) and were incubated for 2h. Brefeldin A (10 µg/ml, Sigma) was added to the wells 15 min after the stimuli were given and further incubated for 4 hours. Cells were collected with a cell scraper, washed with ice cold PBS and pelleted at 300 g. PBMC were fixed and permeabilized with 300 µl BD Cytofix/Cytoperm^{TM} (BD) for 20 min at 4℃ and washed twice with 1 ml BD Perm/Wash^{TM} (BD). Intracellular staining was performed with PE-labeled anti-TNFα (Pharmingen) and APC-labeled anti-CD14 (BD). Flow cytometry analysis was performed on a FACS-Calibur^{TM} cytometer (BD). From each sample 20,000 monocytes were analysed. Data files were analysed using CellQuest^{TM} (BD) software.

6.3.7 PCR

Total RNA was prepared from human PBMC using the High Pure RNA isolation kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Reverse transcription and quantitative real-time PCR of IFNα mRNA and GAPDH mRNA was performed as described previously (262).

6.3.8 Statistics

Data were analysed by GraphPad Prism 3.0 program (GraphPad Software, San Diego, USA). Significant differences were assessed for analysis of variance by repeated measures one-way ANOVA followed by the Dunnett or the Bonferroni test. In the figures *, **, *** represent p-values <0.05, <0.01 and <0.001, respectively.

6.4 Results

6.4.1 Synergistic effect of CpG-ODN and muropeptides in PBMC

In order to investigate a putative synergistic sensing of bacteria by the intracellular receptors TLR9 and NOD2, the well-known TLR9 ligand 2006-CpG and the control ODN 2006-GpC were incubated together with MDP in human PBMC and the release of TNF was measured by ELISA. As shown in figure 18, neither 100 nM MDP, nor 1 or 2 µM 2006-CpG or 2006-GpC alone, induced significant release of TNF. In contrast, co-incubation of 2006-CpG and MDP exerted a strong synergistic effect and led to the release of significant amounts of TNF. The same observations, although only at slightly higher concentrations, were observed for GpC, indicating that the effect is not strictly sequence specific. These results also translate to the release of IL-1β, while no IL-10 release was detectable (data not shown).

In the next step, we aimed to investigate the precise role of ODN sequence for the synergism with MDP. Therefore, 2006-CpG-derived ODN with different sizes (6 nucleotides up to 48 nucleotides), a 24-ODN consisting of polyA and

the K16-CpG, which differs in its flanking regions from the 2006-CpG, were employed and human PBMC were co-stimulated with 1 up to 10 µM of ODN and 100 nM MDP. The potency of 1 µM of the 48-ODN, the 36-ODN and the K16-CpG to synergize with MPD was comparable to the original 24-ODN 2006-CpG (figure 19), while 1 µM of the 12-ODN, the 6-ODN and of the polyA-ODN (data not shown) failed to synergize with MDP. If concentrations were raised to 10 µM, a slight but not significant synergism was observed for 12-ODN and poly A-ODN (data not shown). Again, like for 2006-GpC, the effect of K16-GpC was reduced compared to K16-CpG (figure 19). In addition, a second type of CpG-ODN, the D-type ODN 2216-CpG, which has been reported to specifically activate pDC (264), was analyzed for synergism with MDP. The synergism of 2216-CpG with MDP was similar to the 2006-CpG (TNF release: 2 µM 2006-CpG+100 nM MDP: 1188 ± 281 pg/ml versus 2 µM 2216-CpG+100 nM MDP: 1233 ± 293 pg/ml, n=11, $p<0.01$ for both in comparison to control). The effect of 2216-GpC was comparable to 2006-GpC.

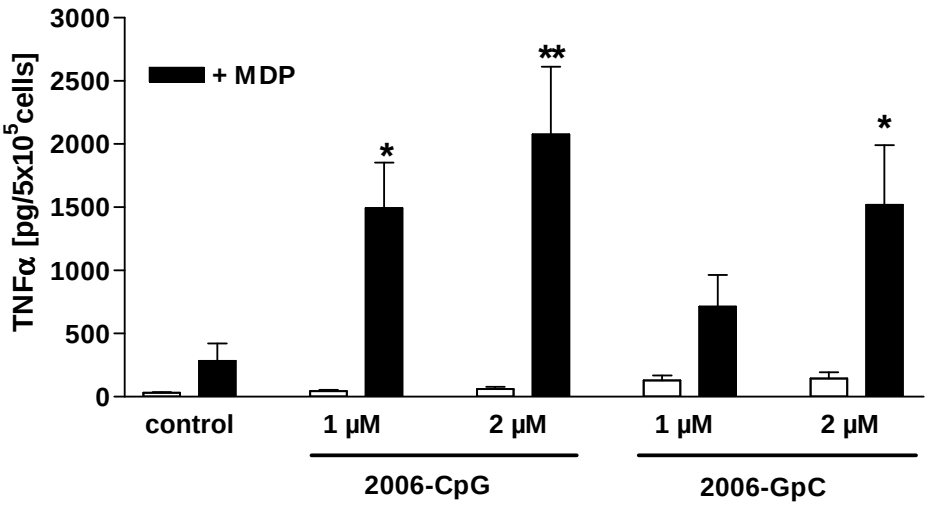

FIGURE 18: CpG and GpC synergize with MDP in stimulation of TNF release. Human PBMC were stimulated over night with 2006-CpG or 2006-GpC in the concentrations indicated in the presence or absence of 100 nM MDP. TNF was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM, n=12. *, ** indicate significant TNF release in comparison to the control.

To investigate the structural requirements of muropeptides for a synergism with CpG-ODN, five muropeptides, which display constituents of the PGN of Gram-negative or Gram-positive bacterial cell walls, and which differ in their amino

acid and sugar composition, were used and compared to MDP (figure 20). We found that the muropeptides M(AγDE), where the glutamine of MDP has been replaced by glutamate as well as M(AγDEK) and M(AγDE*m*Dpm), which posses an additional lysine or diaminopimelic acid respectively, synergized with 2006-CpG in TNF induction. In contrast, the M(DADiQ), where the L-alanine is exchanged by an alanine in D-configuration, and the AM(AγDE*m*Dpm), where an anhydrobound is introduced in the sugar moiety, showed no synergistic effect.

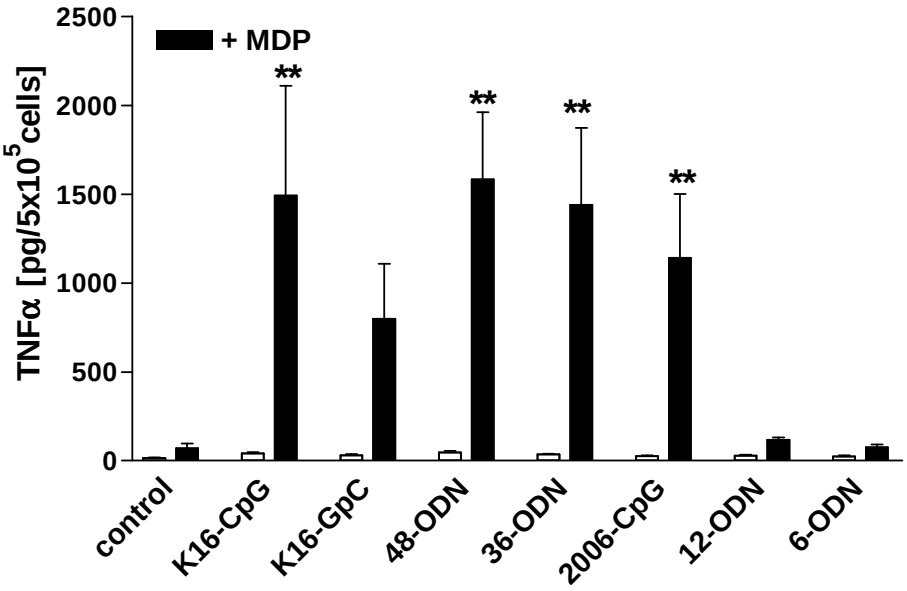

FIGURE 19: Different ODN synergize with MDP in stimulation of TNF release. Human PBMC were stimulated over night with 1 µM K16-CpG, K16-GpC, ODN-48, ODN-36, 2006-CpG, ODN-12 or ODN-6 in the presence or absence of 100 nM MDP. TNF was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM, n=8. ** indicate significant TNF release in comparison to the control.

