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Immunomodulatory Properties of Novel Nucleotide Oligomerization Domain 2 (Nod2) Agonistic Desmuramyldipeptides

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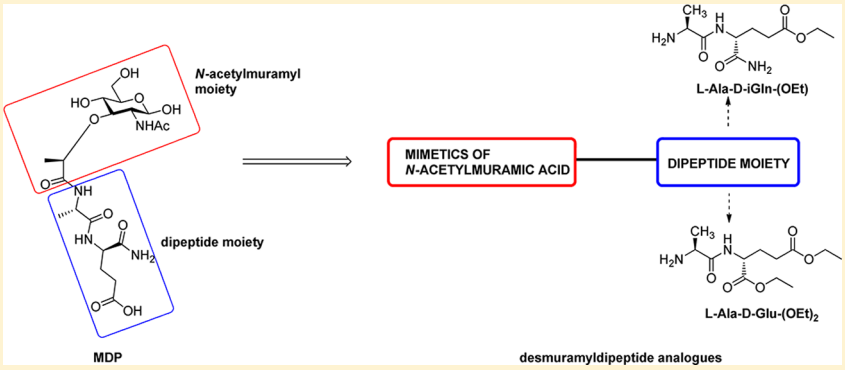
Supporting Information



ABSTRACT: There is a pressing need for the development of novel adjuvants for human use. The minimal bioactive structure of bacterial peptidoglycan (PGN), muramyldipeptide (MDP), and its derivative murabutide (MB) have long been known for their adjuvant activities. For this reason, a series of novel desmuramyldipeptides have been designed and synthesized as part of our search for therapeutically useful MDP analogues. Since nucleotide oligomerization domain 2 (Nod2) is a putative receptor for MDP, we used engineered HEK293 cells overexpressing Nod2 to screen and validate our compounds for their Nod2-agonist activity. Their immunomodulatory properties were subsequently assessed in vitro by evaluating their effect on proinflammatory cytokine production of phorbol 12-myristate 13-acetate (PMA)/ionomycin-stimulated human peripheral blood mononuclear cells (PBMCs). Herein, we present novel desmuramyldipeptides, the most active of them possessing immunoenhancing properties as a result of their potent Nod2-agonistic effect.

1. INTRODUCTION

An important segment of the innate immune system, which is the first line of defense against invading pathogens, comprises pattern recognition receptors (PRRs) such as the Toll-like (TLR), Nod-like (NLR), and retinoic acid inducible gene (RIG)-like receptors that recognize pathogen-associated molecular patterns (PAMP). As part of the innate immune system, recognition of PAMPs plays a major role in instructing appropriate adaptive immune responses.[1−3] Adjuvants act by stimulating the innate immune response of the host. Aluminum salt-based mineral salts, although rather weak adjuvants, have remained the only adjuvants currently approved by the FDA.[2,4] For this reason, the development of novel, safe, and effective adjuvants for human use is strongly encouraged. A thorough understanding of innate immunity and the underlying activity of TLRs and NLRs is expected to contribute significantly to the rational design of novel adjuvants.[5]

The response to bacterial peptidoglycan (PGN) is mediated largely by Nod1 and Nod2, two members of the cytosolic localized nucleotide oligomerization domain (Nod)-like receptor family, which recognize the minimal structural motif of PGN, D-glutamyl-*meso*DAP (iE-DAP), and muramyldipeptide (MDP), respectively.[6−8] Nod1 and Nod2 induce NF-κB activation following recognition of bacterial components, thus playing a role in innate immunity.[9] On the basis of the molecular mechanism of other NLRs, it is generally accepted that during MDP-induced activation, Nod2 undergoes conformational changes that result in self-oligomerization. This is followed by recruitment and activation of the serine threonine kinase RICK (also called RIP2), which is essential for activation of the NF-κB and MAPK signaling pathways that contribute to the Nod2-mediated inflammatory and immune response.[10,11] Furthermore, several studies in vitro have demonstrated that Nod2 and Nod1 agonists have a synergistic effect on production of TLR-mediated inflammatory cytokines, which could potentiate the cellular response against pathogens.[12−14]

Microbial recognition usually triggers the dominant Th1-like proinflammatory cytokine response.[15] The cytokines TNF-α, IL-1β, and IL-6 thus generated are controlled by NF-κB and capable of eliciting expression of chemokines and adhesion molecules, thus playing a key role in the recruitment of neutrophils and orchestrating innate immunity. The innate

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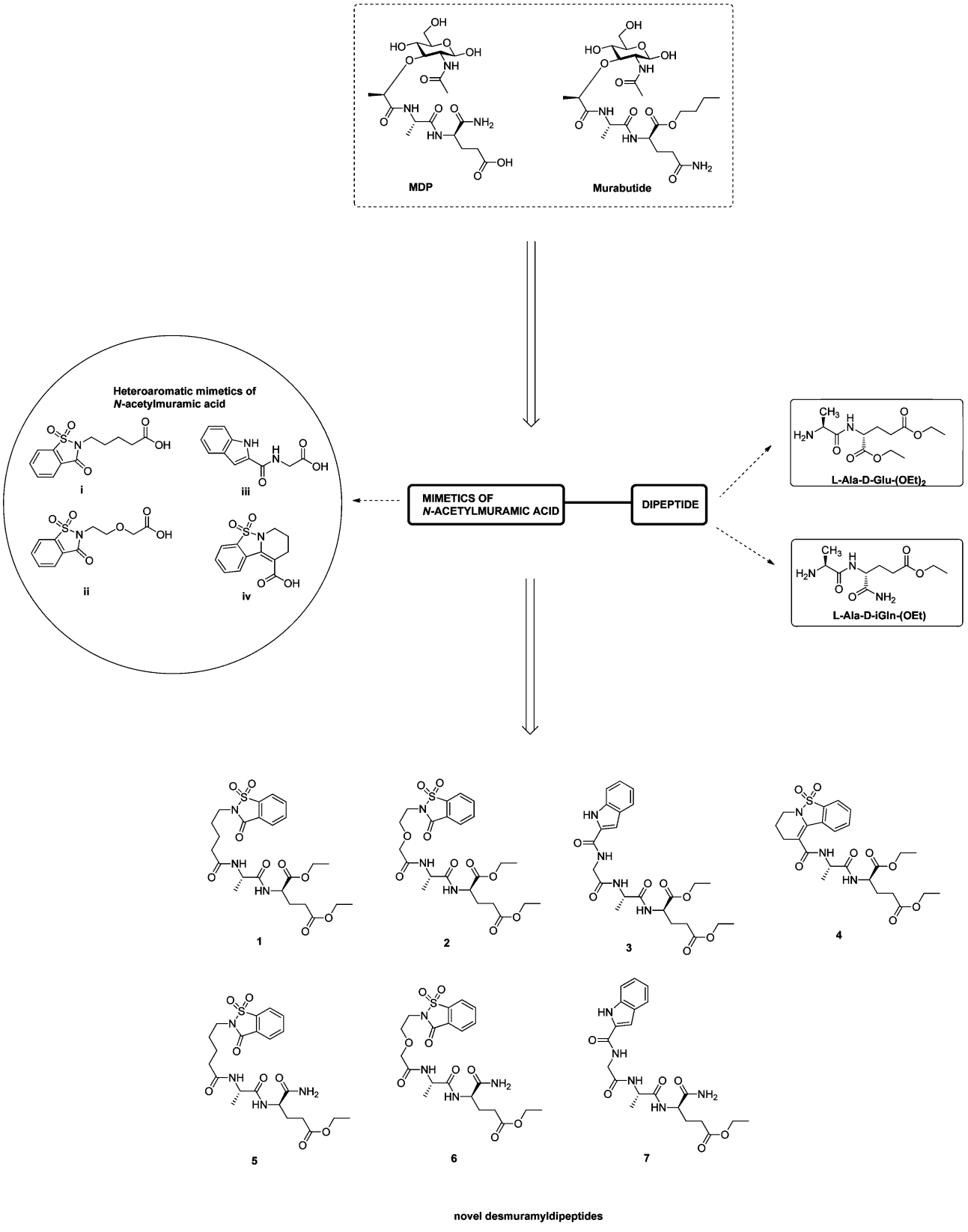

