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

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Muropeptides are breakdown products of peptidoglycan (PGN) of Gram-negative and Gram-positive bacteria. They are released during bacterial growth and division, as part of the host response by lysozyme and amidases, or upon antibiotic treatment. After phagocytosis of bacteria or bacterial breakdown products by host immune cells, the muropeptides trigger intracellular signaling cascades, leading to altered gene expression and activation of the immune response. Numerous muropeptides and derivatives have been synthesized chemically to characterize their immunostimulatory effects and adjuvant activity. Muramyl dipeptide, a natural partial structure of PGN, is the minimal structure with adjuvant activity. This review discusses the structure and occurrence of muropeptides and gives a broad overview of their inflammatory and adjuvant activity and the possible involvement of receptors in these responses.

Keywords: Muropeptides, muramyl dipeptide, immune response, inflammation, adjuvant activity

INTRODUCTION

The recognition of conserved bacterial structures, so-called 'pathogen-associated molecular patterns' (PAMPs) by pattern recognition receptors (PRRs) is a prerequisite for the activation of anti-bacterial host defense reactions. The best-studied recognition pattern is lipopolysaccharide (LPS) of Gram-negative bacteria, which is located in the outer membrane and consists of a lipid A anchor, an inner oligosaccharide core and outer chains of variable repetitive carbohydrates.[1] To a certain extent, lipoteichoic acid (LTA) represents the counterpart to LPS in Gram-positive bacteria. LTA is characterized by a polyol phosphate polymer anchored in the cytoplasmic membrane and projecting through the peptidoglycan (PGN).[2–4] Gram-positive and Gram-negative bacteria both contain 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 amino acids coupled to the muramic acid.[5,6] Muropeptides are breakdown products of PGN that bear at least the MurNAc moiety and one amino acid. One prominent muropeptide is muramyl dipeptide (MDP), which has been known since the 1970s to be the minimal structure that displays adjuvant activity.[7]

PRRs on immune cells play a key role in the recognition and killing of invading bacteria. One prominent family of the PRRs is the Toll-like receptor (TLR) family. Up to now, 13 mammalian TLRs have been identified, 10 in humans and 12 in mice.[8–11] The extracellular domain of all TLR molecules contains leucine-rich repeats. The cytoplasmic Toll/interleukin-1 receptor (TIR) domain shows similarities with a cytoplasmic domain of the interleukin-1 (IL-1) receptor family[12] and was also found in the cytoplasmic protein MyD88 (myeloid differentiation factor 88),[13] which functions as an ubiquitous adapter coupling TLR with downstream signaling kinases. Recent data show that some TLRs also display MyD88-independent

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Abbreviations: CpG-ODN, oligodeoxynucleotide containing cytosine-guanosine; DAP, diaminopimelic acid; GlcNAc, *N*-acetylglucosamine; MDP, muramyl dipeptide; MurNAc, *N*-acetylmuramic acid; NOD, nucleotide-binding oligomerization domain; PBMCs, peripheral blood mononuclear cells; PGN, peptidoglycan; TLR, Toll-like receptor.

pathways.[14] TLR4 is required to induce a cytokine response to LPS, as shown in C3H/HeJ and C57BL/10ScCr mice,[15] which carry spontaneous mutations of the *tlr4* genes, as well as in *tlr4*-knock-out mice.[16] CD14, an LPS receptor which lacks a membrane-spanning domain, and the extracellular adaptor molecules LPS-binding protein (LBP)[17,18] and MD-2[19] are also involved in initiating the cellular response to LPS. The analysis of lipid A structures showed that variations in the molecular composition of lipid A led to either conical-shaped lipid A or cylindrical-shaped lipid A, which are recognized by TLR4 or TLR2, respectively.[1,20]

Although some investigators initially reported that LTA signaling is TLR4 dependent,[21–23] several other groups have meanwhile proven that LTA recognition is instead TLR2-dependent.[3,24–26] This was substantiated further by the receptor dependency of chemically synthesized LTA.[27] The initially contradictory reports are likely due to the discrepant quality of the LTA preparations rather than to conformational variants. Commercial LTA preparations are often contaminated with endotoxin or other substances,[28,29] and the immunostimulatory activity of LTA from *Staphylococcus aureus* is destroyed by hot phenol extraction.[4]

Another potent activator of the immune system is bacterial DNA. Immune cells discriminate between host and bacterial DNA on the basis of unmethylated CpG motifs that occur more frequently in bacterial DNA than in vertebrate DNA. The recognition of bacterial DNA and synthetic CpG oligodesoxynucleotides (ODN), is mediated via TLR9,[30] which is exclusively located intracellularly in the endoplasmic reticulum.[31,32]

Beside LPS, LTA and DNA, the dominant cell wall component PGN also possesses immune activating activity, inducing activation of transcription and release of inflammatory mediators.[33–36] PGN was primarily thought to signal via TLR2.[37–41] This is under controversial discussion at the moment, since recent publications have demonstrated that repurification of Gram-negative PGN preparations to eliminate lipoproteins and repurification of Gram-positive PGN to eliminate LTA both abolished the TLR2-dependent activity of PGN.[26,42] Our group extended these observations by showing that small quantities of LTA are indeed present in commercial PGN preparations and by demonstrating that these small quantities of LTA can account for the immunostimulatory potency of PGN preparations, because the cytokine-inducing activity of LTA is amplified by a factor of up to 1000 when it is presented to immune cells on a scaffold instead of in solution (Traub *et al.*, submitted). This conclusion was further supported by the observation that a novel synthetic bisamphiphilic LTA, *i.e.* a model of two cross-linked LTA, displayed a greater immunostimulatory capacity than the monoamphiphilic molecule (Traub *et al.*, submitted).

Recent reports have identified the cytoplasmic proteins NOD1 and NOD2 as receptors for PGN degradation products. The NOD proteins are subfamily members of the CATERPILLAR family. Prominent members of this subfamily are NOD1 (also called CARD4) and NOD2 (also called CARD15). Both are located in the cytoplasmic compartment and are characterized by three structural domains: (i) a C-terminal domain with multiple leucine-rich repeats (LRR) that recognizes microbial components; (ii) a central nucleotide binding site (NBS), which is important for self-oligomerization of the molecule; and (iii) N-terminal effector motifs, the CARD domains, one or two for NOD1 and NOD2, respectively.[43] NOD1 was shown to recognize a breakdown product of Gram-negative PGN, *i.e.* the dipeptide γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP), of which the latter amino acid is not present in eukaryotes and is, therefore, an effective bacterial signature,[44] while NOD2 recognizes MDP,[45] a natural metabolite of both Gram-positive and Gram-negative PGN.

In this review, we summarize the structure, occurrence and actions of MDP and other muropeptides on the immune system, their strong synergistic effect with other bacterial components as well as the receptors that may be responsible for their recognition and intracellular signaling.