Since low quantities of LPS (in the range of pg/ml) are sufficient to synergize with MDP in the induction of cytokine release, resulting in a shift of the immune stimulatory potency of LPS by three log orders (134), the synergistic effect of 2006-CpG or 2006-GpC plus MDP was tested in the presence of the LPS-blocking substance PolyB or the specific LPS inhibitor LALF (260, 261). Neither the addition of PolyB (5 µg/ml) nor LALF (10 µg/ml) led to a significant reduction (TNF pg/ml: CpG+MDP: 715 ± 116, CpG+MDP+PolyB: 736 ± 159, CpG+MDP +LALF: 530 ± 73, n=7, $p>0.05$). In the same experiment, LPS (1 ng/ml)-induced cytokine release was abrogated by addition of PolyB (5 µg/ml) or LALF (10 µg/ml) (data not shown).

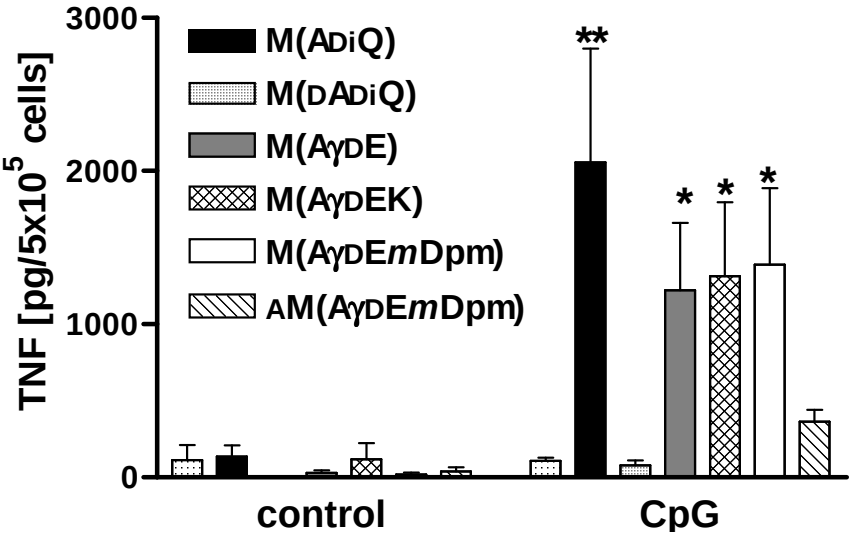

FIGURE 20: Structural requirements of muropeptides to synergize with CpG. Human PBMC were stimulated over night with 2 µM CpG in the presence or absence of 100 nM of the muropeptides indicated in the figure. TNF was measured in the cell-free supernatants by ELISA. Data are given as means ± SEM, n=7. *, ** indicates significant TNF release in comparison to the control. M: N-acetylmuramic acid, A: alanine, Q: glutamine, E: glutamate, K: lysine, Dpm: diaminopimelic acid.

6.4.2 TLR9 and NOD2 mediate the synergistic effect of CpG and MDP

To confirm that the effects of costimulation with CpG and MDP indeed are mediated via synergistic sensing by TLR9 and NOD2, the role of TLR2, TLR4, TLR9 and NOD2 were investigated. When peritoneal macrophages from NOD2+/+ mice were stimulated with 2006-CpG and MDP a significant increase of TNF release was observed compared to stimulation with 2006-CpG alone, indicating that the CpG/MDP synergism also translates to murine cells, even if a CpG sequence, designed to stimulate human cells is used (figure 21). While TNF release induced by 2006-CpG was similar for cells from NOD2+/+ and NOD2-/- mice, the response to 2006-CpG plus MDP was significantly impaired in NOD2-/- cells (figure 21). The same effect was observed when human PBMC from patients with Crohn's disease, which carry the lack of function mutation 3020insC in their NOD2 gene (64, 65) were costimulated and the TNF release was compared to PBMC from Crohn's patients with a wild type NOD2 gene (TNF in pg/ml for costimulation with 1 µM 2006-CpG+100 nM MDP: NOD2+/+: 1380 ± 767, n=3; NOD2 3020insC: 19,5 ± 8, n=4; $p<0.05$). Control experiments

confirmed normal responsiveness of PBMC from NOD2 3020insC donors towards LPS stimulation.

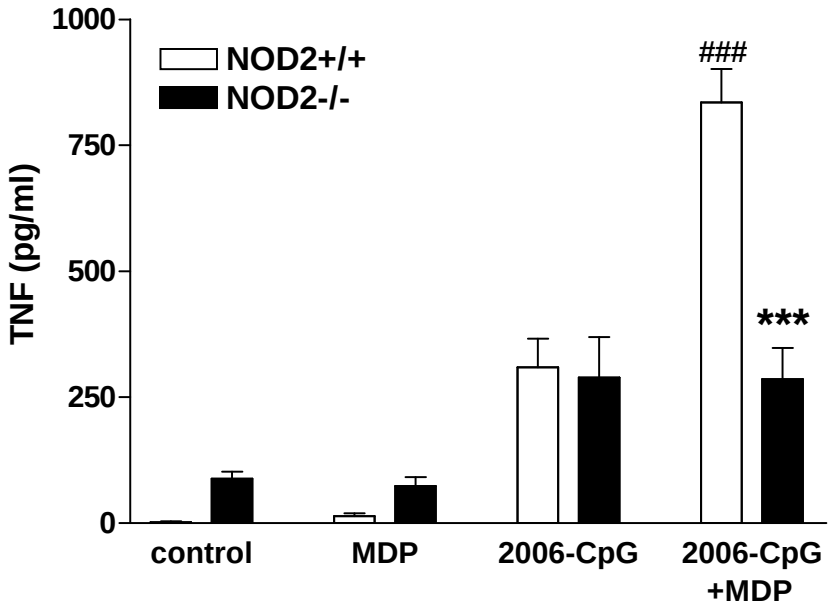

FIGURE 21: Synergism of MDP with 2006-CpG is mediated via NOD2. Peritoneal macrophages from NOD2+/+ and NOD2-/- mice were stimulated over night with 100 nM 2006-CpG in the presence or absence of 500 nM MDP. TNF was measured in the cell-free supernatant by ELISA. Data are given as means ± SEM. The experiment was performed three times with similar results. ### indicates significantly different TNF release in comparison to stimulation with 2006-CpG, *** indicates significantly different TNF release in comparison to stimulation of NOD2-/- cells.

To examine the role of TLR, bone marrow wash out cells from TLR4-mutated, TLR2-/- and TLR9-/- mice were stimulated with 2006-CpG and MDP and the release of TNF was measured. While all cells from wild type mice responded to the costimulation with significant TNF release, the cells from TLR9-/- mice failed to do so (data not shown), but the responses of the cells from TLR4-mutated and TLR2-/- mice remained unchanged (data not shown), indicating that only TLR9 is responsible for the synergistic effect. Interestingly, and in contrast to the human situation, we found that for murine cells the CpG/MDP synergism was strictly dependent on the CpG motive and did not occur with the GpC sequence. Since in preparations of human PBMC only small subpopulations, namely the pDC and B-lymphocytes, express TLR9 (49), we aimed to investigate the target cell population responsible for the synergism. Intracellular TNF staining of PBMC stimulated with 2006-CpG plus MDP followed by FACS analysis to determine the TNF producing cells was performed. As indicated in

figure 22A, not all but a large portion of monocytes released TNF, making it likely that a soluble mediator has been produced from the TLR9 positive cells after contact with the CpG, which in turn enabled TLR9-negative monocytes to release TNF upon MDP stimulation. Corroborating these results, in monocytes that were isolated on the basis of CD14 selection by microbeads, i.e. excluding the CD14 negative pDC, the synergistic effect was significantly impaired (figure 23A).