Figure 1. Design of novel desmuramyldipeptides.

cytokines, including IL-1β, IL-6, IL-12p70, and TNF-α, affect not only the initiation but also the expression of adaptive immunity.[16−18] Modulating the innate arm of the immune system could thus be an interesting approach for regulating the adaptive response. MDP, a component of Freund's complete adjuvant, is a potent adjuvant that induces both humoral and cell-mediated responses in animal models.[19] Identification of MDP as a Nod2 agonist, along with recent results

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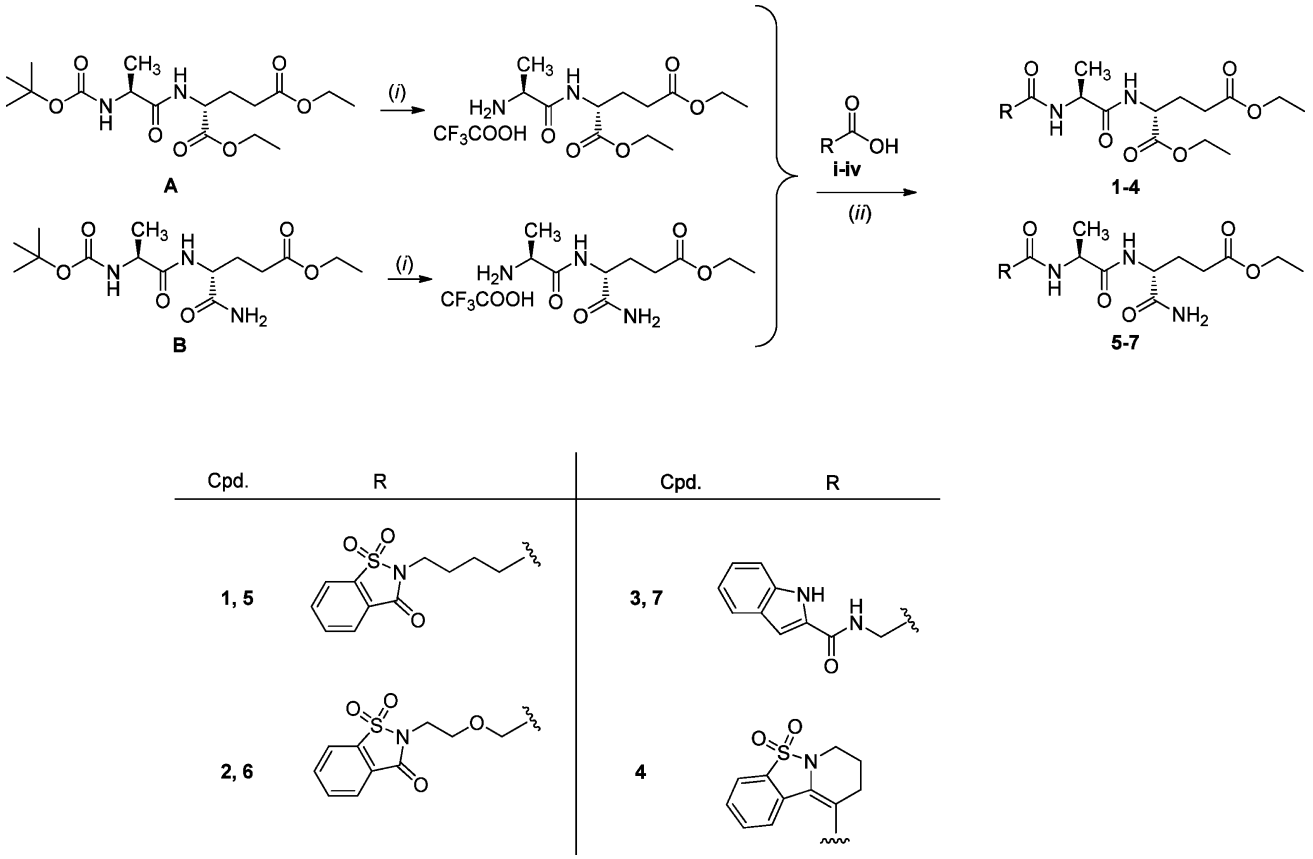
Scheme 1. Synthesis of MDP Derivatives Carrying L-Ala-D-iGln and L-Ala-D-Glu Moiety[a]



[a](i) TFA/dichloromethane (9:1), 0 °C to rt, 3 h; (ii) TBTU, *N*-methylmorpholine, dichloromethane, 0 °C to rt, 3 h.

demonstrating a role for Nod2 in the adaptive immune response, has led to a better understanding of its adjuvanticity, which could be ascribed to Nod2 activation.[8,20,21] However, despite extensive research on MDP, the molecule is too pyrogenic and arthritogenic to be used as an adjuvant in humans.[5]

Numerous derivatives of MDP have therefore been synthesized with the intention of improving the pharmacological properties and lowering the toxicological profile of the parent molecule. These efforts led to the discovery of murabutide (MB), a safe and apyrogenic derivative of MDP. It interacts with cells of the immune system, both innate and adaptive, exerting its effect through activation of Nod2.[22] MB possesses the ability to enhance antibody responses, thus enhancing the immunogenicity of vaccines.[23] Additionally, it enhances host resistance against microbial infections, nonspecific resistance against tumors, and the induction of colony stimulating factors (CSFs) and other cytokines implicated in hematopoesis.[22,24,25] On account of its numerous biological effects, MB has been the subject of several clinical studies and is being developed in the context of nonspecific immune-based therapies and targeting chronic viral diseases.[22] MB would be a useful immunomodulator if certain drawbacks, such as its rapid elimination and lack of oral availability, could be overcome.[22]

Desmuramylpeptides are MDP derivatives lacking the carbohydrate moiety, which renders them less hydrophilic than the parent molecule. Some of them were shown to enhance the host defense against microbial infections, whereas others exhibited strong antiviral activity, adjuvant activity, and remarkable antitumor potency.[26] The absence of structural data for the binding site of MDP makes the design of novel immunomodulators difficult, the structure−activity relationship (SAR) of well-known immunomodulatory compounds being the only route for rational drug design. We recently described the synthesis of desmuramyldipeptides that were shown to possess immunomodulatory properties.[27] In this report, a series of new and improved derivatives were designed, synthesized, and biologically evaluated. Since Nod2 is located in the cytosol, we synthesized desmuramyldipeptides that were more lipophilic to facilitate their passive transport through the cell membrane. Their mechanism of action was established on HEK293 cells overexpressing the Nod2 gene, proving that desmuramyldipeptides exert their actions via activation of Nod2.

2. RESULTS

2.1. Design. The desmuramyldipeptides were shown to enter the cell by passive absorption, which is dependent on their lipophilicity.[28] Moreover, the information obtained from previous studies on SAR of MDP analogues indicates that the introduction of a lipophilic substituent into MDP may increase its adjuvant activity.[29] For these reasons, the carbohydrate moiety was replaced by different heteroaromatic groups (**i**−**iv**) that proved to be appropriate surrogates for the *N*-acetylmuramyl group of the parent molecule in our previous research.[27]

The lipophilic mimetics of the *N*-acetylmuramic part of MDP were coupled with both parent dipeptide moieties, L-Ala-D-iGln and L-Ala-D-Glu, since most of the known MDP analogues

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possess an intact dipeptide moiety, thus affording novel desmuramyldipeptides. Additionally, the carboxylic acid groups of dipeptide moieties were replaced by ethyl ester functionalities to further increase the lipophilicity of the compounds (shown in Figure 1). Taken together, seven novel lipophilic desmuramyldipeptides were synthesized and screened for their immunomodulatory properties. Since Nod2 has been proposed as a receptor for MDP, we used engineered Nod2-transfected HEK-Blue Nod2 cells to screen and validate our compounds for their Nod2-agonistic activity. Their immunomodulatory properties were subsequently assessed in vitro using primary immunocompetent cells. Their effect on the proinflammatory cytokine production of phorbol 12-myristate 13-acetate (PMA)/ionomycin-stimulated human peripheral blood mononuclear cells (PBMCs) was evaluated relative to that of murabutide.