MUROPEPTIDES

Structure

While Gram-negative bacteria possess only a thin layer of PGN (*Escherichia coli* 1 nm[6]), Gram-positive bacteria bear a multilayered PGN cell wall (about 20–40 nm[5]), which encases the cytoplasmic membrane. Figure 1 shows the typical structure of the PGN of a Gram-negative bacterium (*e.g. E. coli*). PGN consists of a glycan backbone with alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). Four to five amino acids are linked to the lactyl group of the MurNAc residue. The amino acids occur in alternating L- and D-isomers and include γ-bonded D-glutamic acid, non-protein amino acids like diaminopimelic acid (DAP), ornithine or lanthionine, in combinations typical for the bacterial species. These amino acids cross-link the glycan backbones, usually via the free amino group of a basic amino acid, such as L-lysine (Lys) or *meso*-DAP, for lys-type or DAP-type PGN, and a terminal amino acid with a free carboxy group, frequently D-alanine.[46]

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.[47] The typical structure of PGN of Gram-positive bacteria like *S. aureus* is L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala, with an interpeptide

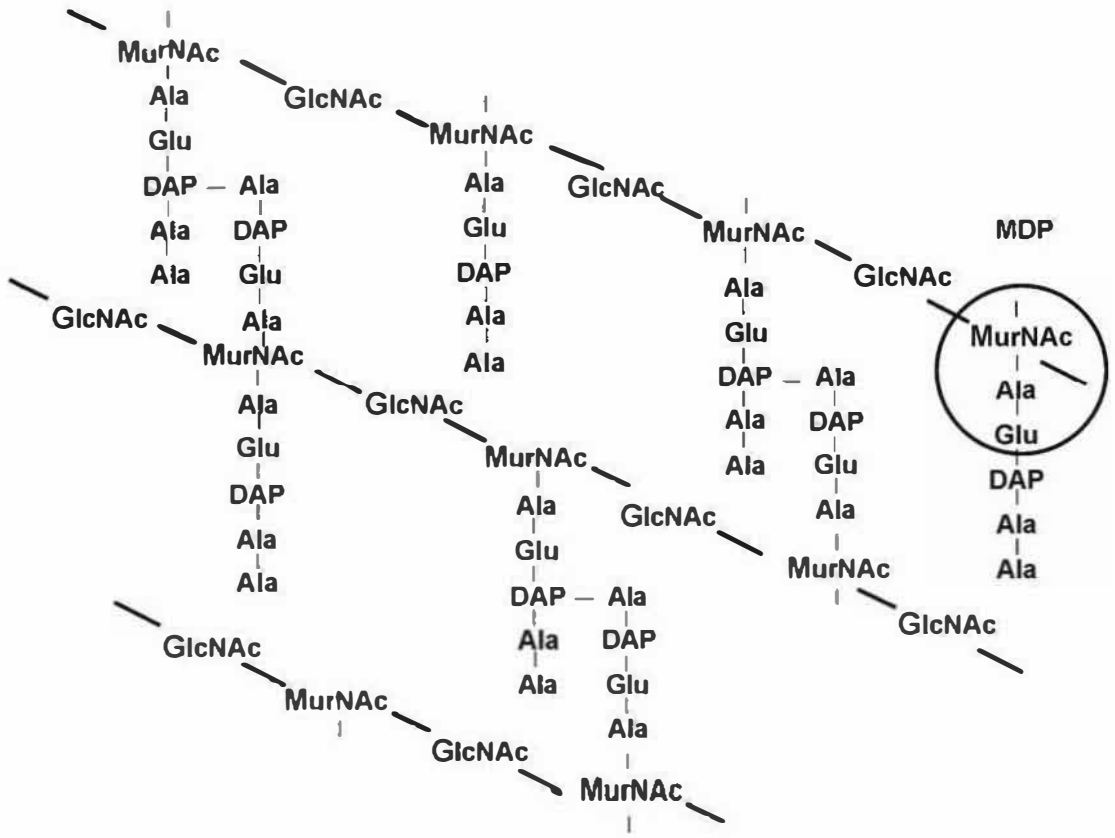

Fig. 1. Structure of the murein sacculus of a typical Gram-negative bacterium (*e.g. Escherichia coli*). Abbreviations: MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; Ala, alanine; Glu, glutamate; DAP, diaminopimelic acid.

bridge often made up of a chain of five Gly between the Lys side chain and the Ala of the neighboring chain.[48] Further variations in the peptide chains can be found in both Gram-negative and Gram-positive PGNs. Usually, the amino acid, which is linked to the muramic acid, is L-Ala, but this can be replaced by Gly or L-Ser. The second position D-Glu can be replaced by amidated Glu, Gly, amidated Gly or amidated Ala. The most common variations occur at position three, where *meso*-DAP or L-Lys are the norm, but L-ornithine, LL-DAP, hydroxy-Lys and others may substitute. Position four and five are almost always occupied by D-Ala with very few variations.[46] Additionally, there are variations of the interpeptide bridges. 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 made up of 2–7 amino acids with various sequences.[46] The well-known muropeptide MDP (N-acetyl-muramyl-L-alanyl-D-isoglutamine) corresponds to the stem peptide found in *Streptococcus pneumoniae*, Ala-isoGln-Lys-Ala-Ala,[49] but in most other PGNs, Glu is found in the second position.

Muropeptides are released during bacterial growth and division. The activity of bacterial lytic transglycosylases mostly produces anhydromuropeptides, which carry the terminal MurNAc residue in the 1,6 anhydro-form.[50] Lytic host enzymes like lysozyme and amidase can digest PGN, resulting in the release of muropeptide fragments. Muropeptides like the 1,6-anhydrodisaccharide tetrapeptide have been detected after cleavage of *E. coli* PGN with amidase from human serum.[51] When PGN from radiolabeled *Bacillus subtilis* cell walls was subjected to digestion by a macrophage cell line, disaccharides with di-, tri- and tetrapeptides (GlcNAc-MurNAc-Ala-isoGln-DAP-Ala and shortened forms) were released.[52] The PGN-hydrolases glucosaminidases also release muropeptides, and endopeptidases like the PGN hydrolase lysostaphin produced by *Staphylococcus simulans*, hydrolyze the peptide cross-bridges.[53–55]

The PGN recognition protein PGRP-L, which is mainly expressed in the liver, is an *N*-acetylmuramoyl-L-alanine amidase that cleaves PGN between the sugar moiety and the peptide moiety.[56] This activity suggested a role of PGRP-L in initiating the innate immune response to PGN. However, *Listeria monocytogenes* also expresses an *N*-acetylmuramoyl-L-alanine amidase, which mainly releases peptidic chains and sugar moieties but not substantial amounts of muropeptides.[57] Thus, the role of this kind of amidase may rather be to terminate immune reactions, not to trigger them.