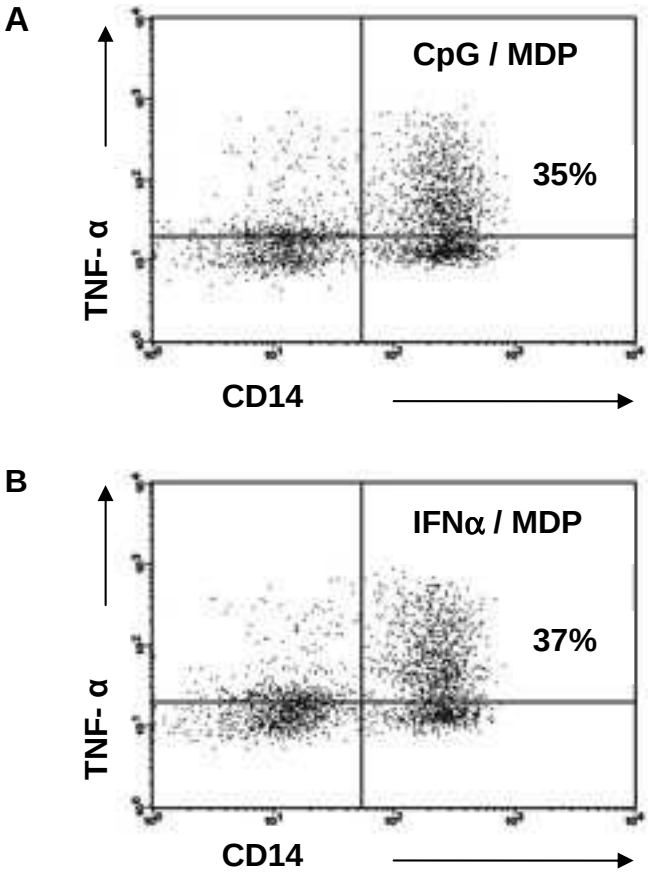

FIGURE 22:CpG and IFNα synergize with MDP in stimulation of TNF release. Human PBMC were stimulated for 4h with (A) 2 µM 2006-CpG or (B) 100 ng/ml IFNα in the presence or absence of 100 nM MDP. Intracellular TNF was stained and measured by FACS analysis. Data are given as dot blots and represent one out of three representative experiments. The percentage of TNF-producing cells is given in the diagram.

However, the TNF response of monocytes, which were isolated from PBMC via negative selection with magnetic beads, was comparable to PBMC (data not shown). As negative selection depletes B-lymphocytes but is not efficient in depletion of pDC, the latter cell type appears to be important for the observed synergism. pDC are strong producers of IFNα upon CpG-DNA stimulation

(264). In line with the above results, CD14-selected monocytes did not display IFNα production, while in PBMC 2216-CpG and to a lesser extent also 2006-CpG increased the expression of the respective transcripts (figure 23B). Furthermore, IFNα in combination with MDP was able to stimulate TNF release from PBMC (figure 22B), while IFNα alone had no effect, making IFNα a likely, although probably not the only soluble mediator of the CpG/MDP synergism.

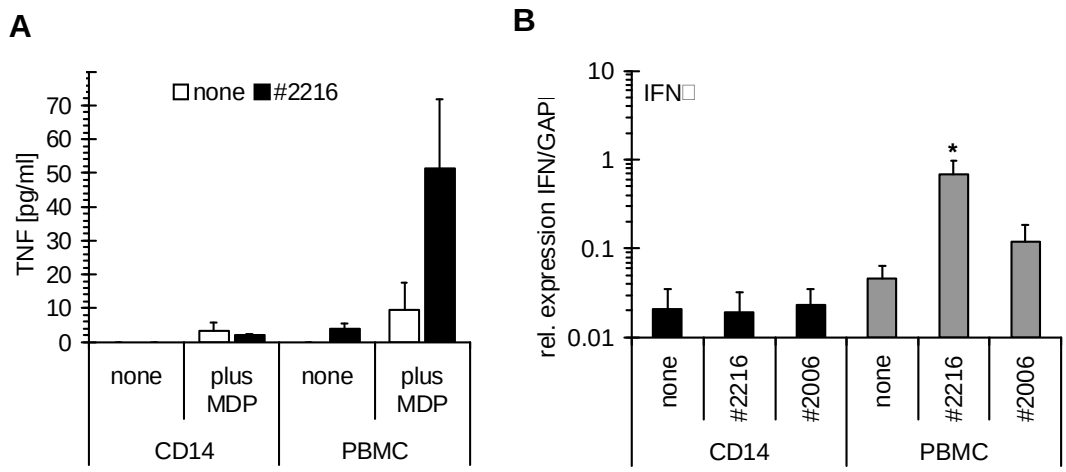

FIGURE 23: CpG/MDP synergism is abrogated in CD14-selected monocytes. Human PBMC or CD14 sorted monocytes were (A) stimulated over night with 1 µM 2216-CpG and 100 nM MDP and TNF was measured in the cell-free supernatant by ELISA, or (B) stimulated with 1 µM 2216-CpG for 4h and IFNα mRNA was determined by real-time PCR. Data are given as means ± SEM, n=3. * indicates significance in comparison to the control.

6.5 Discussion

Immune cells as well as some epithelial cells are equipped with intracellular PRR, which enable intracellular sensing of bacteria. Most of our knowledge about bacterial recognition stems from activation of PRR by isolated, extracellularly presented PAMP. Only little is known about intracellular sensing and even less about the simultaneous stimulation of immune cells by several bacterial stimuli, which much more reflects the physiological situation. The best studied synergism of bacterial components so far is the LPS/MDP synergism, where minor amounts of MDP (nM) suffice to enhance the immunostimulatory potency of LPS by a factor thousand (134). This effect has been clearly attributed to a synergistic sensing by the extracellular PRR TLR4 and the

intracellular NOD2 protein (166, 172). Furthermore, synergistic activity of NOD1 and NOD2 with TLR2 and TLR3 agonists have been shown (166, 172, 205), and there is also some evidence for a synergism between the intracellular, in the endoplasmatic reticulum expressed TLR9 and NOD1/2 (172). The synergism of TLR9 with NOD1/2 has been demonstrated for IL-8 release and NF-κB activation in THP-1 cells, however could not be reproduced in human PBMC (166).

In the present work, we show that common bacterial structures like bacterial DNA and muropeptides synergize in the induction of an inflammatory response, i.e. release of cytokines such as TNF and IL-1β by human PBMC, although they have no such immune stimulatory qualities if applied alone. For the synergism, a role of the intracellular TLR9 and NOD2 as PRR was confirmed by the use of knockout mice and Crohn's disease patients carrying the loss of function mutation 3020insC. We furthermore show that the synergistic effect also translates from MDP to larger muropeptide structures and to the NOD1 agonist M(AγDE*m*Dpm). The potency of the K-type ODN 2006-CpG and K16-CpG and the D-type ODN 2216-CpG to synergize with MDP was equipotent. Furthermore, the synergism was not strictly dependent on the bacterial CpG motif, but occurred also with the respective control ODN with inverted CpG-motif (i.e. GpC), although somewhat higher ODN concentrations were necessary.

Since it is well known that minor amounts of LPS suffice to synergize with MDP (134), LPS contaminations of our muropeptides and ODN have been carefully excluded. All muropeptides have been tested negative by LAL, and LPS contaminants of the CpG- and GpC-ODN, which cannot be tested by LAL, were excluded by the LPS inhibitors LALF and PolyB. Furthermore, the clear dependence on ODN length as well as no dependence on TLR4, support that indeed a direct effect of ODN is studied.

The fact that the human immune system distinguishes bacterial from endogenous DNA via the presence of repetitive unmethylated CpG motifs in the bacterial DNA, has led to the synthesis of various CpG containing ODN, like the D-type and K-type ODN. Those differ in number of the CpG motifs, flanking sequences and phosphorothioate (PTO) modification and can be used to selectively trigger TLR9 to induce different sets of T_H1-based cellular and

humoral immune responses (44, 265, 266). However, as we have shown, these differences do not affect the synergism of CpG-ODN with MDP. In contrast, if the CpG-ODN were shortened to 12 or 6 ODN or contained no CpG motif at all (like polyA), no significant synergistic effect was obtained, even if the concentrations were raised ten-fold. Surprisingly, we observed a synergistic effect of 2006-CpG and MDP in murine bone marrow and peritoneal macrophages, although the CpG motif used was optimized for human and not for murine cells (48, 254, 267). This reduced sequence dependence of the synergism might be explained by the PTO modification of the synthetic CpG-ODN, which not only protects them from rapid digestion by nucleases (40, 41, 268), but also contributes to non-sequence specific immune stimulatory effects (266). In line with this, a recent publication has shown that nonoptimal PTO-modified ODN (i.e. human motif used for murine cells and vice versa) also lead to a TLR9-dependent activation of p38 MAPK kinase, although the activation is delayed and less sustained. The same results were obtained when the CpG motif was inverted into GpC (45). Surprisingly, the synergism in all murine cells was strictly sequence dependent and did not occur with GpC-ODN, indicating that there are still species-specific differences, which are not fully understood.