2.2. Chemistry. The lipophilic mimetics of the *N*-acetylmuramic part of MDP (**i**–**iv**) were synthesized according to the published procedure.[27] The protected dipeptides Boc-L-alanyl-D-glutamic acid diethyl ester **A** and Boc-L-alanyl-D-isoglutamine ethyl ester **B** were also prepared by known procedures.[27] Removal of the Boc-protecting group from Boc-L-alanyl-D-glutamic acid diethyl ester **A**, using trifluoroacetic acid in dichloromethane, gave the corresponding trifluoroacetate salt of the dipeptide which was immediately used in the following reaction. Synthesized acids **i**–**iv** were coupled with diethyl L-alanyl-D-glutamate trifluoroacetate using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as a coupling reagent to give diethyl esters **1**–**4** (Scheme 1). The same strategy was applied to the synthesis of derivatives of L-alanyl-D-isoglutamic acid. Removal of the Boc-protecting group from Boc-L-alanyl-D-isoglutamine ethyl ester **B** was achieved using trifluoroacetic acid in dichloromethane to give the ethyl L-alanyl-D-isoglutaminate trifluoroacetate, which was immediately coupled with acids **i**–**iii**, thus affording the corresponding ethyl esters **5**–**7** (Scheme 1).

2.3. Biological Data. *2.3.1. Determination of Nod2-Agonistic Activity of Desmuramyldipeptides.* Potential Nod2 agonists (compounds **1**–**7**) and MB were examined using the HEK-Blue Nod2 reporter gene assay. Nod2-specific HEK-Blue cells were incubated for 18 h with MB and desmuramyldipeptides at 20 μM. The positive control MB and compounds **3** and **4** significantly increased NF-κB transcriptional activity with respect to untreated cells (Figure 2). Nod2-specific responses were confirmed, using Ramos-Blue cells as the control cell line. These cells express TLR3, TLR7, TLR9, and Nod1 but not Nod2 and do not respond to Nod2 agonists. They stably express an NF-κB/AP-1-inducible secreted embryonic alkaline phosphate (SEAP) reporter construct. These cells were stimulated with the compound of interest (20 μM) for 18 h followed by determination of SEAP activity in the supernatant. None of the desmuramyldipeptides or murabutide induced any NF-κB activity with respect to the untreated cells (data not shown).

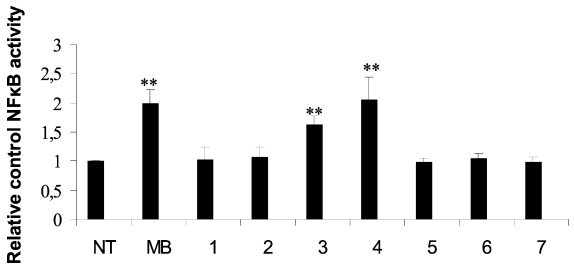

Figure 2. Effects of desmuramyldipeptides **1**–**7** on NF-κB transcriptional activity. SEAP activity was measured in Nod2-specific HEK-Blue cells after incubation for 18 h with murabutide (MB) and other Nod2 agonistic compounds (20 μM). Columns represent mean values of duplicates of three independent experiments. Error bars indicate ±SD: (**) $p < 0.01$ vs untreated cells (NT).

2.3.2. Immunomodulatory Properties of Novel Desmuramyldipeptides and Murabutide on PBMCs. The potential immunomodulatory effects of the desmuramyldipeptides were evaluated using primary immunocompetent cells. PBMCs represent an ideal target cell for assaying our compounds, since they provide a physiologically relevant model system for the identification of a broad range of innate immune stimulants.[30] The combination PMA/ionomycin is the most effective stimulus for the induction of Th1-type cytokines, and most of the current studies have used it for cell activation because unstimulated cells spontaneously produce little or no cytokines.[31,32] Cytokine phenotypes derived upon PMA/ionomycin activation represent the physiologic potential of cellular cytokine production,[33] which prompted us to use a combination of stimuli, a desmuramyldipeptide and PMA/ionomycin. The 20 μM concentration of MB and the tested compounds is in agreement with various studies of MB wherein authors used this concentration to elicit a biological response. The exposure time of 18 h and the concentrations of stimuli, PMA (3.33 ng/mL) and ionomycin (500 nM), were previously determined to be the most optimal.[27]

By use of the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (MTS) metabolic activity assay, the proliferation rates of PBMCs were evaluated in the presence of MB and the novel desmuramyldipeptide analogues. Cells were treated for 18 h with the compound of interest at 20 μM. Comparison of the resulting metabolic activities with that for the untreated control showed that the compounds were well tolerated by PBMCs, since none of their residual metabolic activities fell below 90% at the maximum concentration tested (data not shown). Another experiment was performed in which the cells were stimulated with desmuramyldipeptides and MB in the presence of PMA (3.33 ng/mL) and ionomycin (500 nM). Comparison of metabolic activities relative to those in the PMA/ionomycin-treated control revealed no statistically significant difference (data not shown).

The desmuramyldipeptide compounds **1**–**7** and MB, each at 20 μM, were dissolved in DMSO and further diluted in culture medium so that the final concentration of DMSO did not exceed 0.1%. PMA and ionomycin were then added to the cell culture medium to give final concentrations of 3.33 ng/mL and 500 nM, respectively. The immunomodulatory capacity of synthesized desmuramyldipeptides was assessed on human PBMC relative to that of murabutide using the human inflammatory cytokine kit (IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12p70). Some of the compounds considerably changed the level of cytokine production induced by PMA/ionomycin (Figure 3).

For IL-1β release, a very significant increase was observed with MB and compounds **3** and **4**. For IL-6 release, it was only increased by compound **3**. IL-8 and IL-10 release was not affected by any of the compounds or by MB. IL-12p70 release was modestly affected by MB as well as compounds **3** and **4**. Finally, TNF-α release was increased by MB and compound **3**

