Muropeptides from Salmonella typhi differentially induce pro-inflammatory cytokine production by macrophages and dendritic cells

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Summary

Bacterial peptidoglycan (PG) and its derivatives potently activate mammalian innate immune system and are promising immunomodulators and vaccine adjuvants. However, their effects on human antigen-presenting cells, such as dendritic cells (DCs) and Mphi, are not fully understood. Lysosyme treatment of PG from *Salmonella typhi* yielded three muropeptides, GlcNAc – MurNAc – L-Ala – D-isoGlu – *meso*-DAP (GM-3P), GlcNAc – MurNAc – L-Ala – D-isoGlu – *meso*-DAP – D-Ala (GM-4P), and a dimer $(GM\text{-}4P)_2$, in which two GM-4P monomers are linked through their peptidic moieties. All three muropeptides induced TNF-α and IL-6 production by Mphi (GM-3P > GM-4P > $(GM\text{-}4P)_2$), but failed to trigger TNF-α, IL-6 and IL-12p70 production by immature DCs. At the same time, GM-3P-stimulated DCs abundantly produced inflammatory chemokines IL-8, MIP-1α and MIP-1β, as well as upregulated HLA-DR, CD80, CD86 and CD83 expression. Thus, muropeptide-dependent pro-inflammatory cytokine production is selectively repressed in DCs. While this defect may be partly compensated *in vivo* by muropeptide-activated Mphi, neither Mphi nor DCs produce Th1- or Th17-polarizing cytokines upon muropeptide stimulation, which may contribute to the preferential induction of Th2 responses by muropeptides.

Introduction

Bacterial peptidoglycan (PG), also known as murein, is one of the most potent activators of the mammalian innate immune system. Polymeric PG is recognized by a cell surface receptor, TLR2 [1], whereas the cytosolic receptors NOD1 and NOD2 recognize muropeptides, which are monomeric PG fragments that arise either as a result of PG break-down in the macroorganism, or during PG biosynthesis and/or degradation in the bacteria [2-4]. Typical muropeptides are composed of a carbohydrate and a peptidic part. The carbohydrate part consists of a single N-acetylmuramic acid residue (MurNAc) or a disaccharide N-acetylglucosaminyl (GlcNAc) – MurNAc (abbreviated GM). The peptide part is covalently linked to MurNAc and consists of 2-5 amino-acid residues. After binding muropeptide ligands, NOD1 and NOD2 initiate relatively short intracellular signalling pathways, which result in the activation of nuclear factor kappa B (NF-κB) and expression of NF-κB-dependent genes of the innate immune response, including pro-inflammatory cytokines and antimicrobial peptides [5].

Muropeptides are viewed as promising immunomodulators and vaccine adjuvants, which is due to their defined chemical structure, relatively simple production technologies, and the ability to evoke a number of immunostimulatory effects *in vivo*, for instance to trigger cytokine and chemokine release [6, 7]. However, cellular sources of cytokines and chemokines upon administration of muropeptides have not been conclusively defined. Key cellular targets of muropeptide immunomodulators and adjuvants are likely to be conventional (myeloid) dendritic cells (DCs) and macrophages (Mphi), as both respond to NOD agonists *in vitro* [3, 6, 8-10] and both can serve as antigen-presenting cells (APCs) [11, 12]. Although some effects of muropeptides on DCs, Mphi and their precursors have been investigated [3, 8, 9, 13], variations in

experimental procedures do not allow to compare cytokine, chemokine and surface molecule profiles of DCs and Mphi upon muropeptide treatment, which makes it difficult to estimate contribution of either cell type to the effects of muropeptides.

A considerable amount of work has been done to identify optimal PG-derived NOD1 and NOD2 agonists. By screening a large array of muropeptides, it was shown that NOD1 recognizes muropeptides with terminal *meso*-diaminopimelic acid (*meso*-DAP), which arise mostly from PG of Gram-negative bacteria. Most potent NOD1 agonists are muramyl tripeptide MurNAc – L-Ala – D-isoGln – *meso*-DAP and the corresponding GM-tripeptide (GlcNAc – MurNAc – L-Ala – D-isoGln – *meso*-DAP, followed by the tripeptide L-Ala – D-isoGln – *meso*-DAP, dipeptide D-isoGln – *meso*-DAP and lactoyl tripeptide Lac – L-Ala – D-isoGln – *meso*-DAP [3, 14]. NOD2 detects muramyl dipeptide MurNAc – L-Ala – D-isoGln (MDP), which is a structural component of PG from both Gram-negative and Gram-positive bacteria [2, 14]. Besides MDP, NOD2 recognizes N-acetylglucosaminyl N-acetyl muramyl dipeptide (GlcNAc – MurNAc – L-Ala – D-isoGln) and muramyl tripeptides with terminal L-lysine [2, 14, 15].