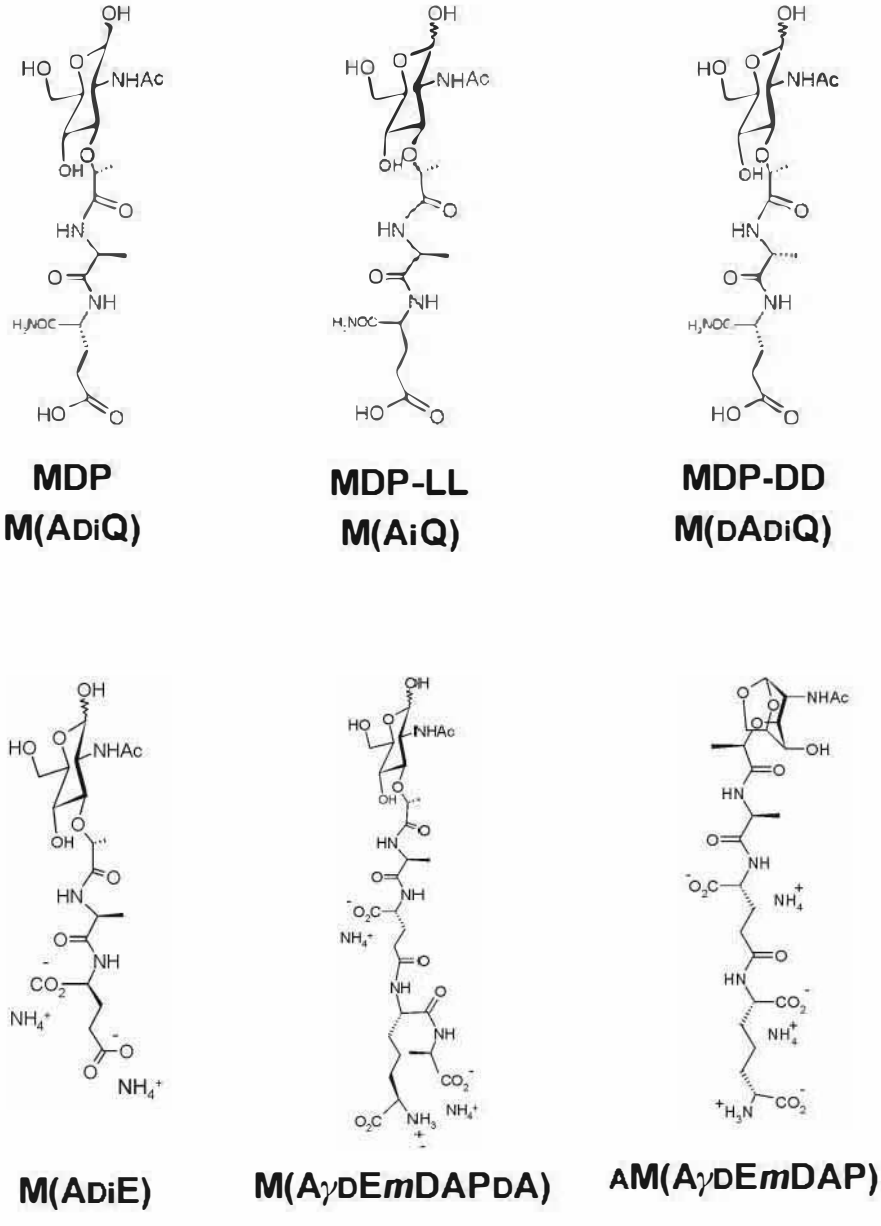

Fig. 2. Different muropeptide structures. Abbreviations: M, *N*-acetylmuramic acid; AM, 1,6-anhydromuramic acid; A, alanine; D, amino acid in D-configuration; L, amino acid in L-configuration; Q, glutamine; E, glutamate; DAP, diaminopimelic acid; γ, linkage of amino acids; *m*, meso

Taken together, a variety of muropeptides occur naturally, depending on the variability in PGN structure and dependent on the mode of PGN digestion. A selection of muropeptide structures is shown in Figure 2.

Occurrence in tissue and body fluids

To investigate whether free muropeptides occur naturally in host tissue and body fluids and to investigate their metabolism and clearance, muramic acid, which is not synthesized by mammalian enzymes, is a suitable marker for bacterial cell wall components. First evidence of muropeptides in tissue came from the discovery of sleep-promoting factor (factor S), later shown to be muropeptides, in cerebrospinal fluid of sleep-deprived healthy animals[58] and in human urine from a healthy donor.[59–61] No muramic acid could be found in brains and spleens of healthy rats,[62,63] while in another study small amounts were detectable in liver, brain and kidney (100–150 pmol/g tissue).[64] Higher muramic acid concentrations were detected in patient samples such as synovial fluid of septic patients (< 250–1700 ng/ml),[65] arthritic patients (220–2000 ng/ml)[66] and in urine of patients with urinal tract infections (about 170 ng/ml).[67] In patients with pneumococcal meningitis, muramic acid

levels between 6.8 and 3890 ng/ml were detected in cerebrospinal fluid.[63] Muramic acid in the intestinal content of healthy humans and in stool samples has been determined to lie in the range of 20–87 μmol/l MDP (~10–45 μg/ml).[68] To explain how bacterial breakdown products are taken up from the gut, Vavricka *et al.*[68] recently hypothesized that the apical di/tri-peptide transporter hPepT1 in the intestinal epithelium may transport MDP.

Muropeptides are highly water soluble and are, therefore, eliminated from the circulation rapidly. Larger, radiolabeled muropeptide structures like the disaccharide pentapeptide (GlcNAc-MurNAc-Ala-isoGln-*meso*-DAP-Ala-Ala) and other structures have been recovered in murine urine after intravenous administration.[69,70] After oral administration of 1.5 mg/kg MDP to rats, a maximal MDP concentration of 20 ng/ml (~0.05%) was detected in the blood plasma after 1 h.[71] Two minutes after intravenous injection of the same dose of MDP into rats, less than 35% of the injected dose was detected; after 2 h, MDP had nearly been eliminated.[71] Similar results were obtained in another study, where 2 mg of MDP was injected into the ear vein of a rabbit.[72]

The investigation of the levels of muramic acid in healthy animals may be hampered by the limitations of the respective chromatographic methods used, which have detection limits in the nanogram per millilitre range. Measurements in infected animals and humans indicate the presence of muropeptides in diseased tissue. In most *in vivo* and *in vitro* studies reported so far, higher concentrations of MDP and other muropeptides (often 10–100 μg/ml) have been used than were ever detected even in very sick patients (up to 4 μg/ml), thus questioning the relevance of some of the results.