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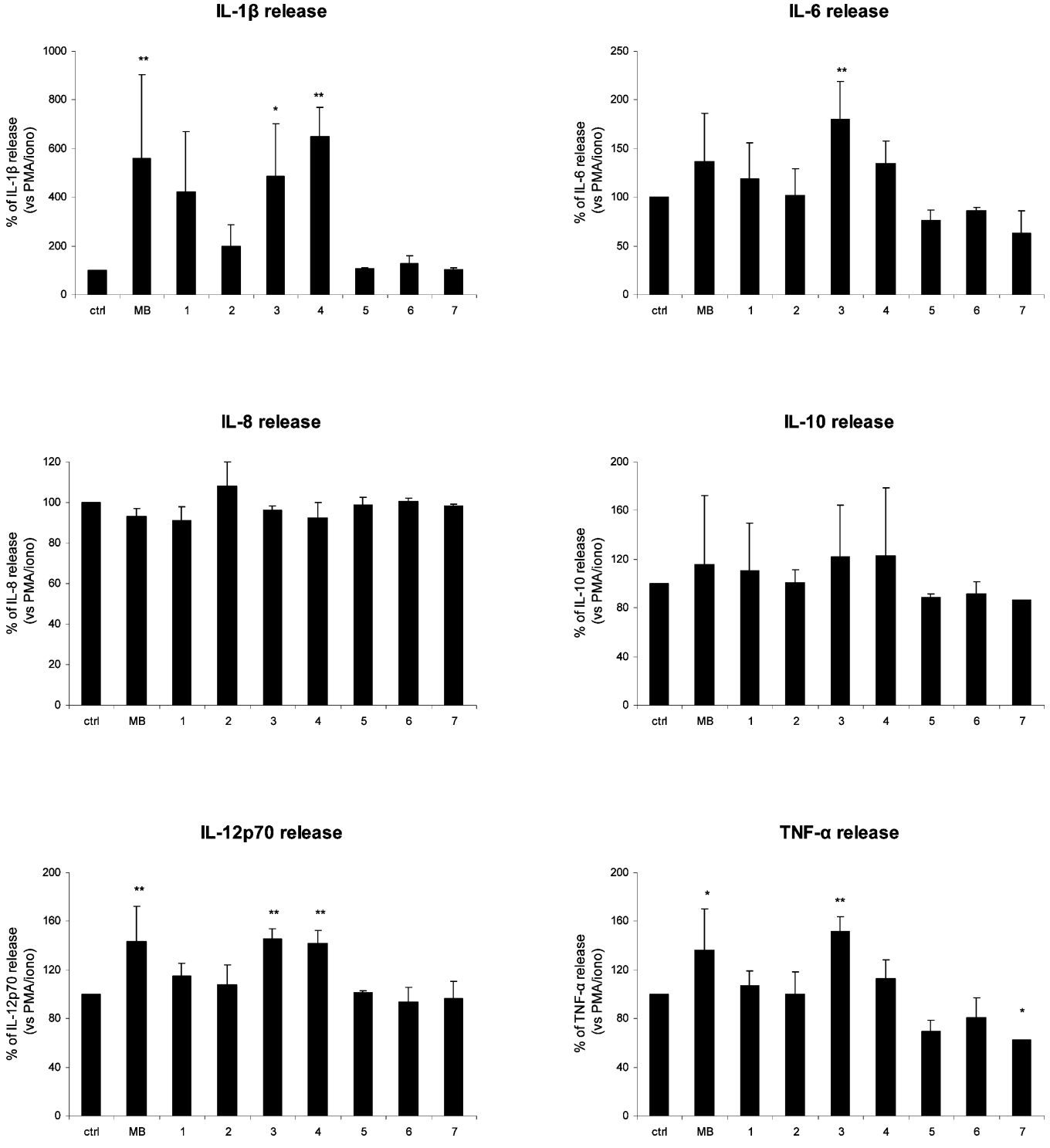

Figure 3. Effect of murabutide (MB) and the novel desmuramyldipeptide analogues on PMA/ionomycin-induced cytokine release in PBMCs. Desmuramyldipeptides or MB was added (20 μM) to PMA/ionomycin-treated cells. Control cells were treated only with PMA (3.33 ng/mL) and ionomycin (500 nM). Cytokine release was determined after 18 h. Values are expressed as % of PMA/ionomycin-induced cytokine release. Columns represent mean values of duplicates ± SD of three independent experiments (data are representative of three independent donors): (*) $p < 0.05$ and (**) $p < 0.01$ vs PMA/ionomycin treated cells.

and significantly reduced by compound **7**. Compound **3** was therefore selected for further evaluation of its dose- and time-dependent effects on cytokine release from PMA/ionomycin-stimulated PBMCs.

*2.3.3. Dose-Dependent Effects of Immunomodulatory Compound **3** and Murabutide on Cytokine Production.* The dependence of dose was also investigated. The results confirmed the influence of increasing concentrations of desmuramyldipeptide **3** and MB on the modulation of cytokine release from PBMCs. Cytokine release was stimulated using four concentrations of compound **3** and MB (0.2, 2, 20, and 200 μM). Both compound **3** and MB induced a dose-

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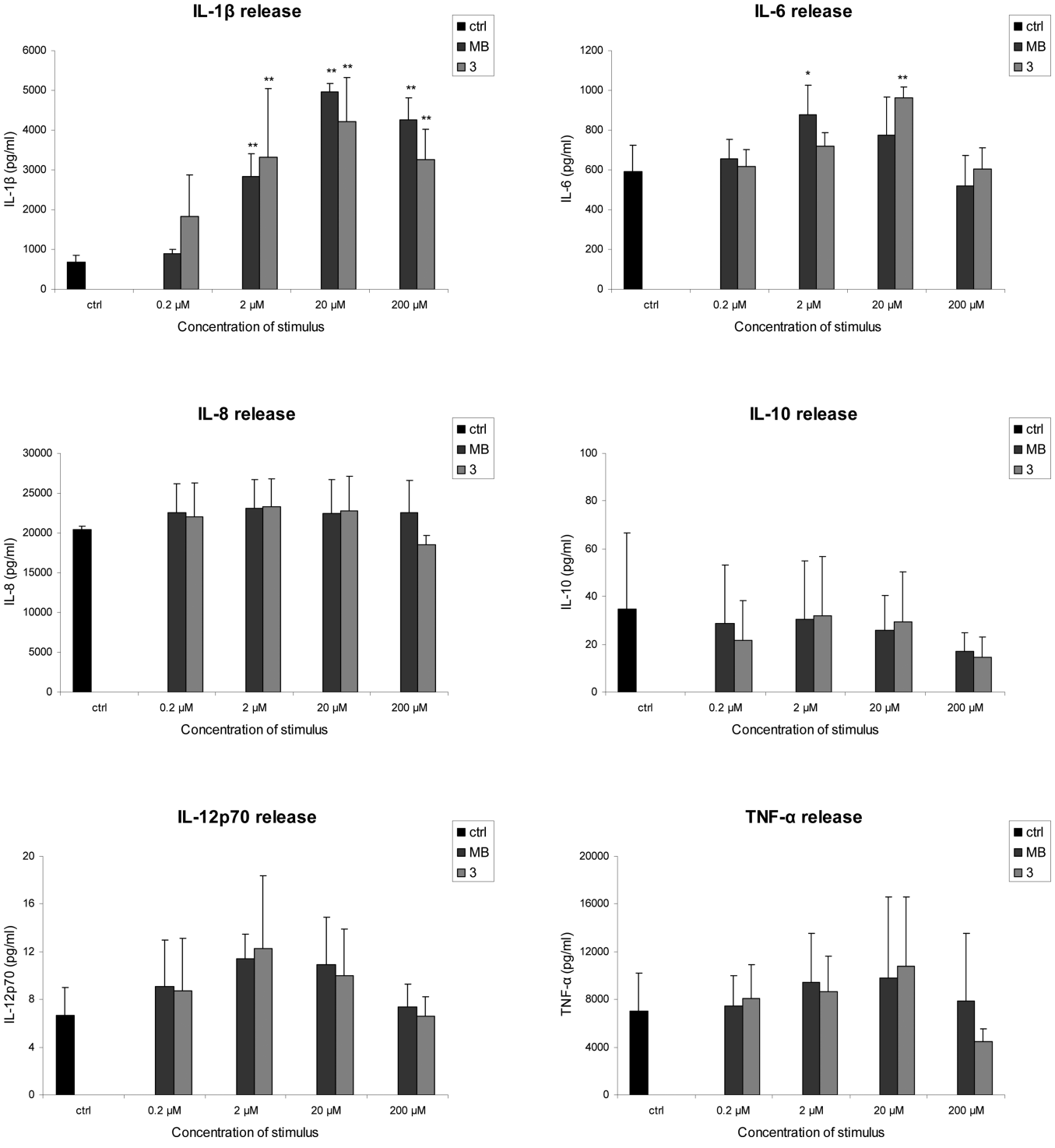

Figure 4. Influence of concentration of compound **3** on cytokine release. Cells were incubated for 18 h with increasing concentrations of compound **3** or murabutide (MB) (0.2–200 μM) in the presence of PMA (3.33 ng/mL) and ionomycin (500 nM). The control cells were treated with the same concentrations of PMA and ionomycin in the absence of compound. Columns represent mean values of two independent experiments in duplicate. Error bars indicate ±SD (data are representative of two independent donors): (*) $p < 0.05$ and (**) $p < 0.01$ vs PMA/ionomycin treated cells.

dependent stimulatory effect on cytokine release (Figure 4). The release of IL-1β was increased significantly by both compounds at 2 μM and more so at 20 and 200 μM. Similarly, IL-6 release was moderately affected by MB at 2 μM and significantly increased by compound **3** at 20 μM. Release of IL-8 and IL-10 was not affected by compound **3** or by MB. IL-12p70 release was elevated by both compounds at 2 and 20 μM; however, the effect was not statistically significant. A similar trend was observed for TNF-α.