The above-mentioned works aimed to find minimal PG fragments still capable of activating NOD1 or NOD2. Peptidic moieties in such compounds consist of 2-3 amino-acid residues. However, in the native PG, peptides can be longer; furthermore, peptidic chains stemming from neighbouring glycan chains often make covalent bonds with each other, so when PG hydrolysis is catalysed by lysosyme, 'linked' muropeptides can arise, in which two GM disaccharides are linked by a peptide bridge consisting of eight or more amino-acid residues. The effects of such compounds on innate immune cells have not been characterized. In the present work, we subjected PG from a prototypic Gram-negative microorganism, *Salmonella typhi (S. typhi)*, to lysosyme hydrolysis, and

generated a set of natural GM-peptides, including a 'linked' GM-peptide. To examine the role of carbohydrate residues in the activity of GM-peptides, corresponding lactoyl peptides (Lac-peptides) were made, which contain a lactate residue instead of the disaccharide. We then directly compared the effects of GM- and Lac-peptides on in-vitro-generated human Mphi and immature DCs.

Materials and Methods

Active compounds

Following compounds were generated: (1) N-acetylglucosaminyl N-acetylmuramyl tripeptide (GM-3P; GlcNAc – MurNAc – L-Ala – D-isoGlu – *meso*-DAP); (2) N-acetylglucosaminyl N-acetylmuramyl tetrapeptide (GM-4P; GlcNAc – MurNAc – L-Ala – D-isoGlu – *meso*-DAP – D-Ala); (3) a 'linked' muropeptide, which is a dimer $(GM\text{-}4P)_2$, where two GM-4P monomers are linked by an amide bond between the HOOC-group of terminal D-alanine of one monomer and ω-amino group of *meso*-DAP of the other monomer; (4) three Lac-peptides (Lac-3P, Lac-4P, $(Lac\text{-}4P)_2$), generated from GM-3P, GM-4P and $(GM\text{-}4P)_2$, respectively, by alkaline β-elimination.

Following procedures were employed to produce active compounds. Initial purification of PG from *S. typhi*, in order to remove bulk of proteins, nucleic acids and lipids, was done by three consecutive treatments of bacterial biomass with 45%:55% phenol:water (70°C, 30 min). The PG pellet was washed, 5 g of PG was resuspended in 100 ml of 0.2 M triethylammonium acetate buffer (pH 7.2) and treated with 0.3% lysosyme (Sigma, St-Louis, MO) for 1 h at 10°C. The reaction mixture was then dialysed for 72 h against the same buffer through a membrane with 5-kDa cut-off (Millipore, Billerica, MA). A mixture of PG hydrolysis fragments was purified from the dialysate by

gel chromatography on Sephadex G-50 (Sigma), with elution by 0.2 M sodium chloride and subsequent de-salting by deionized water on a TSK-40 column. The eluate contained three main components, GM-3P, GM-4P and $(GM\text{-}4P)_2$, which were further isolated as mixtures of α- and β-anomers by high-performance liquid chromatography (HPLC) using a Zorbax ODS column (9.4 mm × 25 cm; Agilent Technologies, Santa Clara, CA) in a gradient of water : 40% aqeous acetonitrile in the presence of 0.1% trifluoroacetic acid. Lac-peptides were produced by treating respective GM-peptides with 4M ammonium hydroxide at 37°C during 5 h [14] and subsequently purified by HPLC in the above conditions. Purity of all substances was confirmed by analytical HPLC, molecular masses were verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and molecular structures were confirmed by ^{13}C nuclear magnetic resonance spectra.

N-acetylglucosaminyl N-acetylmuramyl dipeptide (GM-2P; GlcNAc – MurNAc – L-Ala – D-isoGlu) was bought from Sigma.