The role of TLR9 as important PRR for the CpG/MDP synergism was proven by the use of TLR9-/- mice, where the synergistic effect was completely blunted. This was further confirmed by the finding that human PBMC, which were depleted of pDC, known to be the only blood cell population beside B-cells to express high levels of TLR9 (49), failed to respond to costimulation with CpG/MDP. In contrast, lymphocyte-depleted PBMC showed significant TNF release in response to costimulation. Since intracellular staining of TNF revealed that TNF was produced by CD14-positive and CD14-negative cells, this implicated that probably a soluble mediator was released from TLR9 bearing cells, which in turn enabled monocytes to respond with TNF release if costimulated with MDP. IFNα, which is only released from pDC in response to CpG stimulation (264), was able to substitute for CpG to induce TNF release from monocytes if given together with MDP. Hence, IFNα is likely to be a key mediator of the synergistic CpG/MDP effect.

Although first reports were conflicting, the cytoplasmatic proteins NOD1 and NOD2 are now commonly accepted as intracellular PRR for PGN breakdown

products (61, 192). Thereby MDP, a PGN component common to almost all bacterial cell walls, represents the minimal structure recognized by NOD2 (64, 65), while NOD1 sensing is restricted to muramyl tripeptides containing diaminopimelic acid at the third amino acid position, a motif which mostly occurs in Gram-negative bacteria but only in very few Gram-positive species (63, 189). In case of the CpG/MDP synergism we also confirmed by the use of macrophages from NOD2-/- mice and of PBMC derived from Crohn's patients carrying the 3020insC mutation, NOD2 as MDP receptor. When further muropeptides were investigated, we found that the synergism also translates to a muramyl dipeptide that contains glutamate instead of glutamine, a structure which is more common in Gram-negative PGN, as well as to muramyl tripeptides, which contain lysine or diaminopimelic acid at the third amino acid position. The latter finding suggests, that the synergism also takes place with the NOD1 agonist. However, this could not be finally proven, since no NOD1-/- mice were available. No synergism was observed for MDP containing D-alanine instead of L-alanine, indicating that the recognition process is stereolselective and for the muramyl tripeptide carrying an anhydrobound in the sugar moiety. The same results were obtained for the synergism of LPS with different muropeptides (134), indicating the same structural requirements of muropeptides for synergism of NOD2 with different TLR agonists.

For intracellular sensing, bacteria or bacterial particles need to be taken up by the cell. In case of intracellular replicating bacteria like *Chlamydia* spp. or *Listeria* spp., a direct activation of intracellular PRR is easy to imagine and evidence for recognition of *C. pneumoniae* (269) or invasive *Shigella flexneri* (208) by NOD1 has already been provided. The current concept for TLR9 activation is that CpG-DNA is taken up by endocytosis and transported to a tubular lysosomal compartment. TLR9 moves then from the endoplasmatic reticulum to the CpG containing compartment, where it probably directly binds to the DNA (56). How muropeptides find their way to the cytosolic NOD proteins is not clear yet. For *Helicobacter pylori*, a transfer of muropeptides into the cytosol of the host cell via a type IV secretion system has been suggested (212). If epithelial cells are studied, permeabilization procedures or microinjection of muropeptides are generally used. Professional phagocytes, like in our case, seem to take up the muropeptides via phagocytosis. However,

even after phagocytosis of muropeptides, the route of delivery from the phagosom to the NOD remains to be defined.

Taken together it is of great importance to note that common bacterial products, like DNA and muropeptides, which are exposed to immune cells during each bacterial infection, lead to a potent synergistic activation of immune cells via intracellular receptors. Furthermore, by this synergism, bacterial structures, which are weak immune stimuli at physiological relevant concentrations, are converted into potent immune activators, questioning somewhat results obtained with isolated stimuli. This is an important finding, contributing to the understanding of the complex process of immune activation by bacteria, but also has to be considered in practical terms since CpG-ODN are already used in clinical trials as immunomodulators in asthma (270) and immuno-protective (271) and in anti-cancer therapy (272).

6.6 Acknowledgements

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7 Summarizing discussion

The immune system responds to invading pathogens with a broad spectrum of inflammatory reactions. Important immune mediators are for example cytokines, chemokines and eicosanoids. The pathogens are recognized by monocytes, macrophages, dendritic cells and neutrophils. This recognition takes place via pathogen recognition receptors (PRR). The PRR detect conserved structures of pathogens, the pathogen-associated molecular patterns (PAMPs). The distinction of self and not-self is possible because these conserved structures are not produced in the mammalian body and serve therefore as danger signals. PAMPs are often structures of the bacterial cell wall or some intracellular molecules. Eminent structures from Gram-negative bacteria are lipopolysaccharide (LPS), peptidoglycan (PGN) and bacterial DNA, while conserved structures from Gram-positive bacteria are lipoteichoic acid (LTA) and PGN as well as bacterial DNA. Naturally occurring breakdown products of PGN are called muropeptides. One prominent muropeptide is muramyl dipeptide (MDP, M(ADiQ)), which is known as minimal active principle for adjuvant activity (37). Different bacterial strains use a variety of amino acids to build up their PGN, while the backbone of the PGN chains are conserved and consist always of alternating sugar molecules, namely N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) (34). A broad diversity of muropeptides is therefore possible. Bacterial DNA is discriminated from host DNA through unmethylated CpG motifs, because they are rarely present in the mammalian genome. Oligodeoxynucleotides (ODN) containing CpG motifs have been designed leading to similar innate and acquired immune responses (266). All these conserved structures are recognized by PRR. Very important PRR are members of the toll-like receptor (TLR) family which comprises to date 13 members (52-54). LPS is recognized by TLR4, while LTA and PGN are recognized by TLR2 (10, 76, 273). Intracellular receptors are TLR9, which recognize CpG-ODN and which are mainly expressed in plasmacytoid dendritic cells (pDC) and B-cells (48-50), while in myeloid dendritic cells, natural killer (NK) cells, T-cells and macrophages TLR9 is expressed only at low level (49). Like-wise, the NOD (nucleotide-binding oligomerization domain)-receptors are

located intracellular. Eminent members of the NOD-family are NOD1 and NOD2, which discriminate between muropeptides containing diaminopimelic acid (DAP, Dpm) and other muropeptides like MDP, respectively (59).

In the first part of this work, we have shown that PGN induces cytokine release on its own, but in contrast to MDP (134) it fails to synergize with LPS. In addition, PGN and LTA are relatively weak inducers of TNFα, while both are strong inducers of IL-8. These findings showed that there exist more similarities between PGN and LTA. In other models, this similarity of PGN and LTA has also been observed (218, 219). Furthermore, here we have isolated PGN from *Eschericia coli* (*E. coli*) using the standard SDS-method with subsequent enzyme digestion (274, 275). This PGN from *E.coli* was not able to induce IL-6 release in bone marrow cells from C3H/HeJ mice, carrying a non-functional TLR4. In addition, in combination with the LPS-specific blocking substance LALF, because remaining LPS contaminations (< 1%) have been found, no TLR2-dependency and only little cytokine induction was found in bone marrow cells from wild type and TLR2 knockout mice. Resulting from these findings in absence of any LTA present in PGN of Gram-negative bacteria we have found no TLR2-dependent activity. In literature, commercial PGN from *Staphylococcus aureus* (*S. aureus*) was found to be TLR2-dependent (76, 276). In line with our findings, recently, Travassos et al. (27) and Hoebe et al. (79) reported that signalling of highly purified PGN is not dependent on TLR2 and they showed that TLR2-dependent stimulation of Gram-positive PGN is due to LTA contaminations. In contrast to Travassos et al. and Hoebe et al. (27, 79) which purified the Gram-positive PGN further, we extracted LTA from commercial *S. aureus* PGN with our standardized LTA extraction method (10). The elution profiles showed both relatively low TNFα but strong IL-8 release. In addition, a phosphate content, which is a lead activity for LTA, has been found in the corresponding fractions. From 48 mg of commercial *S. aureus* PGN we were able to extract 10-20 µg of LTA, calculated by cytokine inducing activity and phosphate content. The presence of LTA has been confirmed with NMR, where LTA-typical signals have been found. These findings corroborate the results of Travassos et al. and Hoebe et al. (27, 79) that commercial PGN contain LTA contaminations even if the amount is relatively small. Additionally,