*2.3.4. Compound **3** and Murabutide Cause Time-Dependent Cytokine Production.* Cytokine release from PMA/ionomycin-stimulated PBMCs under the influence of desmuramyldipeptide **3** and MB was measured at 4, 18, and 40 h. Both compound **3** and MB induced time-dependent stimulatory

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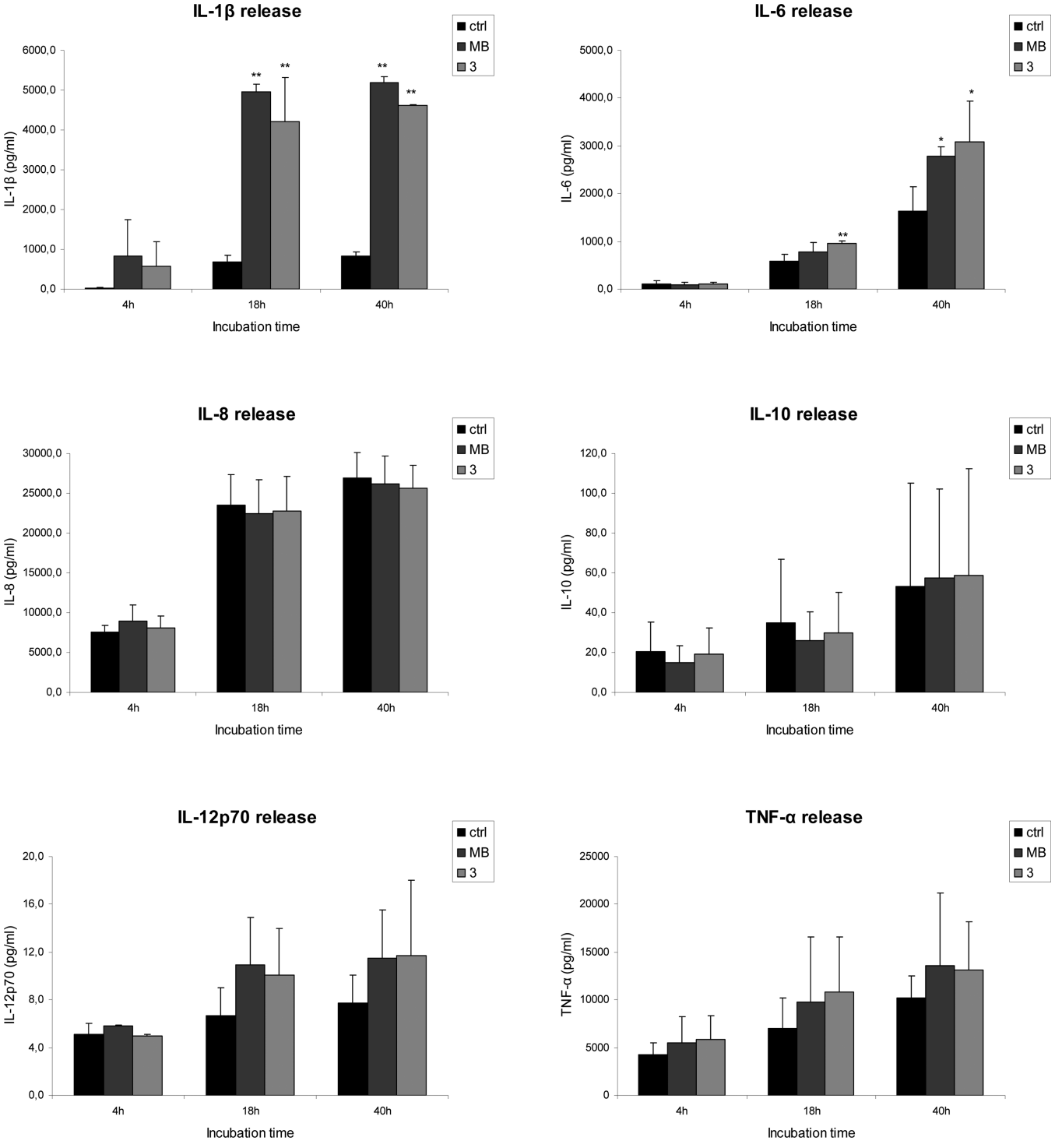

Figure 5. Time-dependent cytokine release from stimulated PBMCs. Cells were incubated for 4, 18, and 40 h with murabutide (MB) or with compound **3** (both at 20 μM) in the presence of PMA (3.33 ng/mL) and ionomycin (500 nM). Control cells were treated with only PMA and ionomycin. Columns represent mean values of two independent experiments in duplicate. Error bars indicate ±SD (data are representative of two independent donors): (∗) $p < 0.05$ and (∗∗) $p < 0.01$ vs PMA/ionomycin treated cells.

effects (Figure 5). The release of IL-1β was increased by compound **3** and MB from the earliest time interval of 4 h. The influence of incubation time on IL-6 release was observed only for compound **3** at 18 h, whereas after 40 h both compounds significantly increased the release of IL-6. Release of IL-8 and IL-10 was not affected by either compound **3** or MB, while IL-12p70 and TNF-α release increased after 18 and 40 h of treatment by compound **3** and MB.

3. DISCUSSION AND CONCLUSIONS

A series of more lipophilic desmuramyldipeptides have been designed and synthesized to facilitate their access to their putative target, Nod2 protein, located in the cytosol. HEK-Blue Nod 2 cells are engineered HEK293 cells that stably coexpress the human Nod2 gene and an NF-κB-inducible SEAP reporter gene. They are designed to provide a rapid, sensitive, and

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reliable method to screen and validate Nod2 agonists by monitoring the activation of NF-κB. Recognition of a Nod2 agonist by its cognate receptor triggers a signaling cascade leading to the activation of NF-κB and the production of SEAP. The experiment confirmed the Nod2-agonistic effect of MB, serving as reference, and of compounds **3** and **4**, which significantly increased NF-κB transcriptional activity with respect to untreated cells. On account of its similarity with MDP, MB can supposedly serve as substrate for the hPepT1 transporter, which enables it to enter the host cell.[34,35] The desmuramyldipeptides do not act as substrates for the hPepT1 transporter. Instead they are absorbed passively and appear to enter the cell by the paracellular route.[28] Moreover, the passive transport of compounds through the cell membrane is dependent on their lipophilicity. This was reflected by a strong correlation between lipophilicity of our compounds and the observed induction of NF-κB transcriptional activity. The more lipophilic L-Ala-D-Glu containing esters **1**−**4** were considerably more effective in inducing NF-κB transcriptional activity, whereas their counterparts incorporating the L-Ala-D-iGln moiety, compounds **5**−**7**, were completely inactive. The most active, and coincidentally the most lipophilic, compound of the series was compound **4**, incorporating a heteroaromatic pyrido[1,2]benzisothiazole ring system[36] in place of carbohydrate moiety, with activity surpassing even that of murabutide. Compound **3**, containing an indole-2-ylcarboxamido moiety as a replacement for the *N*-acetylmuramyl fragment, was also confirmed as a Nod2 agonist. Since HEK293 cells express endogenous levels of TLR3, TLR5, and Nod1, HEK-Blue-Nod 2 cells will respond to their cognate ligands, such as poly(I:C), flagellin, and iE-DAP, respectively. In order to confirm Nod2-specific responses, we used Ramos-Blue cells as a control cell line. Ramos-Blue cells express TLR3, TLR7, TLR9, and Nod1; however, they do not express Nod2 and are thus nonresponsive to Nod2 agonists. The test confirmed that neither the desmuramyldipeptides nor murabutide induced any NF-κB activity in Ramos-Blue cells with respect to the untreated cells.

