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Muropeptides trigger distinct activation profiles in macrophages and dendritic cells

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ABSTRACT

Bacterial peptidoglycan and its muropeptide 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. Lysozyme 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-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-4P)₂), but failed to trigger TNF- α , IL-6 and IL-12p70 production by immature DCs. At the same time, muropeptide-stimulated DCs abundantly produced inflammatory chemokines IL-8, MIP-1 α and MIP-1 β , as well as displayed signs of phenotypic and functional maturation. Thus, muropeptide-dependent pro-inflammatory cytokine production is 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 and should be taken into account when designing muropeptide-based immunomodulators and adjuvants.

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1. Introduction

Bacterial peptidoglycan (PG) is one of the most potent microbial activators of the mammalian innate immune system. While recognition of polymeric PG has been a matter of debate [1,2], it is established that monomeric PG fragments, known as muropeptides, are recognized by the cytosolic receptors NOD1 and NOD2 [3–5]. Muropeptides arise either as a result of PG breakdown in the macroorganism, or during PG biosynthesis and/or degradation in bacteria, and 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. The peptide part is covalently linked to MurNAc and consists of 2–5 amino-acid residues. After binding muropeptide ligands, NOD1 and NOD2 initiate intracellular signalling pathways, which result in the activation of nuclear factor kappa B (NF- κ B) and expression of the innate immune response genes, such as pro-inflammatory cytokines and antimicrobial peptides [6].

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* [7,8]. Therefore, considerable work has been done to identify optimal NOD1 and NOD2 agonists. Thus, NOD1 has been shown to recognize muropeptides with terminal *meso*-diaminopimelic acid (*meso*-DAP), which arise mostly from PG of Gram-negative bacteria. Most potent NOD1 agonists are N-acetylmuramyl tripeptide MurNAc–L-Ala–D-isoGln–*meso*-DAP and the corresponding N-acetylglucosaminyl N-acetylmuramyl tripeptide GlcNAc–MurNAc–L-Ala–D-isoGln–*meso*-DAP (GM-3P), 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 (Lac-3P) [4,9]. NOD2 detects N-acetylmuramyl dipeptide MurNAc–L-Ala–D-isoGln and N-acetylglucosaminyl N-acetylmuramyl dipeptide GlcNAc–MurNAc–L-Ala–D-isoGln (GM-2P), which are structural components of PG from both Gram-negative and Gram-positive bacteria; in addition, NOD2 senses muramyl tripeptides with terminal L-lysine [3,9,10].

The above-mentioned works aimed to find minimal PG motifs still capable of activating NOD1 or NOD2. Peptidic moieties in such compounds consisted of 2–3 amino-acid residues. However, in the native PG, peptides are usually longer; furthermore, peptidic chains stemming from neighbouring glycan chains often make covalent bonds with each other, so when PG hydrolysis is catalysed by lysozyme, dimeric muropeptides can arise, in which two GM disaccharides are

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linked by a peptide bridge consisting of eight or more amino-acid residues. Although such long mucopeptides at low concentrations do not activate NOD receptors transgenically expressed in HEK293 cells [5,9], little is known about their effects on natural immune cells like macrophages (Mphi) or dendritic cells (DCs).

A key activity of mucopeptides is triggering cytokine and chemokine release [7,8]. However, cellular sources of cytokines and chemokines upon mucopeptide administration have not been conclusively defined. Key cellular targets of mucopeptide immunomodulators and adjuvants are likely to be conventional (myeloid) DCs and Mphi, as both respond to NOD agonists *in vitro* [4,7,11–13] and both can serve as antigen-presenting cells (APCs) [14,15]. Knowledge of mucopeptide-induced DC and Mphi activation profiles is important for understanding the mechanisms of action of mucopeptides *in vivo*.