findings, how such small amounts present in commercial PGN-preparation are able to induce the strong cytokine release by PGN, could be explained by a further observation of this work. LTA immobilized on a surface and thereby presented to immune cells has a far greater potency to induce cytokines than soluble LTA molecules. Aiming to model the physiological situation, we preincubated LTA on a polystyrol plate, which led to enhanced cytokine release, i.e. the TNFα-inducing activity of LTA was amplified by a factor of about 1000. Subsequent experiments with rhodamine-labeled LTA showed, that only about 1 to 4% after 2 and 24 hours, respectively, had bound to the plate. These results demonstrated that only small amounts of presented LTA are necessary to induce the strong cytokine release induced by LTA. Taken together, with the result that we have extracted 10-20 µg LTA from 48 mg PGN, the small amount of LTA is sufficient to explain the entire cytokine inducing capacity of PGN. Additionally, the presentation of LTA mirrors the physiological situation, where LTA protrude through the PGN sacculus (8) and lead to the dimerization of the receptor. This concept is supported by a chemically modified LTA, which was available for us, where an additionally fatty acid anchor was coupled to one LTA molecule. An increased cytokine release of this bisamphiphilic LTA similar to monoamphiphilic LTA that is presented on a surface was observed. These findings go in line with earlier reports where LTA molecules, which have been cross-linked by anti-polyglycerophosphate antibodies, resulted in increased activity (30). Taken together, these results support the hypothesis that bound and presented LTA may lead to receptor clustering and therefore increased activity.

Glycols and closely related compounds have earlier been shown to exhibit bactericidal and bacteriostatic properties (221, 222). In addition, we have investigated here the inhibitory properties of polypropylene glycol (PPG) on cytokine induction by Gram-positive immune stimuli and thereby we have found a novel LTA-specific inhibitor, i.e. polypropylene glycol with molecular weight of 1200 (PPG 1200). We have found that UV-inactivated *S. aureus*-, LTA- and PGN-induced cytokine release was inhibited with a similar inhibitory concentration (IC_{50}). The same results have been observed for LTA and PGN from different bacterial species. LPS and UV-inactivated *E. coli* were only partially affected by PPG 1200, and only at a 100-fold higher PPG 1200

concentration. To check if PPG 1200 interferes with LTA or the receptor, nanobeads coated with PPG 1200 have been produced and these experiments have shown that LTA binds to PPG and that this complex binds to the cells, though it cannot activate them any longer. These results, that cytokine release induced by intact *S. aureus*, LTA and PGN is inhibited by the novel inhibitor PPG 1200, strengthen the concept that LTA represents the active principle in PGN and that LTA represent the major immunostimulatory component of *S. aureus*.

Taken together, these studies demonstrate that minimal contaminations with potent and stable structures such as LPS and here LTA lead to misinterpreted results. Contaminations with LPS can be excluded by limulus amebocyte lysate (LAL) assay, as long as interference of the sample with LPS recovery is excluded. No such test is up to now available for LTA. With the newly discovered tool, the LTA-inhibitor PPG 1200, LTA contaminations can be excluded in future. Therefore, previous work on LTA and PGN as well as reports of new ligands of TLR must be reviewed critically.

Taken together, LTA and not PGN represents the active principle of the cytokine inducing activity and is therefore the major immunostimulatory component of Gram-positive bacteria. The identification of a specific inhibitor of LTA activity led to the identification of LTA-mediated actions as well as will allow in the future quality control of bacterial preparations. Additionally, this non-toxic compound may have potential for clinical applications in Gram-positive bacterial disease.

In the second part of this work, we have found that muropeptides as breakdown-products of PGN induced no cytokine release on their own. MDP (muramyl dipeptide, M(ADiQ)) has been found in the seventies to be the minimal active structure for adjuvant activity (37). Resulting from this, muropeptides are rather immune-amplifier and modulators of the immune reaction, than being an immune stimulus on their own. MDP and other muropeptides have been shown to have priming effects towards LPS *in vitro* and *in vivo* (155). Additionally, MDP and muropeptides exerted remarkable synergistic effects with LPS (14, 135). Despite this, no thorough characterization of the mechanism of synergy with LPS had been done and the investigation of the structural requirements of

different muropeptide structures had not been determined. In contrast to our results that MDP and other muropeptides are not able to induce cytokine release on their own (134), in other models MDP and other muropeptides have been shown to induce the release of cytokines (129, 130, 225, 227, 249, 250). However, often very high and therefore unphysiological concentrations have been used in these experiments and in addition these authors have done no exclusions of endotoxin-contaminations. Beside this, in several *in vitro* systems synergistic effects of muropeptides with LPS in inducing cytokines have been shown (14, 127, 133, 135). Here, we used synthetic and endotoxin-free muropeptides in very low concentrations at as little as 20 nM corresponding to 10 ng/ml of the muropeptides. In our human whole blood model, MDP induced a shift to the left of the concentration-response curve by a factor of 1000 and enhanced the TNFα-release about 4-fold. LPS can be contaminated with lipoproteins (246), but using highly purified LPS showed that the synergistic effect is not dependent on the purity of the LPS preparation. In *in vivo* models, muropeptides have priming abilities (127, 156, 159). Therefore, possible effects of preincubation have been determined, but the synergistic effect was unchanged in our model by preincubation of MDP, only 10% increase could be observed in comparison to simultaneous stimulation with LPS and MDP. Additionally, experiments with isolated monocytes showed, that no other cells are necessary for the synergistic effect. The synergy of LPS and MDP not only holds true for TNFα-release, besides this other cytokines like IL-1, IL-6 and IL-10 showed enhanced release. These results are in line with other findings that synergy of LPS and MDP is translated to different cytokines like TNFα, IL-1, IL-6 and IL-8 (14, 164, 165). Lethal toxicity of costimulation by LPS and MDP *in vivo* has been shown earlier (156, 159, 251), but we have shown employing isolated liver perfusion that the synergistic effect of LPS and MDP occurs in addition *ex vivo*. Observing the TNFα-release over time, we have shown that MDP did not alter the kinetics of LPS-induced TNFα-release. In contrast, determination of the TNFα mRNA expression has shown an increase. Using the inhibitor actinomycin D (act D) revealed, that the enhancement of TNFα mRNA is rather due to *de novo* transcription than to increased stability. In line with these findings, in a human cell line, MDP induced minimal expression of TNF

mRNA but coincubation led to an increased transcription, however, the induced mRNA was not translated into protein (135).

Different synthetic muropeptide structures have been made available to us and the structural requirements of muropeptides to synergize with LPS in cytokine induction have been determined. Some structures like MDP (M(ADiQ)), GMDP (GM(ADiQ)), MDP with replacement of isoglutamine in glutamate (M(AγDE)), and this structure extended by lysine (M(AγDEK) synergized with LPS in cytokine induction, as did artificial structures with non-natural fatty acids (C_{18}M(ADiQ) and M(ADiQKεC_{18})) (abbreviations of muropeptides shown in table 5). M(A) exerted no synergistic effect and this panel of structures showed, that muramic acid with two additional amino acids is necessary for the synergistic effect. Others reported similar findings, that MDP is a strong synergist (133, 135, 172), but none of them undertook a comparison of different muropeptide derivatives in costimulation with LPS. Like-wise stereoisomers of MDP (M(AiQ) and M(DADiQ)), where the amino acids are not in the naturally occurring LD-configuration, are commercially available and were assessed with regard to possible synergistic structures to LPS. However, stereoisomers of MDP showed no synergy with LPS in human whole blood. The importance of the configuration of the amino acids with regard to cytokine induction by the muropeptides alone or in costimulation with LPS have been observed by others as well (65, 277, 278). The artificial structures with non-natural fatty acids as chemical modification did not differ with regard to the synergistic effect with LPS. If had been assumed that the fatty acid facilitate the entering into the cell, but they did not improve the efficacy of the muropeptides in our hands. Muropeptides containing diaminopimelic acid (DAP, Dpm) and anhydro-compounds containing DAP as well failed to induce an enhanced LPS-induced cytokine release. In contrast, others reported, that similar structures containing DAP acted in synergy with LPS or lipid A (63, 171, 172), but the used structures are not identical. Antagonistic effects of the small muropeptide (M(A)) containing only one amino acid, muramic acid (M) or anhydro-muramic acid (AM) on the synergy induced by LPS and MDP have not been observed. Taken together, the mechanism of the synergy of LPS and MDP has been studied as well as the structural requirements of different muropeptide structures to synergize with

LPS in cytokine induction. These results demonstrate, that muropeptides are rather natural amplifiers than an immune stimulus on their own.