The synthesized desmuramyldipeptide analogues and MB as a reference compound were screened for their ability to modulate PMA/ionomycin-induced cytokine release from PBMCs. MB significantly increased the release of proinflammatory cytokines IL-1β, IL-12p70, and TNF-α, while compound **3**, which proved to be the most promising compound of the series, significantly increased the release of IL-1β, IL-6, IL-12p70, and TNF-α. Similarly, compound **4** also enhanced the release of IL-1β, IL-6, and IL-12p70. Compounds **1**, **2**, **5**, **6**, and **7** showed no stimulatory effect. In contrast, compound **7** considerably reduced the TNF-α release from PMA/ionomycin-stimulated PBMCs. Compound **3** and MB as a reference were chosen for further characterization. A dose-dependent manner was observed when stimulating PBMCs with increasing concentrations of the compounds (from 0.2 to 200 μM). The cytokine release-increasing effect was seen at concentrations as low as 2 μM and was most pronounced at 20 μM, whereas a decline in cytokine release was observed for both compounds at the highest concentration tested (200 μM) probably because of cell exhaustion. A time-course experiment was performed to evaluate the effects of MB and compound **3** on PMA/ionomycin-induced cytokine release from PBMCs after 4, 18, and 40 h of incubation. The measured cytokine concentrations at three different intervals revealed similar profiles for both compounds. A rise in concentration as a general trend was seen with all proinflammatory cytokines. After 4 h of incubation only the production of IL-1β was increased by MB and compound **3**. The influence of incubation time was evident after 18 h and most pronounced after 40 h.

The results of the HEK-Blue Nod2 reporter gene assay corroborate those obtained with experiments on PBMCs. The compounds that significantly induced NF-κB transcriptional activity (MB and compounds **3** and **4**) also significantly altered the cytokine release from PMA/ionomycin-stimulated cells, whereas compounds that were nonactive in the reporter gene assay did not affect the cytokine release from PBMCs.

The carbohydrate moiety of compounds **3** and **4** was replaced by different heteroaromatic groups which proved to be appropriate surrogates for the *N*-acetylmuramyl group of murabutide. In addition, this replacement rendered them considerably more lipophilic than the parent molecule.[28,29] Since the information obtained from previous studies on SAR of MDP analogues indicates that the introduction of a lipophilic substituent into MDP may increase its adjuvant activity, desmuramyldipeptides **3** and **4** represent interesting candidates for further research of their immunoadjuvant properties.

In conclusion, we synthesized new and improved desmuramyldipeptide analogues of MDP and determined their mechanism of action. We have observed significant immunomodulatory effects of desmuramyldipeptide analogues on PMA/ionomycin-induced cytokine release from PBMCs. The potent Nod2-agonistic effect of MB and desmuramyldipeptides **3** and **4**, established on HEK293 cells overexpressing the Nod2 gene, indicates that these compounds exert their activity via activation of Nod2. On account of their lipophilic character, compounds **3** and **4** are interesting candidates for further studies to determine their usefulness as adjuvants.

4. EXPERIMENTAL SECTION

4.1. General. Chemicals were obtained from Acros, Aldrich Chemical Co., and Fluka and used without further purification. Murabutide (a synthetic muramyldipeptide-derived Nod2 agonist) was obtained from Invivogen, Inc., (San Diego, CA). PMA and ionomycin were from Sigma. Analytical TLC was performed on Merck 60 F254 silica gel plates (0.25 mm), using visualization with ultraviolet light and ninhydrin. Column chromatography was carried out on silica gel 60 (particle size 240−400 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. ^{1}H NMR spectra were recorded at 300 MHz on a Bruker Avance DPX300 spectrometer in DMSO-d_6 solution with TMS as the internal standard. Spectra were assigned using gradient COSY and HSQC experiments. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. HPLC analyses were performed on an Agilent Technologies HP 1100 instrument with G1365B UV−vis detector (220 nm), using a Luna C18 column (4.6 mm × 150 mm) at a flow rate of 1 mL/min. The eluant was a mixture of 0.1% TFA in water (A) and acetonitrile (B). Gradient was 10% B to 80% B in 30 min. The purity of all pharmacologically investigated compounds was >95% as determined by RP-HPLC.

4.2. General Procedures. *4.2.1. Acidolytic Cleavage of Boc Protecting Groups.* To an ice-chilled stirred mixture of trifluoroacetic acid and dichloromethane (5/1, 10 mL) Boc-protected dipeptides (1 mmol) and Boc-L-alanyl-D-glutamic acid diethyl ester **A** or Boc-L-alanyl-D-isoglutamine ethyl ester **B** (1 mmol) were added, and the mixture was allowed to warm to room temperature. After 3 h the reaction was completed and the solvent was evaporated in vacuo. The residue was washed three times with diethyl ether and immediately used for the next step.

4.2.2. TBTU-Mediated Coupling of Carboxylic Acids with Amines. To a stirred solution of the corresponding carboxylic acid derivative (1.1 mmol) and ethyl L-alanyl-D-isoglutaminate trifluoroacetate (1

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mmol) or diethyl L-alanyl-D-glutamate trifluoroacetate (1 mmol) in dry dichloromethane, *N*-methylmorpholine (2.5 mmol) was added at 0 °C. After the mixture was stirred for 15 min, TBTU (1.1 mmol) was added. The mixture was allowed to warm to room temperature, and stirring then continued for 3 h. The solvent was removed under reduced pressure and the residue dissolved in ethyl acetate (20 mL) which was washed with 10% citric acid (2 × 10 mL), water (20 mL), saturated $NaHCO_3$ solution (2 × 10 mL), and water (20 mL) and then dried over anhydrous Na_2SO_4. If necessary, the products were additionally purified using column chromatography to afford sufficiently pure products.

4.3. Characterization of Compounds. *4.3.1. Diethyl (2R)-2-[((2S)-2-{[5-(1,1,3-Trioxo-1,3-dihydro-2H-1,2-benzisothiazol-2-yl)-pentanoyl]amino}propanoyl)amino]pentanedioate (1).* **1** was synthesized from **A** according to the general procedure for coupling with TBTU. White amorphous powder; yield, 399 mg (74%); mp 90−94 °C. ^{1}H NMR (DMSO-d_6): δ = 1.14−1.19 (m, 9H, 2 × CH_2CH_3 and CH_3-Ala), 1.52−1.62 (m, 2H, $CH_2CH_2CH_2$), 1.68−1.78 (m, 2H, $CH_2CH_2CH_2$), 1.80−1.88 (m, 1H, H-β-Glu), 1.93−2.05 (m, 1H, H-β-Glu), 2.18 (t, 2H, J = 7.2 Hz, CH_2-γ-Glu), 2.33 (t, 2H, J = 7.35 Hz, CH_2CO), 3.71 (t, 2H, J = 7.05 Hz, NCH_2), 4.00−4.10 (m, 4H, 2 × CH_2CH_3), 4.20−4.36 (m, 2H, CH-Glu and CH-Ala), 7.94−8.12 (m, 4H, saccharinyl-H), 8.20 (d, 1H, J = 7.8 Hz, NH-Ala), 8.30 (d, 1H, J = 7.8 Hz, NH-Glu). MS (ESI): m/z (%) = 540.2 (M + H)$^+$. IR (KBr): ν = 3334, 2980, 1734, 1633, 1534, 1464, 1381, 1331, 1301, 1275, 1185, 1062, 1032, 991, 962, 862, 786, 752, 676 cm^{-1}. HPLC (254 nm): 95.8%, t_R = 18.04 min. HRMS calcd for $C_{24}H_{34}N_3O_9S$ m/z, 540.2016 (M + H)$^+$; found, 540.2004. $[\alpha]_D^{20}$ −12.1° (c 0.24, MeOH).