By LAL test (EndoSafe KTA; Charles River Laboratories, Wilmington, MA), endotoxin levels in all GM- and Lac-peptides were below 0.01 endotoxin unit (EU) per μg; control lipopolysaccharide (LPS) contained 16000 EU/μg.

Inhibitors

A broad-range protease inhibitor cocktail (PIC), which inhibits serine, cysteine, aspartic, and aminopeptidases, was from Sigma (catalogue number P8340) and contained 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM leupeptin A and 1.4 mM E-64. A broad-range matrix metalloproteinase (MMP) inhibitor (N-hydroxy-1,3-di-(4-methoxybenzenesulphonyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide), which

inhibits MMP-1 (IC_{50} = 24 nM), MMP-3 (IC_{50} = 18.4 nM), MMP-7 (IC_{50} = 30 nM) and MMP-9 (IC_{50} = 2.7 nM), was from Merck (Darmstadt, Germany).

Culture and stimulation of DCs and Mphi

Buffy coats were obtained from a blood transfusion facility at Russian Oncological Research Center (Moscow, Russia). From all samples, paired DC and Mphi cultures were generated by commonly used techniques [16]. Mononuclear cells (MNCs) were isolated by Ficoll-urographin density gradient (ρ = 1.077 g/ml; Paneco, Moscow, Russia), and monocytes were isolated from MNC suspensions by adhesion to plastic. To obtain DCs, monocytes were cultured 6 days in RPMI (PAA, Pasching, Austria) supplemented with 2 mM L-glutamine (Sigma), 10% fetal calf serum (PAA), 80 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml interleukin (IL)-4 (both from Invitrogen, Paisley, UK). Medium was refreshed on day 3, and immature DCs (floating or loosely adherent cells) were collected on day 6. Mphi were obtained using the same procedure, except that IL-4 was not added; on day 6, Mphi were detached from plastic by trypsinization. By immunophenotyping, DCs were $CD1a^{\pm}CD11c^{+}CD14^{-}CD80^{dim}CD83^{-}CD86^{dim}CD206^{-}$HLA-$DR^{+}$, and Mphi were $CD1a^{-}$ $CD11c^{+}CD14^{+}CD80^{dim}CD83^{-}CD86^{dim}CD206^{+}$HLA-$DR^{+}$ (not shown). Lymphocyte contamination was <3% for DC cultures and <1% for Mphi cultures.

DCs and Mphi were washed and plated in 96-well flat-bottom plates (Nunc, Roskilde, Denmark) at 8×10^{4} cells per well. Cells were allowed to settle during 1 h at 37°C and then cultured for 24 h either in the absence of stimuli, or in the presence of GM- or Lac-peptides at 0.1-10 μg/ml. LPS from *E. coli* O111:B4 (0.1 μg/ml; Sigma) was used as a positive control. In some experiments, PIC or MMP inhibitor were added to a final dilution 1:1000 or final concentration 100 nM, respectively, 30 min prior to

stimulation. At concentrations used, neither of the inhibitors adversely affected cell viability. After 24 h, supernatants were collected and frozen at –70°C.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-1β, IL-6, IL-10, interferon (IFN)-α and tumor necrosis factor (TNF)-α in the cell supernatants were determined using ELISA kits from Invitrogen, IL-12p70 was measured by an OptEIA set from BD Pharmingen (San Diego, CA), and IL-23 was measured by an ELISA kit from Bender Medsystems (Vienna, Austria) according to manufacturers' instructions.

Multiplex analysis

Supernatants from unstimulated and GM-3P- or LPS-stimulated DCs and Mphi were obtained as above and processed using a 27-plex Human Cytokine Panel (Bio-Rad, Hercules, CA), a fluorescent microbead-based kit that enables simultaneous detection of 27 analytes (cytokines, chemokines and growth factors) in one sample. Samples were read on a Bioplex 2000 reader (Bio-Rad). For each analyte and stimulation condition, a stimulation index (SI) was calculated (SI = stimulated production / background production). Induction of an analyte was deemed significant if average SI was ≥ 3 and significantly different from 1 ($p < 0.05$ by Wilcoxon matched pair test). To simplify comprehension, SIs were rounded to the nearest integer and presented as a semi-quantitative color diagram, where four individual colors depict significantly induced analytes with SI = 3 to 9, 10 to 99, 100 to 999, and 1000 to 10000.