Taken together, these studies demonstrate that muropeptides are natural amplifiers of immunological and inflammatory effects. We have shown that the response to LPS differs dramatically in the absence or presence of muropeptides from Gram-negative and Gram-positive cells walls, which are naturally present during infections. In addition, the used LAL-negative preparations did not induce any cytokine release on their own in human whole blood even at microgram/ml concentrations. This characterizes the muropeptides rather as synergists, than as independent immune stimuli. However, they play an important role in modulating immune responses.

Taken together, muropeptides are strong immune amplifier. Therefore, this opens new possibilities for pyrogen testing. In the presence of muropeptides even very small LPS contaminations could be determined and thus the sensitivity of testing of pharmaceutical drugs can be strongly enhanced resulting in improved security of patients. In addition, new therapeutic strategies in infection and sepsis can be developed. Antagonizing the effects of muropeptides might represent an interesting anti-inflammatory strategy leaving the LPS-based recognition of pathogens intact. Additionally, based on the findings, that muropeptides modulate immune responses, novel immunostimulators have been and will be developed tailored for patients with immunosuppressive diseases.

In the last part of this work, we observed that a strong synergistic effect occurred with CpG-ODN as well as with LPS with regard to increased cytokine release in PBMC (peripheral blood mononuclear cells). The described LPS/MDP synergism was due to the extracellular TLR4 and the intracellular located NOD2 protein (166, 172). In addition, synergistic activation of NOD1- and NOD2-agonists with TLR2- and TLR3-agonists have been reported (166, 172, 205). In this work we have determined the interplay of the intracellular located receptors NOD1 and NOD2 with TLR9. In PBMC, MDP and other muropeptides as well as CpG-ODN alone induced only little cytokine release. However, these two compounds synergized in cytokine release like TNFα and IL-1β. Others were able to show in the human monocytic cell line THP-1

synergism of a CpG-ODN with MDP for IL-8 release and NF-κB activation (172). While others used the same model like ours, human PBMC, they did not observe our effect (166). To study the synergistic effect of CpG-ODN and MDP, further types of ODN were used. In addition to the commonly used 2006-CpG, which is a K-type ODN, an additional K-type ODN K16-CpG as well as a D-type ODN 2216-CpG have been used (sequences of the CpG-ODN shown in table 7). The same strong synergy with the different CpG-ODN has been observed. Therefore, the synergistic effect of CpG-ODN and MDP is not dependent on the sequence of the CpG-ODN. In line with these findings, we observed that the commonly used control-ODN, where the CpG-motif is inverted into a GpC-motif is able to synergize with MDP, despite higher concentration are necessary to exert synergy. To further elucidate the synergistic effect, the 2006-CpG (24-mer) was extended to a 48-mer and a 36-mer as well as shortened to a 12-mer and a 6-mer. In addition, an ODN containing no CpG-motif (polyA) was used. In our hands, the extended ODN, but not the polyA as well as the 12-mer and the 6-mer, exerted synergy with MDP even if higher concentrations of polyA, 12-mer and 6-mer have been used. This leads to the assumption that a certain length of the used ODN and the presence of a CpG-motif is necessary for an optimal synergistic effect of ODN and MDP. Costimulation of different muropeptide structures with CpG-ODN have confirmed that larger muropeptides structures including a DAP-containing muropeptide are able to synergize with CpG-ODN. The muropeptide structures which were able to synergize with CpG-ODN synergized as well with LPS (134). Additionally, these results clearly indicated that synergy occurs with NOD1- and NOD2-agonists. These findings showed that the same structural requirements of muropeptides to synergize with LPS or CpG-ODN exist. That the synergistic effect is not mediated by TLR2 and TLR4, but instead of TLR9 and NOD2, has been corroborated in knockout mice. In the TLR9 knockout mice, no cytokine release after costimualtion by CpG-ODN and MDP have been found. Interestingly, we have observed, that although the CpG motif used was optimized for human and not for murine cells, in murine bone marrow and peritoneal macrophages we found the same synergistic effects as have been observed in human PBMC. Others have reported that there are optimized structures for human and murine cells (48, 254, 267). In addition, in murine cell systems it holds true, that the control ODN

where the CpG-motif is inverted into a GpC-motif induce no activation of the immune system (263). A possible explanation of these findings could additionally be, that all ODN employed are PTO- (phosphorothioate) modified to be resistant against nuclease digestion (40, 41, 268) but contribute to non-sequence specific immune stimulatory effects (266). In line with these findings, recently Roberts et al. (45) observed that non-optimal PTO-modified ODN also led to a TLR9-dependent activation of p38 MAPK (mitogen-activated protein kinase) despite human motifs have been used for murine cells and vice versa. This occurred additionally in case of the use of GpC-ODN (45). Further experiments have shown that in patients with Crohn´s disease carrying the loss of function mutation 3020insC in NOD2 or in CD14 purified monocytes from wild type donors no synergism could be observed. Only in pDC and B-cells (48-50) TLR9 is highly expressed while in myeloid dendritic cells, natural killer (NK) cells, T-cells and macrophages TLR9 is only expressed at low levels (49). In PBMC, therefore, only pDC and B-cells can be responsible for the TLR9-dependent activation. In CD14 purified monocytes, which were depleted in pDC, no synergism could be observed while in lymphocyte-depleted PBMC the synergistic effect could be observed. These findings suggest, that NOD2 and the pDC, which highly express TLR9, are responsible for the synergistic effect of MDP and CpG-ODN. After intracellular staining of TNFα in PBMC, we observed TNFα-production by monocytes. These findings led to the hypothesis that most probably a soluble mediator was released from TLR9-positive cells that induced a TNFα-release in TLR9-negative cells. Costimulation of IFNα and MDP led to the same TNFα-release by TLR9-positive and -negative PBMC. IFNα is therefore the likely mediator of this bystander effect. LPS contaminations, which have been shown to be able to induce at very little concentrations strong synergistic effects together with MDP (134), were carefully excluded by the use of the LAL (limulus amebocyte lysate) assay and the LPS-specific inhibitors PolyB (polymyxin B) and LALF (LPS-specific binding protein). Costimulation of inhibitors with CpG-ODN and muropeptides led not to a decrease in cytokine-inducing capacity. Additionally, the dependence on length of the synergistic effect and the use of TLR4 knockout mice clearly showed that CpG-ODN and MDP specific effects have been studied.

Taken together, the interplay of common bacterial products like bacterial DNA and muropeptides, which are present during bacterial infection, lead to a dramatically synergistic activation of the immune system. New light is shed on the activation of the immune system through immune responses by interaction of intracellular receptors. In addition, bacterial DNA and muropeptides represent weak immune stimuli with regard to leukocyte activation. However, interaction of these naturally occurring bacterial products at physiologically relevant concentrations leads to strong synergistic immune reactions questioning therefore the role of results obtained with the stimuli alone. Therefore, it is of great importance not only to study the immune stimuli by themselves but also to study the effects of the combination of different immune stimuli on the immune system.

Taken together, strong synergistic activation of the immune system through bacterial DNA and muropeptides takes place. This is of huge concern because CpG-ODN are already used in clinical trials as therapy in cancer (272), asthma (270) as well as immunoprotective agents for microbial sepsis and against antibiotic resistant diseases (265, 279). It is of major importance to notice the strong interplay of two immunomodulators enhancing immune reactions leading to possible over-reaction of the immune system, when naturally occurring muropeptides are present during infections and additionally CpG-ODN are given to patients possibly leading to adverse drug effects.