In the present work, we subjected PG from a prototypic Gram-negative microorganism, *Salmonella typhi* (*S. typhi*), to lysozyme hydrolysis, and generated three GM-peptides: (1) GM-3P; (2) N-acetylglucosaminyl N-acetylmuramyl tetrapeptide GlcNAc–MurNAc–L-Ala–D-isoGlu–*meso*-DAP–D-Ala (GM-4P); and (3) a dimer (GM-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. 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 investigated the effects of GM- and Lac-peptides on *in vitro*-generated human Mphi and immature DCs. We found a clear dichotomy between DCs and Mphi upon mucopeptide stimulation, whereby DCs responded by secreting chemokines and upregulating co-stimulatory molecules, while Mphi secreted pro-inflammatory cytokines. Furthermore, we show that dimeric mucopeptides are weak activators of DCs and Mphi, while the activity of GM-4P is comparable to that of the classical NOD1 agonist, GM-3P.

2. Materials and methods

2.1. Active compounds and their generation

Following procedures were used to generate GM-3P, GM-4P and (GM-4P)₂. 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 thoroughly 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% lysozyme (Sigma, St-Louis, MO) for 18 h at RT. The reaction mixture was then dialysed for 72 h against the same buffer through a membrane with 5-kDa cut-off (Millipore, Billerica, MA); the buffer was changed five times. A mixture of low-molecular-weight PG fragments was isolated from the combined dialysate by gel chromatography on Sephadex G-50 (Sigma), with elution by 0.2 M sodium chloride and subsequent desalting by deionized apyrogenic water on a TSK-40 column (Tosoh Bioscience, Stuttgart, Germany). The eluate contained three main components, GM-3P, GM-4P and (GM-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% aqueous acetonitrile with elution by 0.1% trifluoroacetic acid. Lac-3P, Lac-4P and (Lac-4P)₂ were produced by treating GM-3P, GM-4P or (GM-4P)₂, respectively, with 4 M ammonium hydroxide at 37 °C during 5 h (alkaline β-elimination) [9] 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 ¹³C nuclear magnetic resonance spectra. GM-2P was bought from Sigma. By LAL test (EndoSafe KTA; Charles River Laboratories,

Wilmington, MA), endotoxin levels in all substances were below 0.01 endotoxin unit (EU) per µg; control lipopolysaccharide (LPS) contained 16,000 EU/µg.

2.2. 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 (IC₅₀ = 24 nM), MMP-3 (IC₅₀ = 18.4 nM), MMP-7 (IC₅₀ = 30 nM) and MMP-9 (IC₅₀ = 2.7 nM), was from Merck (Darmstadt, Germany).

2.3. 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]. Briefly, mononuclear cells were isolated by Ficoll density gradient ($\rho = 1.077$ g/ml; Paneco, Moscow, Russia), and monocytes were isolated from mononuclear cell 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[±]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.

The 6-day 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 indicated concentrations. LPS from *E. coli* O111:B4 (0.1 µg/ml; Sigma) was used as a positive control. In some experiments, PIC or MMP inhibitor was added to a final dilution of 1:1000 or a final concentration of 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.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-1β, IL-6, IL-10, transforming growth factor (TGF)-β and tumor necrosis factor (TNF)-α in the culture supernatants were determined using ELISA kits from Biosource/Invitrogen, IL-12p70 was measured by an OptEIA set from BD Pharmingen (San Diego, CA), and interferon (IFN)-α and IL-23 were measured by ELISA kits from Bender Medsystems (Vienna, Austria) according to manufacturers' instructions. Lower detection limits were 2 pg/ml for IL-10, 4 pg/ml for IL-1β, IL-6, TNF-α and IL-12p70, 8 pg/ml for IFN-α, 63 pg/ml for TGF-β, and 156 pg/ml for IL-23.

2.5. Multiplex assay

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 assay 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 cell sample, analyte and stimulation condition, a stimulation index (SI) was calculated ($SI = \text{stimulated production} / \text{background production}$). 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, average 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 10,000.

2.6. Immunofluorescent staining and flow cytometry

The 6-day 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%.

2.7. 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 of 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.