Immunofluorescent staining and flow cytometry

DCs and Mphi were cultured in 24-well plates at 2.5×10^5 cells per well with the above-mentioned stimuli. After 24 h, cells were collected and stained with FITC-labelled mAbs against HLA-DR or CD83, PE-labelled mAbs against CD80 and PE-Cy5-labelled

mAbs against CD86 (all from BD Pharmingen). Control cell aliquots were stained with isotype-matched irrelevant mAbs. Stained cells were analysed using a Cytomics FC500 flow cytometer and CXP software (both from Beckman Coulter, Fullerton, CA). Expression of each marker was measured as geometric mean fluorescent intensity (MFI), minus MFI obtained with isotype control. To minimize inter-donor variations, all data were presented as ratio of MFI in stimulated cells to MFI in unstimulated cells, multiplied by 100%.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from DC and Mphi cultures by TriReagent (Sigma) as prescribed by the manufacturer. 1 μg total RNA was reverse-transcribed using RevertAid reverse transcriptase and oligo-dT primer (both from Fermentas, Vilnius, Lithuania). Specific cDNAs were amplified in an ABI Prism 7300 system (Applied Biosystems, Foster City, CA) using gene expression assays from the same company; assay numbers were Hs00196075_m1 (NOD1), Hs00223394_m1 (NOD2) and Hs00152939_m1 (TLR4). Expression was normalized against β_2-microglobulin (B2M) [17]. Relative expression (RE) of each gene of interest was calculated by the formula:

$$RE = 2^{-\Delta\Delta Ct} = 2^{-((Ct\ \text{gene sample} - Ct\ \text{B2M sample}) - (Ct\ \text{gene reference} - Ct\ \text{B2M reference}))},$$

where Ct is cycle of threshold; gene is any gene of interest (NOD1, NOD2 or TLR4); sample is any cDNA sample, reference is a reference cDNA sample in which the expression of gene of interest is taken for 1.

Statistics

Groups were compared by Wilcoxon matched pair test using Statistica 6.0 (StatSoft Inc, Tulsa, OK). Differences were considered significant if $p < 0.05$.

Results

GM- and Lac-peptides induce cytokine production by human Mphi and not by DCs

When PG from *S. typhi* was subjected to exhaustive lysosyme hydrolysis, three main products were found in, and purified from, the hydrolysate: GM-3P, GM-4P and $(GM\text{-}4P)_2$. The 'linked' dimer $(GM\text{-}4P)_2$ arose because peptidic bridges that link neighbouring polysaccharide chains of PG are not susceptible to lysosyme; the two GlcNAc – MurNAc groups in $(GM\text{-}4P)_2$ initially belonged to two different polysaccharide chains. The other two GM-peptides arose, most likely, from those repeating PG units, in which the peptidic chains either had not been completed (GM-3P) or had not made a bond with an identical peptidic chain stemming from a neighbouring polysaccharide chain (GM-4P). Using alkaline treatment, respective Lac-peptides were generated, which feature a lactoyl residue instead of the GM group.

The obtained GM- and Lac-peptides were tested for the ability to induce TNF-α production by human Mphi and immature DCs. At concentrations 1-10 μg/ml, GM-3P dose-dependently induced TNF-α production by Mphi, an effect comparable with that of LPS (Fig. 1A). GM-4P was slightly less potent TNF-α inducer in Mphi; even less active were $(GM\text{-}4P)_2$, Lac-3P, Lac-4P and $(Lac\text{-}4P)_2$, which induced TNF-α only when given at 10 μg/ml.

At the same time, none of the GM- and Lac-peptides tested, except for GM-3P, induced TNF-α production by DCs generated from the same donors, while GM-3P induced minimal production of this cytokine (background production, 35±27 pg/ml, in

the presence of GM-3P at 10 μg/ml, 129±91 pg/ml, $p < 0.05$) (Fig. 1B). When stimulated by LPS, DCs and Mphi produced comparable amounts of TNF-α.