Effects on the immune system

In 1974, MDP was discovered as the minimal structure responsible for the improved reaction to mycobacteria in Freund's complete adjuvant.[7] Since then, much effort has been made to isolate, synthesize and characterize the activity of MDP and other muropeptides.[73,74] In this context, direct effects as well as priming and synergistic properties of muropeptides were discovered and researched independently of the studies on adjuvant activity, although the mechanisms of these activities appears to be at least partially inter-related. In the following, we shall discuss the vaccine adjuvant effects of muropeptides separately from the direct effects and the priming effects, in which pretreatment with muropeptides augments immune responses to a later challenge, and synergistic effects, in which concentrations of muropeptides that are ineffective alone combine to induce a strong host response.

Direct effects

Numerous reports suggest that MDP and other muropeptides directly induce cytokines, thus activating and modulating immune responses and inflammation. However, these reports are controversial and seem to depend on the cell-type and species. For example, in the human Monomac-6 cell line, MDP was reported to induce a weak induction of TNF-α mRNA but not protein,[75] while in human monocytic OCT-differentiated THP-1 and U937 cells induction of IL-8 protein was observed in response to MDP.[22] For human primary cells, IL-1β and TNF-α release from *in vitro* MDP-stimulated monocytes has been shown;[76,77] some authors suggest that activation of the inflammasome complex (caspase-activating complex)[78,79] is responsible for this activity. In contrast, we and others have reported no cytokine release from human whole blood or isolated monocytes by MDP or related compounds.[80,81] Susceptibility towards *in vitro* MDP stimulation has been shown for macrophages from guinea pigs, rats and mice.[82–84] Possible explanations for the controversial results could be different origin and purity of muropeptides, lack of exclusion of contamination (*e.g.* with the strong immune activator LPS), as well as use of often unphysiologically high MDP concentrations.

Although *in vivo*, mice are rather insensitive to muropeptides (LD_{50}, ~2200 mg/kg i.p.[85]),[85] MDP is considered to enhance non-specific resistance of the immune system. This has been proven in several *in vivo* models, where MDP stimulation decreased survival of intracellular *Salmonella typhimurium*,[87] stimulated host resistance against *Klebsiella pneumoniae*[88] and *Candida albicans* infections[89] as well as killing of *Leishmania donovani*.[90]

As mentioned above, MDP has been discovered to be a sleep-promoting factor in the 1970s.[90] The somnogenic and sleep regulatory properties of MDP and derivatives have been demonstrated by transfer experiments, in which substances isolated from sleep-deprived animals induced sleep in the receiver animal.[58,59,91] This might be explained by an indirect action of muropeptides via the induction of endogenous pyrogens like IL-1, TNF and NO, which are probable sleep factors and might, therefore, play a role in sleep regulation.[92–94] Furthermore, MDP and related muropeptides have been shown to induce some behavioral changes like hyperthermia,[95] hypermetabolism, weight loss and suppression of food intake during bacterial infection.[96] However, in order to reduce food intake, very high doses of MDP (> 1 mg/kg) were necessary.[97] There is some evidence that, again, MDP may rather act indirectly via cytokines than having direct anorectic effects.[96,98,99]

To determine the receptor-dependence of the anorectic effect, the role of CD14, TLR2 or TLR4 has been investigated. MDP reduced food intake in wild-type mice of all respective genotypes, also in TLR4 deficient mice,

Table 1. Synergy of LPS or lipid A with MDP and other muropeptides

Model	Stimulus (concentration)	Muropeptide (concentration)	End-point	Synergy	Reference
Human whole blood	LPS, *E. coli* ●26:B7 (10 ng/ml)	MDP (1 μg/ml)	TNF IL-6 IL-10	3-fold 2-fold –	80,124
Human whole blood (20%)	LPS, *S. abortus equi* (10 pg–100 μg/ml	MDP (10 ng/ml)	TNF IL-1β IL-6 IL-10	400-fold 300-fold 7-fold 3-fold	81
PBMCs	LPS, *E. coli* ●111:B4 (10 pg/ml)	MDP (1–100 ng/ml)	IL-ra IL-1β TNF	10-fold 5-fold ±	125
hu MDM	LPS, *E. coli* ●111:B4 (10–100 ng/ml)	MDP (10 μg/ml)	IL-1β mRNA HLA-DR mRNA	+ +	127
Isolated monocytes	LPS, *S. abortus equi* (100 pg/ml)	MDP (10 ng/ml)	TNF	2-fold	81
Mononuclear cells (MNCs)	LPS, *E. coli* (1 ng/ml)	MDP (10 μg/ml	TNF	2-fold	126
Monomac-6	LPS, *E. coli* ●55:B5 (30 ng/ml)	MDP (30 min pre-incubation; 100 μg/ml)	TNF TNF mRNA	2 fold additive	75
●CT-diff. THP-1	LPS, *S. abortus equi* (1–100 ng/ml)	MDP (1–100 ng/ml)	IL-8	8-fold	22
●CT-diff. U937	LPS, *S. abortus equi* (0.1–10 ng/ml)	MDP (0.1–10 ng/ml)	IL-8	3-fold	22
Rat *in vivo*	LPS, *E. coli* ●111:B4 (25 μg/kg)	MDP (0.4 mg/kg)	Anorexia	+	130
Rat liver macrophages	LPS, *E. coli* ●127:B8 (5 ng/well)	MDP (5 μg/well)	Macrophage-mediated cytolysis	2–3-fold	128
RAW264.7 cells	LPS, *E. coli* (10 ng–1 μg/ml)	MDP (40 ng–25 μg/ml)	TNF	3–10-fold	129
Human whole blood (20%)	LPS, *S. abortus equi* (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 – –	81
●CT-diff THP-1	Lipid A, *E. coli* (LA-15-PP) (1–100 ng/ml)	MDP FK 156 FK 565 iE-DAP (1–100 μg/ml)	IL-8	5-fold 7-fold Similar (ns) –	132
BMDM (NOD1$^{-/-}$)	LPS, *S. typhimurium* (100 ng/ml)	iE-DAP 1 ng/ml 1 μg/ml	IL-6	100-fold 500-fold	44
HTE derived from hamster tracheal tissue	LPS, *E. coli* (100 EU/ml; 1–10000 EU/ml)	TCT	IL-1α IL-1amRNA NO Inhibition of DNA synthesis	5.5 fold + 2-fold	131 80%

TCT: tracheal cytotoxin, GlcNAc-1,6 anhydro MurNAc-Ala-Glu-meso-DAP-Ala) (0.09–9.21 μg/ml).

thus indicating that this effect does not stem from possible LPS contamination. The anorectic effect of MDP was blunted in CD14- and TLR2-knockout mice, indicating that CD14 and TLR2 are involved in the signaling pathway of MDP-induced anorexia.[100]

In addition to muropeptide effects like immune activation or anorexia, which have been studied in greater detail (although far from being completely understood), many diverse effects, like induction of leukocytosis, protein influx into cerebrospinal fluid or brain edema in a rabbit model of meningitis[101] have been described. In renal cells, muropeptides have been shown to induce apoptosis[102] and a recent publication suggests that this is mediated via calreticulin,[103] which has also shown to be a binding protein for MDP and PGN.[104]