2.8. Naïve CD4⁺ T cell isolation and mixed leukocyte reaction (MLR)

The 6-day DCs were cultured for an additional 24 h with either no stimuli, GM-3P (10 $\mu\text{g}/\text{ml}$) or LPS (100 ng/ml), whereafter extensively washed. Naïve CD4⁺ T cells were isolated from allogeneic donors to a purity of >95% using naïve CD4⁺ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). These naïve CD4⁺ T cells (2.5×10^4 per well) were co-cultured with the differently treated DCs at DC:T ratios from 1:10 to 1:1000 in 96-well round-bottomed plates for 96 h, in

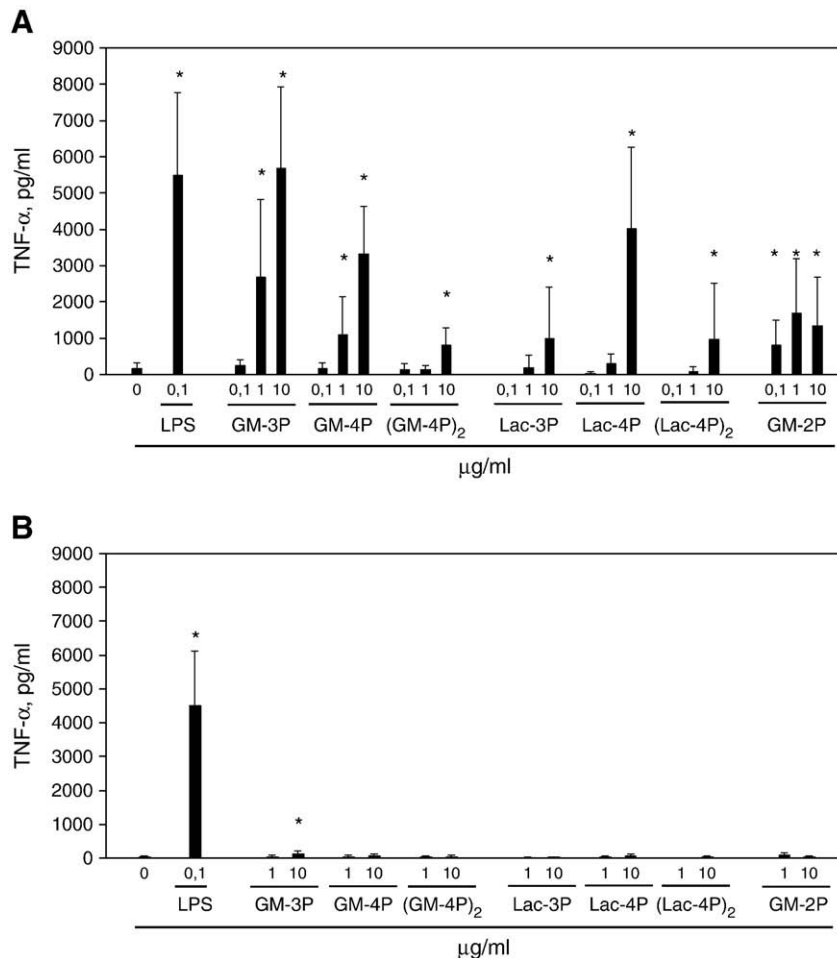


Fig. 1. TNF- α production by Mphi (A) and DCs (B) in the presence of indicated concentrations of GM-peptides, Lac-peptides and LPS, as measured by ELISA (mean \pm SD, $n = 7$). * $p < 0.05$ compared with background TNF- α production.

triplicates; [^3H]-thymidine (0.5 $\mu\text{Ci}/\text{well}$) was added for another 16 h, after which radioactive label incorporation was measured on a liquid scintillation β -counter. Cultures containing only T cells (2.5×10^4 per well) or only LPS-treated DCs (2.5×10^3 per well) were used as negative controls.

2.9. Statistics

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

3. Results

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

When PG from *S. typhi* was subjected to lysozyme hydrolysis, three GM-peptides were found in, and purified from, the hydroly-

sate: GM-3P, GM-4P and (GM-4P) $_2$. Using alkaline treatment, respective Lac-peptides were generated, which contain a lactoyl residue instead of the disaccharide group. These GM- and Lac-peptides were tested for the ability to induce TNF- α production by human Mphi and immature DCs. At concentrations 1–10 $\mu\text{g}/\text{ml}$, 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-4P) $_2$, Lac-3P, Lac-4P and (Lac-4P) $_2$, which induced TNF- α only when given at 10 $\mu\text{g}/\text{ml}$.