All above-mentioned GM- and Lac-peptides contained *meso*-DAP and were presumably recognized by NOD1. To investigate whether NOD2 agonists would differentially affect DCs and Mphi as well, cells were stimulated with a NOD2 ligand, GM-2P [15]. Again, only Mphi made TNF-α, whereas DCs did not respond (Fig. 1).

We then examined production of additional cytokines (IL-1β, IL-6, IL-10, IL-12p70, IL-23, IFN-α) by DCs and Mphi stimulated by GM-3P, GM-4P and $(GM\text{-}4P)_2$. These three GM-peptides were chosen as strong, intermediate and weak TNF-α inducers in Mphi. IL-6 followed the same tendencies as TNF-α, with Mphi producing this cytokine in response to both LPS and GM-peptides, and DCs only in response to LPS (Fig. 2). Similarly, IL-10 was induced by LPS in both cell types (although this did not reach statistical significance due to large variance), but only Mphi produced IL-10 in response to GM-peptides. IL-12p70, again, was produced by DCs in response to LPS and not GM-peptides; however, Mphi did not make this cytokine under any condition tested (Fig. 2 and not shown). IL-1β was induced in Mphi by both GM-peptides and LPS, but not produced by DCs. Finally, IFN-α and IL-23 were not produced by neither cell type under any stimulation (not shown). In all, it can be concluded that GM-peptides effectively induce pro-inflammatory cytokines and IL-10 in Mphi and not in DCs.

Expression of NOD1 and NOD2 mRNA in Mphi and DCs

A weak response of DCs to GM- and Lac-peptides could be due to low expression of NOD1 and/or NOD2. Therefore, expression of NOD1 and NOD2 mRNA in DCs and Mphi was examined by RT-PCR; expression of TLR4 mRNA served as a control. All

three mRNAs were detected in all samples. Expression of NOD1, the main putative receptor for *meso*-DAP-containing muropeptides, was equal in DCs and Mphi, whereas expression of NOD2 and TLR4 in DCs was somewhat lower than in Mphi (Table 1). It should be noted that although DCs expressed lower levels of TLR4 than Mphi, both cell types responded equally well to a TLR4 agonist, LPS. Together, these data suggest that the differences in NOD1/NOD2 expression do not explain the profound discrepancy between DCs and Mphi in cytokine responses to GM- and Lac-peptides.

GM-3P induces chemokine production by DCs

Since DCs expressed same levels of NOD receptors as Mphi, we used multiplex analysis to search for other factors that could be induced by muropeptides in DCs. GM-3P was chosen for these experiments as the most potent cytokine inducer among the muropeptides tested. LPS, used as a positive control, induced a diverse set of pro-inflammatory cytokines, chemokines and growth factors in DCs (Fig. 3, see also Supplemental Table 1). In keeping with our ELISA data, GM-3P was not efficient at inducing pro-inflammatory cytokines in DCs; the induction of IL-6 and IFN-γ, although statistically significant, was 1-2 orders of magnitude lower than in the presence of LPS and probably insignificant biologically (Supplemental Table 1). Surprisingly, however, the chemokines IL-8, MIP-1α and MIP-1β were induced by GM-3P to an extent comparable with LPS. Furthermore, GM-3P triggered production of IP-10 and RANTES by DCs, though did this less efficiently than LPS.

In the case of Mphi, GM-3P and LPS induced similar sets of pro-inflammatory cytokines and growth factors, including IL-6, TNF-α, IFN-γ, G-CSF and GM-CSF (Fig. 3). GM-3P appeared to be more efficient than LPS at inducing IL-10, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), but absolute levels

of these factors were low (Supplemental Table 1). Mphi produced high basal levels of chemokines, which were not further enhanced by GM-3P or LPS, except for RANTES, which was augmented by both stimuli.

GM-peptides induce DC maturation

To more fully compare the effects of GM-peptides on DCs and Mphi, we examined the influence of GM-3P, GM-4P and $(GM\text{-}4P)_2$ on the expression of HLA-DR and co-stimulatory molecules by DCs and Mphi, as well as on the expression of DC maturation marker CD83. LPS was taken as a positive control. GM-3P dose-dependently enhanced the expression of HLA-DR, CD80, CD86 and CD83, albeit not as potently as LPS (Fig. 4). GM-4P displayed a similar, but less pronounced activity. Even less potent was $(GM\text{-}4P)_2$, which enhanced HLA-DR, CD80 and CD83 only at 10 μg/ml and did not affect CD86. The influence of GM-peptides and LPS on the surface phenotype of Mphi was minor (Fig. 4); none of the compounds influenced CD86 and HLA-DR expression by Mphi, whereas CD80 expression was marginally augmented.