Adjuvant activity

As an immunogenic adjuvant, MDP increases phagocytic and anti-microbial activity[105,106] by enhancing the expression of surface markers that are involved in cellular adhesion processes and co-stimulation for antigen presentation.[107,108] In addition, MDP enhances antigen processing and presentation by antigen-presenting cells, thereby leading to increased antibody-mediated cytotoxicity,[109] which thus improves the induction of antibodies by vaccine antigens.[110,111] Furthermore, MDP as well as other muropeptides (tripeptides and disaccharide tri- and tetrapeptides) induce cellular immune defenses, as evidenced by delayed-type hypersensitivity skin reactions.[7,112] MDP has been shown to augment the effect of other immunomodulators like IFN-γ[113] and to synergize with cytokines to stimulate the differentiation and proliferation of lymphocytes.[114]

Priming effects

Muropeptides prime for enhanced susceptibility of animals towards anaphylactic reactions and lethal toxicity in response to bacterial endotoxins.[115] LPS species of low toxicity were found to lead to anaphylactic reactions and reactions to high-toxicity LPS were further augmented.[85] The priming ability of MDP occurred in endotoxin-sensitive and -resistant strains like C3H/HeJ mice, but variations were observed among different mouse strains; for example, C3H/HeN mice are highly and C57BL/6 are less susceptible.[116] To achieve optimal priming effects, distinct time schedules (4 h before and after administration of LPS[116–118] and administration routes (intravenous or intraperitoneal injection) of MDP must be followed.[116]

Structural requirements of muropeptides to prime for induction of anaphylactic reactions by LPS have been investigated in C3H/HeJ mice using the priming conditions mentioned above. While the DAP-type muropeptide GlcNAc-MurNAc-pentapeptide induced no anaphylactic reactions, the disaccharide-tetrapeptide and the MurNAc-tetrapeptide showed marginal reactions, but the disaccharide-tripeptide and the MurNAc-tripeptide led to death of the animals.[119] 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 of L-glutamic acid, L-glutamine or L-isoglutamine were inactive.[119] Furthermore, a synthetic lactyl peptide (desmuramylpeptide) D-lactyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly (FK156) also exhibited priming activity for anaphylactic reactions.[119] Thus, MDP is the minimal structure for priming effects; other structures are also effective, but apparently not fragments that are larger than the disaccharide-tetrapeptide. These observations are in line with a recent publication, which shows that in mice defective for the protein NOD2 (recently identified as the MDP receptor), endotoxic shock induced by pre-treatment with MDP was prevented.[120] As a mechanism for the anaphylactic reaction, a complement-dependent degradation of platelets, which have accumulated in liver and lung, resulting in acute inflammation with severe tissue destruction has been suggested.[121–123]

Synergistic effects with LPS

MDP and other muropeptides are strong immune amplifiers. In contrast to priming effects, where one stimulus is administered before the second stimulus, synergism is defined as a situation in which the combination of two weak stimuli, leads to an enhanced response. Synergistic actions of MDP and LPS have been described in many studies using different cell types and stimulus concentrations. Although different end-points, mostly cytokines, have been measured, all studies consistently describe strong synergistic effects of LPS and muropeptides. Table 1 gives an overview of these studies with regard to cellular models, stimulus concentrations and end-points. Synergistic results were obtained *in vitro* with human primary cells like whole blood,[80,81,124] PBMCs,[125] isolated monocytes,[125–127] and various human monocytic[22,127] and rodent cell lines[128,129] as well as *in vivo* in a rat model of anorexia.[130] Only a few studies have investigated mRNA levels, but have shown for TNF-α and IL-1α that the LPS/muropeptide synergism already affected the mRNA level[75,81] and experiments using actinomycin D demonstrated that this effect is due to increased *de novo* transcription, rather than to an increase in mRNA stability.[81] Several studies have extended these investigations from MDP to further muropeptides with different structures, which were extracted and purified or synthesized, and have defined structural prerequisites and minimal active structures.[44,81,131,132]

Synergistic effects with other TLR agonists

Muropeptides exert remarkable priming and synergistic effects with the TLR4 agonists LPS or lipid A. These

Table 2. Synergy of different stimuli with MDP and other muropeptides

Receptor	Stimulus (concentration)	Muropeptide (concentration)	End-point	Synergy	Model	Ref.
TLR2	LTA, *S. aureus* (0.1–10 μg/ml)	MDP (1–100 μg/ml)	IL-8	7-fold	●CT-diff. THP-1	22
TLR2	LTA, S. aureus (0.1 μg/ml)	GMDP MDP MDP (LL) MDP (DD)	N●	2-fold 2-fold 1.5 fold –	J774.2	134
	LTA, *B. subtilis* (0.1 μg/ml)	GMDP (1 μg/ml, 10 μg/ml)		3-fold		
TLR2	LTA, *S. aureus* (100 ng/ml)	MDP (10 ng/ml)	TNF	–	Human whole blood (20%)	See Fig. 3
TLR2	LTA, *S. aureus* (10 μg/ml)	MDP (50 ng/ml)	TNF	–	PBMCs (10% serum)	Unpub. observ.
TLR2	LTA, *S. aureus* (10 μg/ml)	MDP (50 ng/ml)	TNF	5-fold	PBMCs (without serum)	See Fig. 3
TLR2	LTA, *S. aureus* *S. pneumoniae* (0.04–1 μg/ml)	MDP (30 min preincubation; 100 ng/ml)	TNF	5-fold 5-fold	Human monocytes (2.5% serum)	133
TLR2	MALP-2 (10 ng/ml)	MDP (50 ng/ml)	TNF	5-fold	PBMCs (without serum)	See Fig. 3
TLR2	MALP-2 (1 μg/l)	MDP (20 μg/l)	TNF IL-1β	2.1-fold 3.5-fold	PBMCs	135
TLR2	MALP-2 (1 μg/ml)	MDP (10 μg/ml)	TNF IL-1β IL-10	2-fold 6-fold 6-fold	MNCs	126
TLR2	PGN (100 μg/ml)	MDP (30 min pre-incubation; 100 μg/ml	TNF TNF mRNA	2-fold additive	Monomac-6	75
TLR2	Pam_3Cys (1–100 pg/ml)	MDP FK 156 FK 565 (1–100 μg/ml)	IL-8	4-fold 4-fold Similar (data not shown)	●CT-diff. THP-1	132
TLR2	Pam_3Cys (1 μg/ml)	MDP (10 μg/ml)	TNF IL-1β IL-10	2-fold 8-fold 6-fold	MNCs	126
TLR2	Pam_3Cys (10 μg/l)	MDP (20 μg/l)	TNF IL-1β	1.8-fold 3.2-fold	PBMCs	135
TLR2	Pam_3Cys (2 μg/ml)	MDP (1, 10 μg/ml)	IL-6	2-fold	BMDM	120
TLR3	Poly(I:C) (50 μg/ml)	MDP (10 μg/ml)	TNF	2-fold	MNCs	126
TLR3	Poly(I:C) (100 μg/ml)	MDP (1, 10 μg/ml)	IL-6 IL-12 p40	3-fold 2-fold	BMDM	120
TLR5	Flagellin (10 ng/ml)	MDP (10 μg/ml)	TNF	Additive	MNCs	126
TLR5	Flagellin 50 (μg/l)	MDP (20 μg/l)	TNF IL-1β	3.4-fold 3.9-fold	PBMCs	135
TLR7	Loxoribin (5 μg/ml)	MDP (10 μg/ml)	TNF	–	MNCs	126
TLR7/8	R848 (1 mg/l)	MDP (20 μg/l)	TNF IL-1β	1.7-fold 2-fold	PBMCs	135