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 $\mu\text{g}/\text{ml}$, 129 ± 91 pg/ml, $p < 0.05$) (Fig. 1B). Same weak response of DCs was observed even if GM-3P, GM-4P or (GM-4P) $_2$ were present at an unphysiologically high concentration of 100 $\mu\text{g}/\text{ml}$ (not shown). When stimulated by LPS, DCs and Mphi produced comparable amounts of TNF- α .

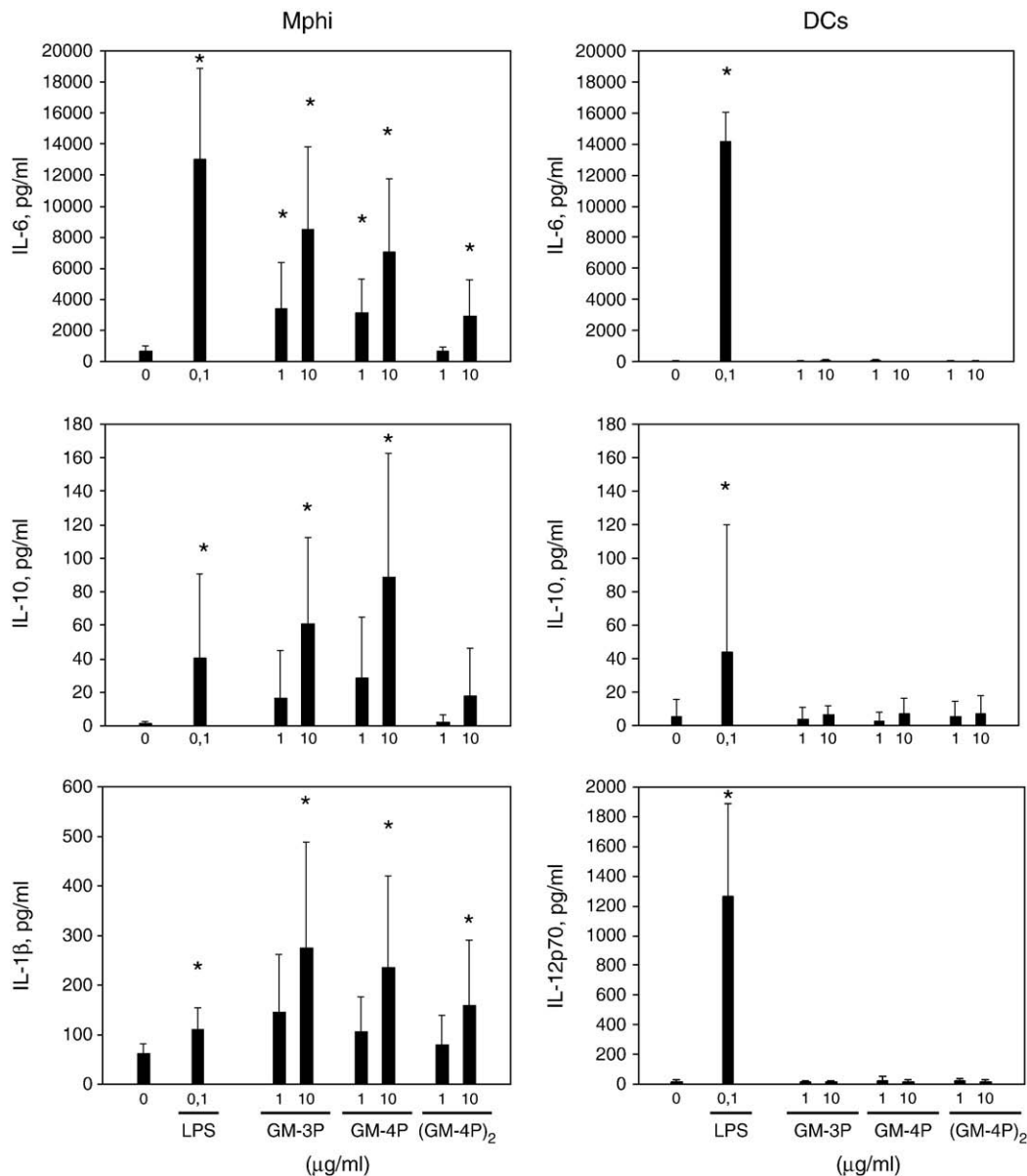


Fig. 2. Production of IL-6, IL-10, IL-1 β and IL-12p70 by Mphi and DCs in the presence of GM-peptides and LPS, as measured by ELISA (mean \pm SD, $n = 5$). * $p < 0.05$ compared with background production of respective cytokines.

All above-mentioned GM- and Lac-peptides contained *meso*-DAP and were presumably recognized by NOD1. To investigate whether a NOD2 agonist would differentially affect DCs and Mphi as well, cells were stimulated with a NOD2 ligand, GM-2P [10]. Again, only Mphi made TNF- α , whereas DCs did not respond (Fig. 1).

We then examined production of additional cytokines (IL-1 β , IL-6, IL-10, IL-12p70, IL-23, and IFN- α) by DCs and Mphi stimulated by GM-3P, GM-4P and (GM-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, 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 (Fig. 2); however, Mphi did not make this cytokine under any condition tested (not shown). IL-1 β was weakly 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 used, while TGF- β 1 was secreted at low levels in the absence of stimuli and not augmented by LPS or muropeptides (not shown). In all, it can be concluded that GM-peptides efficiently induce pro-inflammatory cytokines and IL-10 in Mphi and not in DCs.

3.2. 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 are unlikely to explain the discrepancy between DCs and Mphi in cytokine responses to GM- and Lac-peptides.

3.3. GM-3P induces chemokine production by DCs

Since DCs expressed same levels of NOD receptors as Mphi, we used multiplex assay 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 increase of IL-6 and IFN- γ , although statistically significant, was 1–2 orders of magnitude lower than that induced by LPS and probably insignificant biologically (Supplemental Table 1). Surprisingly, however, the chemokines IL-8, MIP-1 α and MIP-1 β 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.

Table 1

Relative expression of mRNA encoding NOD1, NOD2 and TLR4 by DCs and Mphi (mean \pm SD, $n=4$).