The activity of $(GM\text{-}4P)_2$ is mediated by its proteolytic breakdown products

While GM-3P is known to directly activate NOD1 [4], less is known about the activity of larger GM-peptides like GM-4P and $(GM\text{-}4P)_2$. The experiments presented above showed that GM-4P and $(GM\text{-}4P)_2$ were able to activate DCs and Mphi, although both (especially $(GM\text{-}4P)_2$) were inferior to GM-3P. One possibility is that such larger GM-peptides could be 'trimmed down' by peptidases (either extracellularly or in the lysosomes) to generate NOD1 and/or NOD2 ligands like GM-3P or GM-2P. To examine this, Mphi were stimulated with GM-3P, GM-4P, $(GM\text{-}4P)_2$ or LPS in the absence or presence of a broad-range PIC or MMP inhibitor. Both inhibitors profoundly suppressed

$(GM\text{-}4P)_2$-induced TNF-α production by Mphi, but did not affect GM-3P-, GM-4P- or LPS-induced production (Fig. 5).

Discussion

Mphi and conventional (myeloid) DCs are two related cell types that share a set of surface markers, arise from common progenitors (circulating monocytes and $CD11c^+lin^-$ pre-DCs) and can differentiate into each other depending on the cytokine milieu [16, 18-20]. Both cell types can serve as APCs. For DCs, however, presentation of antigens to T cells is the single most important function; mature DCs, which express high levels of co-stimulatory molecules, are the only cell type that can efficiently activate naïve T cells and induce primary adaptive immune responses [12]. Mphi can also activate T cells, but primarily act as effector cells of innate and adaptive immunity, as well as participate in different aspects of inflammation and tissue remodelling [11]. Both Mphi and DCs can respond to a large set of pathogen-derived molecular patterns (PAMP), including LPS and muropeptides [3, 8], and are key targets for immunomodulators and adjuvants.

In the present work, Mphi and DCs showed distinct response patterns to muropeptides and LPS. Mphi responded by pro-inflammatory cytokine production to a number of muropeptides, including NOD1 and NOD2 agonists, whereby GM-3P at 10 μg/ml was as potent as LPS (Fig. 1-3). DCs, by contrast, produced pro-inflammatory cytokines only in response to LPS (making same amounts of TNF-α and IL-6 as LPS-stimulated Mphi), and not to GM-3P or other muropeptides. At the same time, GM-3P triggered DCs to produce a number of chemokines that can recriut diverse leukocyte populations; in particular, GM-3P- and LPS-stimulated DCs produced similar levels of IL-8, MIP-1α and MIP-1β. Furthermore, GM-3P and other tested muropeptides

augmented HLA-DR, CD80, CD86 and CD83 expression by DCs. Thus, it appears that NOD-dependent production of pro-inflammatory cytokines is selectively repressed in DCs. The mechanism of such repression is presently unclear. One possibility is that in DCs, unlike in Mphi, the signalling pathways downstream of NOD receptors lack components that are necessary for optimal pro-inflammatory cytokine induction. LPS may overcome this defect in DCs, resulting in a well-known synergism between muropeptides and LPS [8].

Upon classical maturation, induced by LPS, DCs simultaneously upregulate MHC molecules, co-stimulatory molecules, pro-inflammatory cytokines and chemokines that attract T cells. Thus, GM-3P and other muropeptides trigger only some aspects of DC maturation. Deficient TNF-α production by DCs in response to muropeptides would result in the lack of the TNF-mediated autocrine positive-feedback loop, which is normally required for optimal induction of other inflammatory mediators and DC maturation [21]. To some extent, this deficiency may be compensated by muropeptide-activated Mphi, which produce abundant TNF-α. When muropeptides are used as adjuvants, the two cell types may cooperate at activating T cells; thus, DCs may provide obligatory activation signals for naïve T helper cells and recruit inflammatory cells by producing chemokines, and Mphi may create an inflammatory milieu, augment DC maturation and turn off Treg cells by means of IL-6 production [22]. However, neither DCs nor Mphi produce Th1- or Th17-polarizing cytokines (IL-12p70 and IL-23, respectively) upon muropeptide stimulation. Mature DCs that do not secrete Th1- and Th17-polarizing cytokines (IL-12p70, IL-1β, IL-23) trigger Th2-type differentiation of T helper cells [23], which is in agreement with a recent publication showing that NOD1 agonists, when used as adjuvants *in vivo*, favor Th2-responses [7, 24]. When Th1- or