4.3.2. Diethyl (2R)-2-{[(2S)-2-({2-[2-(1,1,3-Trioxo-1,3-dihydro-2H-1,2-benzisothiazol-2-yl)ethoxy]acetyl}amino)propanoyl]amino}-pentanedioate (2). was synthesized from **A** according to the general procedure for coupling with TBTU. White amorphous powder; yield, 353 mg (65%); mp 73−76 °C. ^{1}H NMR (DMSO-d_6): δ = 1.15−1.22 (m, 9H, 2 × CH_2CH_3 and CH_3-Ala), 1.74−1.87 (m, 1H, H-β-Glu), 1.92−2.06 (m, 1H, H-β-Glu), 2.32 (t, 2H, J = 7.5 Hz, CH_2-γ-Glu), 3.78 (t, 2H, J = 5.1 Hz, NCH_2), 3.92−3.98 (m, 4H, NCH_2CH_2O and OCH_2CO), 4.01−4.10 (m, 4H, 2 × CH_2CH_3), 4.15−4.23 (m, 1H, CH-Glu), 4.29−4.39 (m, 1H, CH-Ala), 7.59 (d, 1H, J = 7.8 Hz, NH-Ala), 7.98−8.12 (m, 3H, saccharinyl-H), 8.26−8.35 (m, 2H, saccharinyl-H and NH-Glu). MS (ESI): m/z (%) = 542.2 (M + H)$^+$. IR (KBr): ν = 3587, 3394, 3312, 3074, 2986, 2944, 1735, 1655, 1596, 1540, 1458, 1383, 1368, 1335, 1317, 1300, 1273, 1191, 1128, 1085, 1062, 1028, 952, 890, 860, 787, 750, 678, 646 cm^{-1}. HPLC (254 nm): 97.4%, t_R = 17.26 min. HRMS calcd for C23H31N3O10S m/z: 542.1808 (M + H)$^+$, found 542.1782. $[\alpha]_D^{20}$ = +3.8° (c 0.13, MeOH).

4.3.3. Diethyl (2R)-2-{[(2S)-2-({2-[(1H-Indol-2-ylcarbonyl)amino]-acetyl}amino)propanoyl]amino}pentanedioate (3). was synthesized from **A** according to the general procedure for coupling with TBTU. Orange amorphous solid; yield, 378 mg (80%); mp 79−83 °C. ^{1}H NMR (DMSO-d_6): δ = 1.08−1.25 (m, 9H, 2 × CH_2CH_3 and CH_3-Ala), 1.83−1.89 (m, 1H, H-β-Glu), 1.97−2.06 (m, 1H, H-β-Glu), 2.34 (t, 2H, J = 7.2 Hz, CH_2-γ-Glu), 3.94 (t, 2H, J = 5.6 Hz, NCH_2), 3.99−4.12 (m, 4H, 2 × CH_2CH_3), 4.22−4.28 (m, 1H, CH-Glu), 4.34−4.41 (m, 1H, CH-Ala), 7.04 (t, 1H, J = 7.2 Hz, Ar-H), 7.15−7.21 (m, 2H, Ar-H), 7.44 (d, 1H, J = 7.6 Hz, Ar-H), 7.63 (d, 1H, J = 7.6 Hz, Ar-H), 8.17 (d, 1H, J = 7.6 Hz, NH-Ala), 8.32 (d, 1H, J = 8.0 Hz, NH-Glu), 8.77 (t, 1H, J = 5.6 Hz, $CONHCH_2$), 11.56 (s, 1H, indole-NH). MS (ESI): m/z (%) = 475.2 (M + H)$^+$. IR (KBr): ν = 3299, 3061, 2982, 2936, 2365, 1735, 1639, 1550, 1448, 1420, 1376, 1340, 1311, 1205, 1020, 856, 818, 772, 749, 669 cm^{-1}. HPLC (254 nm): 95.1%, t_R = 18.42 min. HRMS calcd for C23H31N4O7 m/z: 475.2193 (M + H)$^+$, found 475.2189. $[\alpha]_D^{20}$ = −9.3° (c 0.15, MeOH).

4.3.4. Diethyl (2R)-2-[((2S)-2-{[(5,5-Dioxo-5,7,8,9-tetrahydro-5λ6-pyrido[1,2-b][1,2]benzisothiazol-10-yl)carbonyl]amino}propanoyl)-amino]pentanedioate (4). **4** was synthesized from **A** according to the general procedure for coupling with TBTU. White amorphous powder; yield, 345 mg (66%); mp 166−168 °C. ^{1}H NMR (DMSO-d_6): δ = 1.12−1.31 (m, 9H, 2 × CH_2CH_3 and CH_3-Ala), 1.83−2.10 (m, 4H, CH_2-β-Glu and $NCH_2CH_2CH_2$), 2.38 (t, 2H, J = 7.5 Hz, CH_2-γ-Glu), 2.45 (t, 2H, J = 6.0 Hz, CH_2C═), 3.48 (t, 2H, J = 5.1 Hz, NCH_2CH_2), 4.02−4.14 (m, 4H, 2 × CH_2CH_3), 4.23−4.34 (m, 1H, CH-Glu), 4.46−4.55 (m, 1H, CH-Ala), 7.62−7.69 (m, 2H, Ar-H), 7.83−7.86 (m, 1H, Ar-H), 7.97−8.02 (m, 1H, Ar-H), 8.44 (d, 1H, J = 7.8 Hz, NH-Ala), 8.70 (d, 1H, J = 7.5 Hz, NH-Glu). MS (ESI): m/z (%) = 522.2 (M + H)$^+$. IR (KBr): ν = 3307, 2979, 1750, 1721, 1654, 1622, 1541, 1472, 1380, 1311, 1264, 1209, 1182, 1062, 1020, 895, 862, 821, 764, 746, 630 cm^{-1}. HPLC (254 nm): 97.9%, t_R = 16.75 min. HRMS calcd for $C_{24}H_{32}N_3O_8S$ m/z, 522.1910 (M + H)$^+$; found, 522.1921. $[\alpha]_D^{20}$ −9.2° (c 0.19, MeOH).

4.3.5. Ethyl (4R)-5-Amino-5-oxo-4-[((2S)-2-{[5-(1,1,3-trioxo-1,3-dihydro-2H-1,2-benzisothiazol-2-yl)pentanoyl]amino}propanoyl)-amino]pentanoate (5). **5** was synthesized from **B** according to the general procedure for coupling with TBTU. White amorphous powder; yield, 432 mg (85%); mp 150−154 °C. ^{1}H NMR (DMSO-d_6): δ = 1.14−1.21 (m, 6H, CH_2CH_3 and CH_3-Ala), 1.52−1.62 (m, 2H, $CH_2CH_2CH_2$), 1.67−1.79 (m, 3H, $CH_2CH_2CH_2$ and H-CH_2-β-iGln), 1.95−2.04 (m, 1H, H-CH_2-β-iGln), 2.15−2.20 (m, 2H, CH_2CONH), 2.27 (t, 2H, J = 7.8 Hz, CH_2-γ-iGln), 3.71 (t, 2H, J = 5.1 Hz, NCH_2), 4.04 (q, 2H, J = 6.9 Hz, CH_2CH_3), 4.11−4.25 (m, 2H, CH-iGln and CH-Ala), 7.08 (s, 1H, NH_2-iGln), 7.27 (s, 1H, NH_2-iGln), 7.97−8.12 (m, 5H, 3 saccharinyl-H and NH-Ala and NH-iGln), 8.29−8.31 (m, 1H, saccharinyl-H4). MS (ESI): m/z (%) = 511.2 (M + H)$^+$. IR (KBr): ν = 3412, 3291, 3067, 2978, 2936, 2739, 2678, 2364, 1735, 1673, 1633, 1545, 1442, 1320, 1258, 1184, 1061, 1029, 992, 876, 786, 751, 722, 675 cm^{-1}. HPLC (254 nm): 96.0%, t_R = 15.54 min. HRMS calcd for $C_{22}H_{31}N_4O_8S$ m/z, 511.1863 (M + H)$^+$; found, 511.1877. $[\alpha]_D^{20}$ −10.9° (c 0.22, MeOH).