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

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

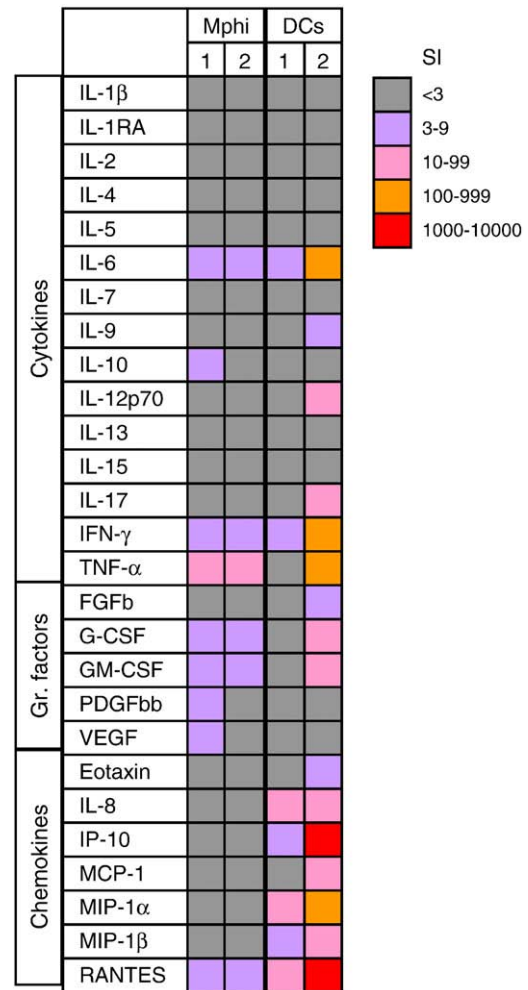


Fig. 3. Production of 27 cytokines, chemokines and growth factors by DCs and Mphi. Paired cultures of DCs and Mphi were obtained from five different donors in five independent experiments, and either left unstimulated, or stimulated with 10 μ g/ml GM-3P (1) or 0.1 μ g/ml LPS (2). Supernatants were collected after 24 h and assessed by Multiplex analysis. Colors depict analytes with particular average SIs (see Materials and methods for more details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 , granulocyte colony-stimulating factor (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.

3.4. 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-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-4P)₂, which enhanced HLA-DR, CD80 and CD83 only at 10 μ g/ml and did not affect CD86.

To examine whether muropeptides induced not only phenotypic, but also functional maturation of DCs, we examined the ability of