(continued on next page)

Table 2. (*continued*) Synergy of different stimuli with MDP and other muropeptides

Receptor	Stimulus (concentration)	Muropeptide (concentration)	End-point	Synergy	Model	Ref.
TLR9	CpG (10–1000 nM)	MDP FK 156 FK 565 (1–100 µg/ml)	IL-8	4-fold 6-fold Similar (ns)	●CT-diff. THP-1	132
TLR9	CpG (2 µM)	MDP MDP (DD) MurNAc-Ala-Gln MurNAc-Ala-Gln-Lys MurNAc-Ala-Gln-DAP Anhydro-MurNAc-P Ala-Gln-DA (50 ng/ml)	TNF – 1200-fold 1300-fold 1300-fold 350-fold	2000-fold	PBMCs	Traub *et al.*, submitted
TLR9	CpG (5 µg/ml)	MDP (10 µg/ml)	TNF	–	MNCs	126
T-cell/ MHCs	SEB (100 ng/ml)	MDP (10 ng/ml)	IL-2	–	Plasma-free blood	See Fig. 3
CD3	●KT-3 (5 ng)	MDP (50 ng/ml)	IL-2	–	Plasma-free blood	See Fig. 3
PKCs	PMA (10 ng/ml)	MDP (10 ng/ml)	TNF	9-fold	Human whole blood (20%)	See Fig. 3

MNCs, mononuclear cells; PBMCs, peripheral blood mononuclear cells.

findings raised the question, whether muropeptides also act synergistically with other TLR agonists. An overview, focusing on stimulus concentrations, receptor dependence, end-points and cellular models is given in Table 2. TLR2 agonists like LTA from different Gram-positive bacteria, MALP-2, PGN and Pam_3CSK were found to synergize with MDP in human PBMCs[133] and various monocytic cell lines.[22,75,120,126,132,134,135] In contrast, in human whole blood, no synergy of LTA and MDP was found (Fig. 3) and the synergistic effect observed in human PBMCs under serum-free conditions was reduced in the presence of 10% autologous serum (authors' unpublished results). In line with this, the experiments of Schröder *et al.*[133] were performed with human monocytes under low serum conditions (2.5%). This might indicate that serum components blunt the synergistic effect of LTA and MDP, but this requires further study.

Fewer and, furthermore, controversial results are available for MDP synergism with TLR3, TLR7 and TLR9 agonists. The TLR3 agonist poly(I:C) has been shown to act synergistically with MDP in human primary monocytic cells,[120,126] while in the same study, flagellin (TLR5-agonist) as well as loxoribin (TLR7-agonist) and CpG-●DN (TLR-9-agonist) did not.[126] In contrast, others reported that flagellin as well as the TLR7-agonist R848 (Resiquimod)[135] and CpG-●DN (Fig. 3 and Traub *et al.*, submitted) induce synergistic cytokine release with MDP and other muropeptides.[132] Unpublished data from our group obtained with non-TLR agonists show that the membrane-permeable, protein kinase C activator phorbol myristate acetate (PMA) acts synergistically with MDP, while the superantigen staphylococcal enterotoxin B (SEB) and anti-CD3 antibody ●KT-3, do not (Fig. 3).

Taken together, MDP and other muropeptides show synergistic activity with a broad variety of different TLR agonists, indicating a possible interplay of the signal transduction pathways of the different TLR receptors and the cytosolic N●D proteins.

Receptors of muropeptides

For many years, the receptor for MDP and other muropeptides was unknown. Various receptors were discussed as possible candidates, such as the 5-HT receptor, CD14 and the TLR receptors. ●nly recently, two intracellular proteins of the N●D family, N●D1 and N●D2, were discovered as receptors for MDP and other muropeptides.

5-HT receptor

MDP has neuropharmacological activities, such as effects on sleep, analgesic properties and influence on behavior.[92,136] Previous reports from Sevcik and Masek *et*

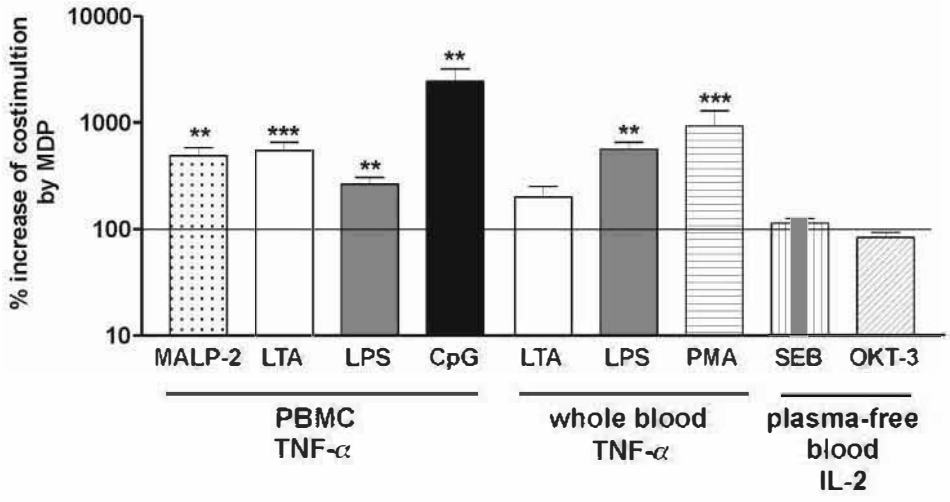

Fig. 3. Synergistic effect of MDP with different immune stimuli. PBMCs were stimulated with MALP-2 (10 ng/ml), LTA (*Staphylococcus aureus*, 10 µg/ml), LPS (*Salmonella abortus equi*, 10 ng/ml) or CpG-ODN 2006 (2 µM) and incubated for 18 h. Human whole blood was stimulated with LTA (*Staph. aureus*, 100 ng/ml), LPS (*S. abortus equi*, 100 pg/ml) or phorbol myristate acetate (PMA, 10 ng/ml) and incubated for 24 h. Plasma-free blood was stimulated with staphylococcal enterotoxin B (SEB, 100 ng/ml) or OKT-3 (5 ng) and incubated for 48 h. All incubations were carried out in the presence or absence of 10 ng/ml MDP (human whole blood) or 50 ng/ml MDP (PBMC and plasma-free blood). Cytokines were determined in the cell-free supernatants by ELISA. Cytokine release induced by the respective stimuli alone was set to 100%. Data are mean ± SEM of 4–8 donors. ** and *** indicate significance versus the respective stimulus alone.