Taken together, comparative characterizations of whole *S. aureus*, LTA, CpG-ODN, PGN and muropeptides have been carried out. This work shows that LTA is the main immunostimulatory principle of *S. aureus*. MDP and other muropeptides synergize in cytokine induction with LPS and CpG-ODN. The structural requirements and the mechanism of the synergy of LPS and MDP have been characterized. The plasmacytoid dendritic cells (pDC) as well as IFNα are probable responsible for the bystander effect which is necessary for the strong synergistic effect of MDP and other muropeptides with different CpG-ODN in PBMC.

This work shows that it is crucial to control the quality of used bacterial stimuli in immunological research in order to understand the complex interplay and thereby the complex process of immune activation by bacteria.

8 Summary

The immune system is able to respond after bacterial recognition with production and secretion of cytokines. Whole Gram-negative and Gram-positive bacteria display this entire spectrum of cytokines. Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, mirrors all the cytokine-inducing activities of whole Gram-negative bacteria and LPS is supposed to be their immunostimulatory principle. Whether lipoteichoic acid (LTA) or peptidoglycan (PGN) is the counterpart of Gram-positive bacteria is still controversially discussed. LTA and PGN induce similar cytokine responses and the activation takes place via the toll-like receptor 2 (TLR2), even though PGN has no lipid anchor, as all other TLR2-agonists. Besides this, muropeptides, which are breakdown products of PGN, induce no cytokine release on their own and show no TLR2-dependent activation. Their intracellular recognition takes place via the NOD1- (nucleotide-binding oligomerization domain) and the NOD2-receptor. The most prominent muropeptide is muramyl dipeptide (MDP, M(ADiQ)), known as the minimal active principle for adjuvant activities. CpG-oligodeoxy-nucleotides (-ODN) are immune stimuli as well and mimic bacterial DNA. They induce T_H1-responses with regard to cellular and humoral immune reactions and are recognized via the intracellular located TLR9.

- Using the human whole blood model, a comparative characterization of whole *Staphylococcus aureus* (*S. aureus*), LTA, PGN and muropeptides has been carried out.
- PGN from Gram-negative bacteria has been isolated and characterized with regard to TLR dependency. PGN from Gram-negative bacteria displays no TLR2-dependent activity.
- LTA contaminations in commercially available PGN preparations have been proven by chemically detection methods, although only small amounts could be detected.
- Presentation on surfaces increased the potency of LTA dramatically and can explain that such small amounts present in PGN preparation lead to the strong signal and, additionally, this indicates that the presentation of

LTA in the PGN sacculus is sufficient to be the main immunostimulatory principle of Gram-positive bacteria.

- A bisamphiphilic LTA, that represents a cross-linkage of two LTA molecules similar as LTA presented on a surface induced a higher cytokine release than monoamphiphilic LTA.
- The discovery of a novel LTA-specific inhibitor polypropylene glycol 1200 (PPG 1200), that blocks cytokine induction by *S. aureus*, LTA and PGN with a similar inhibitory concentration (IC_{50}), shows that inhibition of the immunostimulatory principle abrogates the cytokine response.

- Muropeptides induce no cytokine release on their own, but are strong synergists to LPS increasing the tumour necrosis factor α (TNFα) releasing capacity of LPS about 4-fold and induce a shift of the concentration response curve by a factor of 1000.
- The synergy is not dependent on the purity of LPS preparations, shown with highly purified LPS.
- A preincubation of MDP leads not to a further increase of the enhanced LPS-induced cytokine release.
- The synergistic effect not only occurs with regard to TNFα-release, but other cytokines like interleukin-1β (IL-1β), IL-6 and IL-10 also show increased cytokine release when simultaneously stimulated with LPS and MDP.
- No bystander effect by other immune cells is necessary for the synergistic effect of LPS and MDP; isolated monocytes are sufficient to induce the increased cytokine release.
- In addition, the synergistic effect of LPS and MDP occurs *ex vivo*, shown with isolated liver perfusion indicating a synergistic endotoxin liver injury such as *in vivo*.
- Kinetics of LPS-induced TNFα-release is not altered by addition of MDP, but enhancement of LPS-induced TNFα mRNA is rather due to an increased *de novo* transcription, than an increased mRNA stability.
- The structural requirements of muropeptides to synergize with LPS in cytokine induction have been determined. MDP (M(ADiQ)), GMDP (GM(ADiQ)), artificial structures with non-natural fatty acids (C_{18}M(ADiQ)

and M(ADiQKεC$_{18}$)), MDP with replacement of isoglutamine by glutamate (M(AγDE)), and this structure extended by lysine (M(AγDEK) synergize with LPS in cytokine induction. Stereoisomers of MDP (M(AiQ) and M(DADiQ)) as well as muropeptides containing diaminopimelic acid (DAP, Dpm) and anhydro-compounds containing DAP fail to induce an enhanced LPS-induced cytokine release (abbreviations of muropeptides shown in table 5).

- The small muropeptides containing only one amino acid (M(A)), muramic acid (M) or anhydro-muramic acid A(M) are no antagonists of the synergy of LPS and MDP.

- CpG-ODN are only weak inducers of TNFα and other cytokines in PBMC (peripheral blood mononuclear cells), however the simultaneous stimulation of MDP and CpG-ODN leads to a strong synergistic TNFα-release.
- The synergistic effect of MDP and CpG-ODN occurs with different types of K- and D-type CpG-ODN, as well as with GpC-ODN although higher concentrations are necessary (sequences of ODN shown in table 7).
- Additionally, synergy occurs with different specified muropeptide structures.
- The synergistic effect of MDP and different CpG-ODN translates also to other cytokines like IL-1β while no IL-10 was released.
- The synergistic effect of CpG-ODN and MDP is not mediated by TLR2 and TLR4, but is mediated by TLR9 and NOD2 shown with knockout mice.
- No synergism in PBMC from patients with the 3020insC mutation in NOD2 or in CD14 purified monocytes from wild type donors, which lack the expression of TLR9, has been observed. Therefore, the plasmacytoid dendritic cells (pDC) are probable responsible for the synergistic effect.
- A large part of monocytes, which secreted the observed TNFα, induced by the simultaneously stimulation of MDP and CpG-ODN, leads to the assumption that a soluble mediator secreted by TLR9-positive cells after contact with CpG is responsible for the TNFα-release by TLR9-negative monocytes. IFNα is the likely mediator of this bystander effect.

Taken together, whole *S. aureus*, LTA, CpG-ODN, PGN and muropeptides have been characterized as to the induction of cytokine release in blood leukocytes. Comparative studies have shown, that LTA is the main immunostimulatory principle of *S. aureus*. Synergistic effects of LPS and CpG-ODN with MDP and derivatives have been observed. In addition, structural requirements of the different muropeptides to synergize with LPS and CpG-ODN and a characterization of the mechanism of synergy have been undertaken. These findings add to our understanding of bacterial immune recognition by the innate immune system.

9 Zusammenfassung

Nachdem Bakterien durch das Immunsystem erkannt werden, reagiert dieses mit der Produktion und Sekretion von Zytokinen. Intakte Gram-negative und Gram-positive Bakterien induzieren das gesamte Spektrum möglicher Zytokine. Ebenso wie Gram-negative Bakterien hat Lipopolysaccharid (LPS), ein Zellwandbestandteil von Gram-negativen Bakterien, die Fähigkeit, all diese Zytokine zu induzieren. Darum wird von LPS angenommen, dass es das immunstimmulatorische Prinzip ist. Ob Lipoteichonsäure (LTA) oder Peptidoglykan (PGN) das Gegenstück auf Gram-positiver Seite ist, wird noch kontrovers diskutiert. LTA und PGN induzieren ähnliche Zytokin-Antworten und die Aktivierung findet über den Toll-like Rezeptor 2 (TLR2) statt, obwohl PGN keinen Lipidanker besitzt wie alle anderen TLR2-Agonisten. Außerdem induzieren Muropeptide, welche Bruchstücke des PGN sind, keine Zytokine und zeigen keine TLR2-abhängige Aktivierung. Ihre intrazelluläre Erkennung findet über den NOD1- (Nukleotid bindende Oligomerisierende Domänen) und den NOD2-Rezeptor statt. Ein bekanntes Muropeptid ist Muramyl-Dipeptid (MDP, M(ADiQ)), welches als Minimalprinzip für Adjuvanz-Aktivität gilt. CpG-Oligodesoxynukleotide (-ODN) sind ebenfalls Immunstimuli und ahmen bakterielle DNA nach. Sie induzieren T_H1-Antworten im Bezug auf zelluläre und humorale Immunreaktionen und werden durch den intrazellulär lokalisierten TLR9 erkannt.