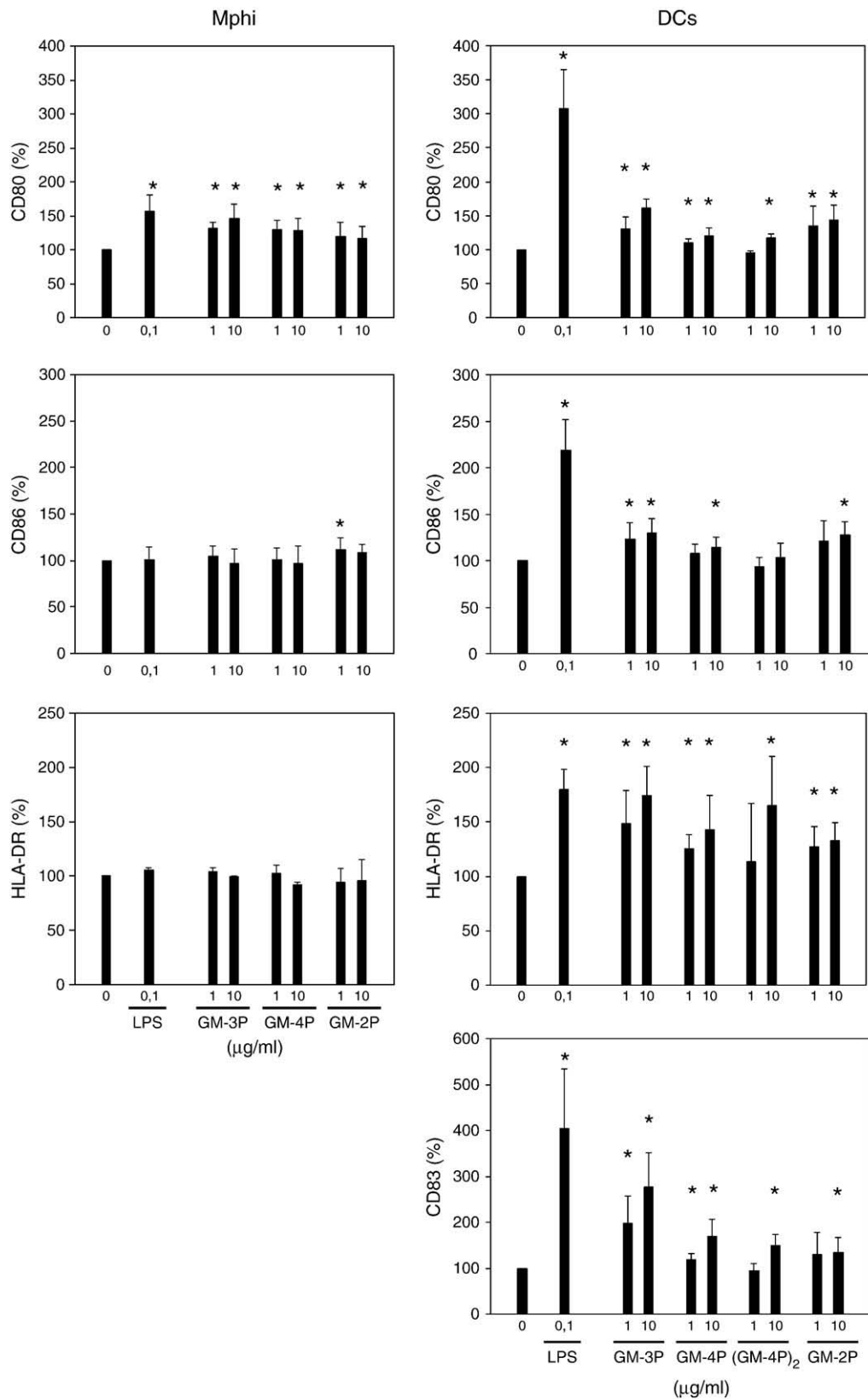


Fig. 4. Expression of surface maturation markers by DCs and Mphi after a 24-h stimulation with GM-peptides or LPS (flow cytometry). The Y-axis depicts the ratio of MFI in stimulated cells to MFI in unstimulated cells, expressed in percents. Mean \pm SD, $n = 5$. * $p < 0.05$ compared with the expression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

untreated, GM-3P-treated or LPS-treated DCs to induce proliferation of allogeneic naïve CD4⁺ T cells. Indeed, GM-3P treatment significantly augmented the allostimulatory capacity of DCs, which was especially obvious at DC:T ratio of 1:100 (Fig. 5). However, this effect

of GM-3P was not as potent as that of LPS. These results conspicuously correlate with the data on phenotypic maturation of DCs (Fig. 4).

The influence of GM-peptides and LPS on the surface phenotype of Mphi was minor (Fig. 4); none of the compounds influenced CD86 and

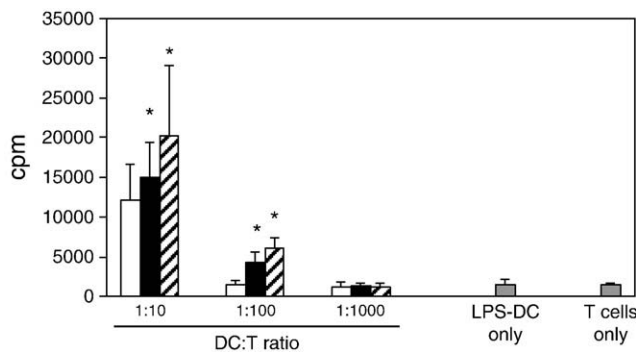


Fig. 5. Allostimulatory capacity of untreated, GM-3P- or LPS-treated DCs. Purified naïve CD4⁺ T cells (2.5×10^4 per well) were co-cultured in triplicates at indicated ratios with allogeneic DCs that had either been untreated (open bars) or treated for 24 h with GM-3P (10 µg/ml; closed bars) or LPS (0.1 µg/ml; hatched bars). As a negative control, LPS-treated DCs alone (2.5×10^3 per well) or naïve CD4⁺ T cells alone (2.5×10^4 per well) were cultured. After 96 h of co-culture, [³H]-thymidine incorporation was measured. Results are mean cpm \pm SD of 4 independent experiments. *, $p < 0.05$ by paired *t*-test compared with untreated DCs at the same DC:T ratio.

HLA-DR expression by Mphi, whereas CD80 expression was marginally augmented.

3.5. The activity of (GM-4P)₂ is mediated by its proteolytic breakdown products

While GM-3P is known to directly activate NOD1 [5], less is known about the activity of larger GM-peptides like GM-4P and (GM-4P)₂. The experiments presented above showed that GM-4P and (GM-4P)₂ were able to activate DCs and Mphi, although both (especially (GM-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-4P)₂ or LPS in the absence or presence of a broad-range PIC or MMP inhibitor. Both inhibitors profoundly suppressed (GM-4P)₂-induced TNF- α production by Mphi, but did not affect GM-3P-, GM-4P- or LPS-induced production (Fig. 6).