al.[137,138] suggested that these activities might be mediated by interactions with the serotoninergic system via the 5-HT receptor. However, recent reports from the same group showed that this is not the case when physiologically relevant concentrations in the nanomolar range are employed and no interaction of MDP with either the 5-HT_4 or the 5-HT_{1A} receptor was observed.[139,140]

CD14

Membrane CD14 (mCD14) is a glycosylphosphatidylinositol-anchored cell-surface molecule found on cells of the myeloid lineage. Since it lacks a cytoplasmic domain, it cannot transmit signals into the cell. The anchor is missing in soluble CD14 (sCD14), which is present in human serum as an acute phase protein. CD14 is thought to be an adaptor molecule accepting complexes made up of LBP and pathogen-derived ligands and passing the pathogen-derived ligand on to a TLR, which bears a transmembrane domain, resulting in intracellular signaling.

The role of CD14 in MDP-dependent cell activation is controversial. Several findings argue in favor of CD14 as an MDP receptor. Anorectic effects of MDP were abrogated in CD14-knockout mice.[100] In human monocytes, the cytokine release induced by the disaccharide-pentapeptide (GlcNAc-MurNAc-Ala-isoGln-*meso*-DAP-Ala-Ala) was enhanced by pre-incubation with sCD14, while anti-CD14 mAb blocked these effects.[141] MDP was also shown to bind to mCD14, preventing the binding of soluble PGN.[142] In human gingival epithelial cells, CD14 and LBP, but not LBP alone, enhanced MDP-stimulated activation[143] arguing for a role of CD14 as a possible MDP receptor. In line with human gingival fibroblasts expressing high CD14 levels, MDP-induced IL-8 release was inhibited by anti-CD14 mAb, while in human periodontal ligament fibroblasts, expressing low CD14, this was not the case. These results indicate that other CD14-independent pathways must exist as well.[144] In line with this, MDP did not induce reporter gene expression in constitutively CD14 expressing CHO cells[145] and in monocytic cell lines, cytokine induction by LPS/MDP[75] or MDP alone[22] could not be inhibited by anti-CD14 mAb. Others have shown that monomeric MDP does not bind to sCD14, while MDP or GlcNAc-MDP immobilized on agarose does.[146] These investigators concluded that solid-bound MDP is needed for CD14 binding.

So far, these results point to a possible interaction of MDP with CD14. Since results of transfection experiments are strongly affected by transfection efficacy and proper protein surface expression and the use of blocking antibodies is limited by the antibody blocking capacity, all of which are not always sufficiently controlled, further experiments with cells from CD14 knockout mice could shed some more light on this subject. Again, the lack of exclusion of LPS contaminations as well as the use of different MDP concentrations, ranging from 1 µg/ml to 100 µg/ml might be a reason for some of the controversy.

TLRs

Each TLR recognizes one or more specific PAMPs. Ligand recognition initiates a signaling cascade, activat-

ing the immune response. The role of TLR2 and TLR4 in MDP signaling has been addressed in several studies. In human gingival epithelial cells, MDP-induced IL-8 release could be reduced by pre-treatment with an anti-TLR2 mAb[143] and reduced food intake caused by MDP was not restored in TLR4-deficient mice, but in TLR2 knockout mice.[100] However, neither TLR4 nor TLR2 dependence was observed when CHO cells were transfected with CD14/TLR2, CD14/TLR4 or CD14/TLR4/TLR2 and stimulated with MDP.[145,147] In line with this, no inhibitory effect of anti-TLR2 or anti-TLR4 mAb on cytokine release was found in monocytic cell lines,[22,75] human periodontal ligament or gingival fibroblasts as well as in IFN-γ-primed oral epithelial cells.[23,144] Additionally, MDP-stimulated cytokine release in monocytic OCT-differentiated U937 cells, which do not express TLR2, indicates TLR2-independent activity.[22] Some stimuli such as Pam_3CSK and MALP-2 require dimerization of TLR2 with TLR1 or TLR6 for signal transduction.[148,149] Since neither expression of TLR2 with TLR1 nor TLR6 enabled MDP to induce NF-κB activation, heterodimerization did not appear to be the missing link.[150] MDP stimulation enhanced the basal transcription of MyD88 mRNA in THP-1 cells, an important adaptor molecule for TLRs, which might provide a possible mechanism for synergistic actions with TLR agonists.[22] Although LPS induced up-regulation of MyD88 mRNA as well, no clear synergistic up-regulation of MyD88 mRNA could be observed with LPS plus MDP.[22]

Taken together, most results argue against a role of TLR2 or TLR4 as the MDP receptor in the initiation of immune responses, although TLR2 seems to play a role in the anorectic effects; not all TLRs have been investigated so far.

NOD proteins

Recently, another family of PRRs, the cytoplasmic nucleotide-binding oligomerization domain (NOD) family of proteins, which seems to play an important role in intracellular immune defense, has been identified. The NOD proteins share homology with a class of plant proteins (R proteins) that recognize invading pathogens and mediate a defense response, resulting in plant-disease resistance.[151] The NOD family of proteins has several members, two of them, NOD1 and NOD2, were initially shown to recognize LPS,[152] but recent studies evidenced that NOD1 and NOD2 actually do not detect LPS, but PGN fragments. It was shown that such fragments, which had co-extracted with LPS during purification in the original study, had led to the false assumption in the first report.[44,153]

NOD1

NOD1 consists of three domains: (i) a centrally located nucleotide oligomerization domain (NOD); (ii) C-terminal leucine-rich repeats (LRR) for bacterial recognition; and (iii) the N-terminal effector domain, which contains one caspase recruitment domain (CARD). NOD1 is expressed in multiple tissues.[154,155] Carneiro *et al.*[156] hypothesized that, when no stimuli are present, NOD proteins are negatively regulated by their LRR by folding. NOD1 detects only PGN fragments containing DAP at the third position, thereby distinguishing between PGN-derived compounds from Gram-negative and Gram-positive bacteria.[153] The precise structure of the PGN-derived compound was identified as a DAP-containing naturally occurring muropeptide, the GlcNAc-MurNAc-tripeptide.[153] Furthermore, NOD1 detects the DAP-containing muropeptides MurNAc-tripeptide, which has only one sugar moiety, as well as a UDP-MurNAc-tripeptide, which is a precursor of the PGN biosynthesis pathway. MurNAc-L-Ala-D-Glu or MurNAc-tetrapeptide are not detected, pointing to the importance of the terminal *meso*-DAP.[157] The dipeptide γ-D-glutamyl-*meso*-DAP has been identified as the minimal active motif,[157] indicating that NOD1 sensing relies only on the peptidic moiety. However, the tripeptide L-Ala-γ-D-Glu-*meso*-DAP showed higher activity, suggesting that the L-Ala residue is required for optimal detection by NOD1.[157] NOD1 also detects a lactoyl-tripeptide containing DAP, though only weakly in comparison to the GlcNAc-MurNAc-tripeptide, MurNAc-tripeptide or the tripeptide alone.[157] Another synthetic dipeptide (*i.e.* γ-D-glutamic-*meso*-DAP) can also stimulate NOD1.[44]