- Mit dem humanen Vollblut-Modell wurde eine vergleichende Charakterisierung von intakten *Staphylococcus aureus* (*S. aureus*), LTA, CpG-ODN, PGN und Muropeptiden vorgenommen.
- PGN aus Gram-negativen Bakterien wurde isoliert und charakterisiert, wobei keine TLR2-Abhängigkeit festgestellt wurde.
- Mit chemischen Detektionsmethoden wurden LTA-Kontaminationen in kommerziell erhältlichen PGN-Präparationen nachgewiesen, obwohl nur sehr geringe Mengen gefunden wurden.

- Die Wirksamkeit der LTA wird durch die Präsentation auf Oberflächen drastisch gesteigert. Dieses Phänomen kann erklären, wie solch geringe Mengen an LTA zu einem starken Signal führen können. Außerdem erklärt dies, dass die Präsentation der LTA in der PGN-Hülle ausreichend ist, um das immunstimulatorische Prinzip von Gram-positiven Bakterien darzustellen.
- Eine bisamphiphile LTA, die eine Vernetzung von zwei LTA-Molekülen darstellt, induziert ebenso wie LTA, die an einer Oberfläche präsentiert wird, eine höhere Zytokin-Freisetzung als monoamphiphile LTA.
- Die Entdeckung des neuen LTA-spezifischen Inhibitors, Polypropylen Glykol 1200 (PPG 1200), der die Zytokin-Induktion durch *S. aureus*, LTA und PGN mit einer ähnlichen inhibitorischen Konzentration (IC_{50}) blockiert, zeigt, dass die Inhibition des immunstimulatorischen Prinzips zu einem Verschwinden der Zytokin-Antwort führt.

- Muropeptide induzieren keine Zytokin-Freisetzung, stellen aber starke Synergisten zu LPS dar, die Tumor Nekrose Faktor α (TNFα)-Freisetzung durch LPS wird vierfach gesteigert und die Konzentrations-Wirkungskurve um den Faktor 1000 verschoben.
- Die Synergie ist nicht abhängig von der Reinheit der LPS-Präparation, was mit hoch-gereinigtem LPS gezeigt wurde.
- Die Vorinkubation von MDP führte nicht zu einer Steigerung der durch LPS und MDP induzierten TNFα-Freisetzung.
- Der synergistische Effekt kommt nicht nur in Bezug auf eine TNFα-Freisetzung vor. Außerdem zeigen andere Zytokine wie Interleukin-1β (IL-1β), IL-6 und IL-10 eine gesteigerte Zytokin-Freisetzung durch gleichzeitige Stimulation mit LPS und MDP.
- Für den synergistischen Effekt von LPS und MDP ist die Mitwirkung anderer Immunzellen nicht notwendig, isolierte Monozyten sind ausreichend für eine gesteigerte Zytokin-Freisetzung.
- Der synergistische Effekt von LPS und MDP zeigt sich auch *ex vivo*; dies wurde mit der Methode der isoliert-perfundierten Leber gezeigt und verdeutlicht, dass ebenso wie *in vivo* ein synergistischer Endotoxin-Leberschaden entsteht.

- Die Kinetik der LPS-induzierten TNFα-Freisetzung ist durch die Zugabe von MDP nicht verändert, jedoch zeigt sich eine Steigerung der LPS-induzierten TNFα-Transkription, die eher an einer gesteigerten *de novo* Transkription als an einer erhöhten mRNA-Stabilität liegt.
- In Bezug auf die Zytokin-Freisetzung wurden die strukturellen Vorraussetzungen der Muropeptide untersucht, um synergistische Effekte mit LPS zu induzieren. MDP (M(ADiQ)), GMDP (GM(ADiQ)), artifizielle Strukturen mit nicht-natürlichen Fettsäuren (C_{18}M(ADiQ) und M(ADiQKεC_{18})), MDP, bei dem Isoglutamin durch Glutamat (M(AγDE)) ersetzt wurde, und dieselbe Struktur mit Lysin verlängert (M(AγDEK), zeigen synergistische Effekte mit LPS im Bezug auf die Zytokin-Induktion. Stereoisomere von MDP (M(AiQ) und M(DADiQ)) und zusätzlich Muropeptide, die Diaminopimelinsäure (DAP, Dpm) enthalten, sowie Anhydro-Verbindungen, die ebenso DAP enthalten, induzieren keine gesteigerte durch LPS-induzierte Zytokin-Freisetzung (Abkürzungen der Muropeptide in Tabelle 5).
- Das kleinste Muropeptid, das nur eine Aminosäure enthält (M(A)), Muraminsäure (M) oder Anhydromuraminsäure A(M) stellen keine Antagonisten der Synergie dar, die durch LPS und MDP induziert wird.

- CpG-ODN induzieren nur wenig TNFα und andere Zytokine in PBMC (mononukleäre Zellen des periphären Blutes). Jedoch führt die gleichzeitige Stimulation durch MDP und CpG-ODN zu einer starken, synergistischen TNFα-Freisetzung.
- Der synergistische Effekt von MDP und CpG-ODN konnte auch mit anderen Typen von K- und D-Typ-ODN beobachtet werden, als auch mit GpC-ODN, wobei höhere Konzentrationen nötig waren (Sequenz der ODN in Tabelle 7).
- Zusätzlich konnte der Synergieeffekt mit unterschiedlichen Muropeptid-Strukturen beobachtet werden.
- Der synergistische Effekt von MDP und unterschiedlichen CpG-ODN ist auf andere Zytokine wie IL-1β übertragbar, jedoch wurde kein IL-10 freigesetzt.

- Der synergistische Effekt ist nicht durch TLR2 und TLR4 vermittelt, allerdings durch TLR9 und NOD2, was in Knockout-Mäusen gezeigt wurde, und TLR9 und NOD2 wurden somit als Rezeptoren identifiziert.
- In PBMC von Patienten mit der 3020insC-Mutation in NOD2 oder in CD14-gereinigten Monozyten von Wildtyp-Spendern, welche keine Expression von TLR9 zeigen, konnte kein Synergismus gezeigt werden. Deswegen sind wahrscheinlich die plasmazytoiden dendritischen Zellen (pDC) für den synergistischen Effekt verantwortlich.
- Ein großer Teil der Monozyten zeigt durch die gleichzeitige Stimulation von MDP und CpG-ODN eine TNFα-Freisetzung. Dies führt zu der Annahme, dass ein löslicher Mediator, der von TLR9-positiven Zellen nach Kontakt mit CpG-ODN sekretiert wird, verantwortlich ist für die TNFα-Freisetzung durch TLR9-negative Monozyten. IFNα ist wahrscheinlich einer der Mediatoren, die für diesen Effekt verantwortlich sind.

Zusammengefasst wurde eine vergleichende Charakterisierung von ganzen *Staphylococcus aureus*, LTA, CpG-ODN, PGN und Muropeptiden durchgeführt. Diese Arbeit zeigt, dass LTA das immunstimulatorische Prinzip von *Staphylococcus aureus* ist. MDP und andere Muropeptide induzieren bei gleichzeitiger Stimulation mit LPS und CpG-ODN eine synergistische Zytokin-Freisetzung. Die strukturellen Voraussetzungen und der Mechanismus der Synergie von LPS und MDP wurden charakterisiert. Für den starken synergistischen Effekt von MDP und CpG-ODN in PBMC sind wahrscheinlich die plasmazytoiden dendritischen Zellen sowie IFNα verantwortlich. Diese Ergebnisse tragen zu unserem Verständnis der Immunerkennung von Bakterien durch das Immunsystem bei.

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