Th17-type responses are desired, muropeptides should probably be combined with other substances that trigger production of respective instructive cytokines.

Another issue addressed in this study is the immunostimulatory activity of ‘optimal’ and ‘non-optimal’ *meso*-DAP-containing muropeptides. GM-3P with terminal *meso*-DAP was predictably the most potent activator of DCs and Mphi [4, 14]. However, the activity GM-4P, in which the *meso*-DAP residue is next-to-the-last, was qualitatively similar, though quantitatively inferior to that of GM-3P (Fig. 1, 2, 4). This contrasts the data by Girardin and co-authors, in whose hands muramyl tetrapeptide (MurNAc – L-Ala – D-Glu – meso-DAP – D-Ala) did not activate human NOD1 or NOD2 transgenically expressed in HEK293T cells [9]. Considering that broad-range peptidase inhibitors did not affect GM-4P-induced TNF-α production by Mphi, it is unlikely that that GM-4P is active because of its ‘trimming down’ to GM-3P or GM-2P by lysosomal peptidases. An alternative explanation is that native NOD1 expressed in Mphi, unlike transgenic NOD1, might be activated by GM-4P, despite the non-optimal position of *meso*-DAP. Another option is that GM-4P might activate an additional, as yet uncharacterized receptor. $(GM\text{-}4P)_2$ was a weak activator of DCs and Mphi; moreover, experiments with protease inhibitors suggested that even this weak activity was almost totally mediated by proteolytic fragments of $(GM\text{-}4P)_2$, possibly GM-4P, GM-3P or GM-2P. Most likely, key amino-acid residues in $(GM\text{-}4P)_2$ are masked, which prevents its recognition by NOD receptors. Lac-3P and Lac-4P retain some ability to induce TNF-α production by Mphi (Fig. 1); thus, loss of the carbohydrate part reduces, but not eliminates, the ability of *meso*-DAP-containing muropeptides to activate human innate immune cells, which is in agreement with previously published data [13, 14].

In all, we show here that muropeptides are unable to induce pro-inflammatory cytokine production by DCs, yet trigger other aspects of DC maturation and induce pro-inflammatory cytokine production by Mphi. Cytokine profiles of muropeptide-activated Mphi and DCs are important for the understanding of biological activity of muropeptides. The mechanisms behind the differential influence of muropeptides on Mphi and DCs, as well as its biological consequences deserve further investigation.

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Tables

Table 1. Relative expression of mRNA for NOD1, NOD2 and TLR4 by DCs and Mphi (mean ± SD, n = 4)*.

	NOD1	NOD2	TLR4
DCs	0.71±0.55	0.24±0.16	0.35±0.35
Mphi	0.73±0.21	0.67±0.55	0.76±0.22

*The expression was assessed by semi-quantitative RT-PCR as described in Materials and Methods; one of the Mphi cDNA samples was selected as a reference.

Figure legends

Figure 1. TNF-α production by Mphi (A) and DCs (B) in the presence of indicated concentrations of GM-peptides, Lac-peptides and LPS, as measured by ELISA (mean ± SD, n = 7). * $p < 0.05$ compared with background TNF-α production.

Figure 2. Production of IL-6, IL-10, IL-1β and IL-12p70 by Mphi and DCs in the presence of GM-peptides and LPS, as measured by ELISA (mean ± SD, n = 5). * $p < 0.05$ compared with background production of respective cytokines.

Figure 3. Production of 27 cytokines, chemokines and growth factors by DCs and Mphi. Paired cultures of DCs and Mphi were obtained from five different donors in five independent experiments, and either left unstimulated, or stimulated with 10 μg/ml GM-3P (1) or 0.1 μg/ml LPS (2). Supernatants were collected after 24 h and assessed by multiplex analysis. Colors depict analytes with particular SIs indices (see Materials and Methods for more detail).