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 cells, granulocytes and to a lesser extent in T-lymphocytes.[158] Recent publications have shown by immunostaining of intestinal tissues that NOD2 is highly expressed in Paneth cells in the terminal ileum.[159–161] Furthermore, NOD2 mRNA was found in unstimulated epithelial cells from normal colon at low levels, but expression was increased in macrophages and intestinal epithelial cells from patients with Crohn's inflammatory bowel disease.[158,162] LPS and TNF induce up-regulation of NOD2 mRNA in myeloblastic and epithelial cells. The authors suggested that the activation of NF-κB by pro-inflammatory stimuli by enteropathogens or pathogenic bacteria could induce expression of NOD2 and lead to a positive feedback loop, resulting in secretion of pro-inflammatory cytokines and chemokines from epithelial cells.[158,163]

A large number of studies also point to a role of NOD2 as a susceptible gene involved in Crohn's disease.[135,164–166] The most common associated mutation is a frame-shift mutation resulting in a NOD2 protein with a truncated terminal LRR that cannot detect MDP.[164]

NOD2 mutations may disable normal local responses in the intestinal mucosa, hindering control of bacterial infection and eventually leading to systemic responses and aberrant inflammation.[167]

Since MDP is a common PGN motif, NOD2 is a broad sensor for Gram-negative and Gram-positive bacteria. Girardin *et al.*[157] have extensively studied the structural requirements of muropeptides to serve as NOD2 agonists. They found that GlcNAc-MurNAc-L-Ala-D-isoGln, MurNAc-L-Ala-D-Glu, as well as muramyl tripeptides with lysine or ornithine and to a lesser extent structures with amidated DAP at the third position, are recognized by NOD2. PGN precursors like UDP-MurNAc-tripeptides are also recognized by NOD2. As minimal structure, an intact MurNAc group substituted with a peptide chain is necessary for NOD2 signaling, because peptides lacking the MurNAc sugar moiety do not activate NOD2.[157] Synthetic MDP-analogues, in which the conformation of the L-Ala had been changed to D-Ala or D-isoGln to L-isoGln revealed that the recognition process is stereoselective.[150] A positive interaction of the signaling pathways of NOD1 and NOD2 with different TLRs is probably the basis for the observed synergism of muropeptides with TLR agonists,[120,126,132] but the precise molecular basis of the interaction is not known to date. A recent report has shown that NOD2 can inhibit TLR2-mediated NF-κB activation,[168] while others reported a modulation of the TLR2 pathways by NOD2.[169] Furthermore, NOD proteins might be important for cells in which TLR are absent or down-regulated.

Internalization of muropeptides

Although there is evidence that NOD proteins play a role in recognition of PGN breakdown products, it is not clear how the muropeptides come into contact with the cytosolic NOD proteins. In most experiments, the investigators used transfection reagents or digitonin-permeabilized cells, which allow the muropeptides to enter the cytosol directly.[153] In the case of immune cells, phagocytosis of bacteria is probably the physiological process. After phagocytosis, the bacteria become degraded by lysosomal proteases and muropeptides may become available to intracellular proteins. How muropeptides reach the cytosol from the phagosome remains unclear.[156]

In non-phagocytic cells, direct bacterial invasion into the cell or by a bacterial transfer apparatus is imaginable. Entero-invasive bacteria like *Salmonella*, invasive *E. coli*, *Listeria*, *Shigella* or *Yersinia*, for example, induce their own uptake by epithelial cells of the intestinal mucosa.[170] Girardin *et al.*[171] demonstrated, that NF-κB activation by *Shigella flexneri* was inhibited by overexpression of dominant-negative NOD1. Furthermore, NF-κB activation induced by *Streptococcus pneumoniae*, was dependent on NOD2, as well as NOD1, and NOD2 mRNA expression was up-regulated after pneumococcal infection.[172] Two studies indicate a direct anti-bacterial activity of NODs. The number of viable, internalized *Salmonella typhimurium* in Caco-2 cells, that were stably transfected with NOD2, was lower than in vector-transfected cells.[173] In line with this, infection with entero-invasive *E. coli* was avoided in dominant-negative NOD1 colon epithelial cells.[174]

Extracellular, non-invasive bacteria, such as cagPAI-positive *Helicobacter pylori*, are recognized by NOD1; also, NOD1-deficient and NOD1-knockout mice were more susceptible to infection with cagPAI-positive *H. pylori* strains.[175] CagPAI genes are proposed to encode a type IV secretion apparatus, and the secretion of PGN fragments by the type IV secretion system, was necessary for NOD1-dependent activation of NF-κB. Digestion and identification of the high-performance liquid chromatography fraction of the PGN of *H. pylori* showed the GlcNAc-MurNAc-tripeptide as the active component.[175] Another hypothesis is the transport of MDP by the intestinal epithelial apical di-/tripeptide transporter hPepT1, which in turn has been shown to activate the cell via NOD2.[68]

Although a direct interaction of muropeptides with NOD proteins has not been shown yet and the route of entering the cell cytoplasm is not fully clarified, there is strong evidence that the NOD proteins are muropeptide receptors or at least possible downstream molecules in muropeptide signaling.

Conclusions

Free muropeptides are present in the body during infection. They are naturally released during bacterial growth and division, by antibiotic treatment or the activity of lytic host enzymes. The diversity of PGNs from different bacterial strains leads to a variety of possible muropeptide structures. Muramyl dipeptide (MDP) is a prominent motif and represents the minimal biologically active structure. Surprisingly, larger structures do not exert enhanced biological activity. The cytosolic proteins NOD1 and NOD2 have been identified as important muropeptide receptors, although possible contributions of CD14 and TLR cannot be fully excluded. Remarkably, these NOD proteins are intracellular receptors suggesting a role for phagocytosed or intracellular pathogens. Muropeptides have diverse effects on the immune system: somnogenic effects, reduction of food intake and, most important, they are potent immune amplifiers. Direct effects on immune cells appear to be rare, *i.e.* they are often only observed at very high concentrations or when proper exclusion of LPS-contamination is missing, but they show potent priming and synergistic effects with other immune stimuli. Taken

together, this illustrates that, during bacterial infection, it is most probably a combined sensing of immune stimuli by different immune receptors that orchestrates the immune response.

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