4. Discussion

Mphi and conventional (myeloid) DCs are two related cell types that share a set of surface markers, arise from common progenitors

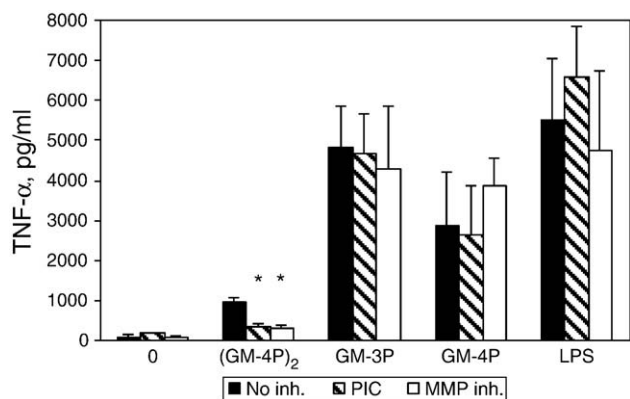


Fig. 6. Levels of TNF- α in Mphi supernatants (ELISA) after a 24-h culture with medium alone, (GM-4P)₂ (10 µg/ml), GM-3P (10 µg/ml), GM-4P (10 µg/ml) or LPS (0.1 µg/ml) in the absence or presence of PIC (1:1000) or an MMP inhibitor (100 nM). Mean \pm SD, $n = 5$. *, $p < 0.05$ compared to culture with the same stimulus in the absence of inhibitors.

(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 [15]. Mphi can also activate T cells, but act primarily as effector cells of innate and adaptive immunity, as well as participate in different aspects of inflammation and tissue remodelling [14]. Both Mphi and DCs can respond to a large set of pathogen-derived molecular patterns (PAMP), including LPS and muropeptides [4,11], 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, as well as to LPS (Figs. 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 recruit diverse leukocyte populations; in particular, GM-3P- and LPS-stimulated DCs produced similar levels of IL-8, MIP-1 α and MIP-1 β . Furthermore, GM-3P and other muropeptides (if tested) augmented HLA-DR, CD80, CD86 and CD83 expression by DCs, as well as increased the ability of DCs to activate naïve CD4⁺ T cells. Thus, it appears that NOD-dependent production of pro-inflammatory cytokines is selectively repressed in DCs. The mechanism behind 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 the well-known synergism between muropeptides and LPS [11].

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. In the absence of Th1- and Th17-polarizing cytokines, mature DCs 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 [8,24]. 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 among the muropeptides studied [5,9]. However, GM-4P and even (GM-4P)₂, despite the non-terminal position of *meso*-DAP, showed an activity qualitatively similar, albeit quantitatively inferior to that of GM-3P (Figs. 1, 2, and 4). This contrasts the data by Girardin and co-authors, in whose hands GM-4P and (GM-4P)₂ did not activate human NOD1 or NOD2 transgenically

expressed in HEK293T cells [5,12]. One explanation for this discrepancy may be that immune cells, unlike HEK293T, can modify such non-optimal muropeptides, for instance trim them down by peptidases, yielding conventional NOD ligands. Such a mechanism is likely for (GM-4P)₂, whose weak activity towards Mphi is indeed virtually blocked by broad-range peptidase inhibitors. However, these inhibitors did not influence the activity of GM-4P. It is possible that native NOD1 expressed in Mphi or DCs, unlike transgenic NOD1, might still be activated by GM-4P, despite the non-optimal position of *meso*-DAP, or that GM-4P might activate an additional, as yet uncharacterized receptor. Given these data, it would be of interest to evaluate GM-4P as an immunomodulator and/or adjuvant *in vivo*. Finally, 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 does not eliminate, the ability of *meso*-DAP-containing muropeptides to activate human innate immune cells, which is in agreement with previously published data [9,25].

In all, we show here that there exists a dichotomy between DC and Mphi responses to muropeptide compounds. Muropeptides are unable to induce pro-inflammatory cytokine production by DCs, yet trigger other aspects of DC maturation and chemokine release. In Mphi, muropeptides trigger pro-inflammatory cytokine production. Cytokine and chemokine profiles of muropeptide-activated Mphi and DCs are important for the understanding biological and therapeutic activities 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.intimp.2010.04.025.

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