Figure 4. Expression of surface maturation markers by DCs and Mphi after a 24-h stimulation with GM-peptides or LPS (flow cytometry). The Y-axis depicts the ratio of MFI in stimulated cells to MFI in unstimulated cells, multiplied by 100%. Mean ± SD, n = 5. * $p < 0.05$ compared with the expression in the absence of stimuli.

Figure 5. Levels of TNF-α in Mphi supernatants (ELISA) after a 24-h culture with medium alone, $(GM\text{-}4P)_2$ (10 μg/ml), GM-3P (10 μg/ml), GM-4P (10 μg/ml) or LPS (0.1 μg/ml) in the absence or presence of PIC (1:1000) or an MMP inhibitor (100 nM). Mean ± SD, n = 5. *, $p < 0.05$ compared to culture with the same stimulus in the absence of inhibitors.

Supplemental Table 1. Raw data of multiplex analysis. Mean ± SD, n = 5. For details, see Figure 3 legend and Materials and Methods.

		Mphi			DCs		
	Units	No stim.	GM-3P	LPS	No stim.	GM-3P	LPS
IL-1β	pg/ml	39±38	117±116	60±34	11±8	19±2	26±17
IL-1RA	ng/ml	14.5±9.4	15.4±8.0	15.2±8.5	2.4±1.4	1.6±0.8	2.8±0.9
IL-2	pg/ml	15±9	34±11	34±8	12±1	13±2	28±19
IL-4	pg/ml	5±3	16±5	14±6	18±9	15±7	19±14
IL-5	pg/ml	22±1	26±1	24±1	21±1	21±1	23±3
IL-6	ng/ml	0.64±31	4.9±1.0	5.6±1.0	0.009±0.006	0.06±0.03	5.1±3.8
IL-7	pg/ml	24±14	40±11	34±14	18±9	29±2	36±19
IL-9	pg/ml	48±6	67±5	66±9	11±7	25±8	59±24
IL-10	pg/ml	10±11	68±54	54±59	8±7	20±1	42±51
IL-12p70	pg/ml	28±9	37±15	37±14	15±10	28±4	358±274
IL-13	pg/ml	2±1	3±2	3±2	1±1	2±1	3±2
IL-15	pg/ml	20±14	36±24	34±24	13±6	18±3	39±18
IL-17	pg/ml	34±24	84±38	67±31	13±5	31±10	149±80
IFN-γ	pg/ml	184±122	781±273	760±317	6±7	54±31	840±491
TNF-α	ng/ml	0.48±0.26	19.3±9.1	17.3±10.1	0.08±0.05	0.24±0.15	14.9±5.4
FGFb	pg/ml	38±33	90±24	89±24	15±19	33±14	85±36
G-CSF	pg/ml	88±41	272±61	352±87	13±4	32±10	271±66
GM-CSF	pg/ml	28±8	197±163	112±29	18±5	20±1	276±61
PDGFbb	pg/ml	27±19	87±76	51±27	88±112	205±216	130±103
VEGF	pg/ml	14±12	72±51	57±63	12±8	21±2	74±87
Eotaxin	pg/ml	19±8	52±10	55±13	10±1	8±3	60±16
IL-8	ng/ml	24.5±9.0	29.2±5.2	31.4±11.9	1.1±0.6	12.7±6.3	17.1±7.0
IP-10	ng/ml	4.9±2.7	6.8±6.0	5.3±3.0	0.005±0.003	0.49±0.25	62.1±34.5
MCP-1	ng/ml	8.0±5.2	6.5±2.4	8.8±4.1	0.06±0.04	0.11±0.07	1.9±1.6
MIP-1α	ng/ml	3.2±1.4	4.8±0.2	4.4±1.2	0.03±0.03	1.2±1.9	5.7±1.5
MIP-1β	ng/ml	5.6±1.3	6.8±0.5	6.1±1.9	0.65±0.7	4.2±1.7	6.5±1.3
RANTES	ng/ml	2.7±0.5	8.2±1.8	9.2±7.6	0.01±0.01	0.76±0.5	16.5±11.9