4.3.6. Ethyl (4R)-5-Amino-5-oxo-4-{[(2S)-2-({2-[2-(1,1,3-trioxo-1,3-dihydro-2H-1,2-benzisothiazol-2-yl)ethoxy]acetyl}amino)-propanoyl]amino}pentanoate (6). **6** was synthesized from **B** according to the general procedure for coupling with TBTU. White amorphous powder; yield, 420 mg (82%); mp 182−185 °C. ^{1}H NMR (DMSO-d_6): δ = 1.15−1.21 (m, 6H, CH_2CH_3 and CH_3-Ala), 1.65−1.79 and 1.90−2.01 (2 m, 1H each, CH_2-β-iGln), 2.26 (t, 2H, J = 8.1 Hz, CH_2-γ-iGln), 3.78 (t, 2H, J = 5.1 Hz, NCH_2), 3.92−3.98 (m, 4H, NCH_2CH_2O and OCH_2CO), 4.04 (q, 2H, J = 6.9 Hz, CH_2CH_3), 4.11−4.17 (m, 1H, CH-iGln), 4.23−4.34 (m, 1H, CH-Ala), 7.08 (s, 1H, NH_2-iGln), 7.26 (s, 1H, NH_2-iGln), 7.68 (d, 1H, J = 7.2 Hz, NH-Ala), 7.98−8.13 (m, 3H, 2 saccharinyl-H and NH-iGln), 8.30−8.33 (m, 1H, saccharinyl-H4). MS (ESI): m/z (%) = 513.2 (M + H)$^+$. IR (KBr): ν = 3396, 3292, 1725, 1656, 1542, 1331, 1269, 1187, 1129, 752 cm^{-1}. HPLC (254 nm): 100.0%, t_R = 15.25 min. HRMS calcd for $C_{21}H_{29}N_4O_9S$ m/z, 513.1655 (M + H)$^+$; found, 513.1653. $[\alpha]_D^{20}$ +16.3° (c 0.17, MeOH).

4.3.7. Ethyl (4R)-5-Amino-4-{[(2S)-2-({2-[(1H-indol-2-ylcarbonyl)-amino]acetyl}amino)propanoyl]amino}-5-oxopentanoate (7). **7** was synthesized from **B** according to the general procedure for coupling with TBTU. Orange amorphous powder; yield, 380 mg (85%); mp 229−233 °C. ^{1}H NMR (DMSO-d_6): δ = 1.15 (t, 3H, J = 7.2 Hz, CH_2CH_3), 1.24 (d, 3H, J = 7.2 Hz, CH_3-Ala), 1.70−1.83 and 1.95−2.06 (2 m, 1H each, CH_2-β-iGln), 2.28 (t, 2H, J = 8.1 Hz, CH_2-γ-iGln), 3.93 (AB system, J = 3.3 Hz, J = 2.4 Hz, 2H, $NHCH_2CO$), 4.02 (q, 2H, J = 7.2 Hz, CH_2CH_3), 4.14−4.21 (m, 1H, CH-iGln), 4.27−4.36 (m, 1H, CH-Ala), 7.03 (dt, 1H, J = 6.8 Hz, J = 1.2 Hz, Ar-H), 7.11 (s, 1H, NH_2-iGln), 7.14 (d, 1H, J = 1.6 Hz, Ar-H), 7.18 (dt, 1H, J = 6.8 Hz, J = 1.2 Hz, Ar-H), 7.28 (s, 1H, NH_2-iGln), 7.43 (dd, 1H, J = 8.4 Hz, J = 0.8 Hz, Ar-H), 7.62 (d, 1H, J = 8.0 Hz, Ar-H), 8.12 (d, 1H, J = 8.4 Hz, NH-iGln), 8.18 (d, 1H, J = 6.8 Hz, NH-Ala), 8,76 (t, 1H, J = 5.6 Hz, $NHCH_2$), 11.55 (s, 1H, indole-NH). MS (ESI): m/z (%) = 446.2 (M + H)$^+$. IR (KBr): ν = 3424, 3302, 3058, 2981, 2366, 1733, 1653, 1630, 1595, 1569, 1544, 1426, 1366, 1342, 1316, 1246, 1212, 1182, 1059, 1021, 939, 816, 780, 747, 691 cm^{-1}. HPLC (254 nm): 96.8%, t_R = 15.92 min. HRMS calcd for $C_{21}H_{28}N_5O_6$ m/z, 446.2040 (M + H)$^+$; found, 446.2040. $[\alpha]_D^{20}$ +17.3° (c 0.18, MeOH).

4.4. Cell Culture. Human PBMC from healthy blood donors were isolated by density gradient centrifugation with Ficoll-Paque (Pharmacia, Sweden). The cells were cultured in RPMI 1640 (Sigma, Germany) supplemented with 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 2 mM L-glutamine (Sigma), 50 μM 2-mercaptoethanol (Sigma), and 10% heat-inactivated fetal bovine

serum (Gibco, U.S.). 1 ×10^6 cells were plated on 24-well culture plates (Nunc, Denmark) treated with a desmuramyldipeptide compound or medium alone, stimulated with 500 nM ionomycin and 3.33 ng/mL PMA, and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cell-free supernatants were collected at different time points (4, 18, and 40 h) and stored at −78 °C before being evaluated for cytokines by a commercially available kit.

4.5. Metabolic Activity Assay. PBMCs (1 × 10^6 cells/mL) were treated with the appropriate amounts of compounds or with the corresponding vehicle (control cells), then seeded in triplicate in 96-well plates. After 18 h metabolic activity was assessed using the CellTiter 96 Aqueous One solution cell proliferation assay (Promega; Madison, WI, U.S.), in accordance with the manufacturer's instructions.

4.6. Multiplexed Cytokine Assays. Cytokine release from PBMCs was measured in cell-free supernatants obtained by centrifugation at 1200 rpm for 5 min and stored at −78 °C until measurement. Flow cytometric analyses were performed using a FACSCalibur flow cytometer with sorting option: four-color and CELLQuest software (BD Biosciences; San Diego, CA, U.S.). Cytokine production was assessed by BD cytometric bead array (CBA) human inflammatory cytokine kit (content: IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12p70). Standard curves were generated using recombinant cytokines provided in the kit. The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Results are expressed in pg/mL.

4.7. Measurement of NF-κB Transcriptional Activity (Quanti-Blue Assay). HEK-Blue Nod 2 cells and Ramos-Blue cells (Invivogen; San Diego, CA, U.S.) were cultured in accordance with the manufacturer's instructions. HEK-Blue Nod 2 cells and Ramos-Blue cells were assayed for NF-κB transcriptional activity changes upon incubation (1 × 10^6 cells/mL) with murabutide and other Nod2 agonistic compounds (20 μM) for 18 h. Secreted embryonic alkaline phosphatase (SEAP) activity was determined in the supernatant in accordance with the manufacturer's instructions. An amount of 20 μL of cell supernatant was added to 200 μL of QUANTI-Blue reagent and incubated at 37 °C for 3 h. Absorbance was measured on a Tecan Safire 2 microplate reader (Reading, U.K.) at 640 nm.

4.8. Statistics. All experiments were performed at least twice, with average values expressed as the mean ± SD. Statistical significance was determined by the Dunnet multiple comparison test. Differences were considered significant for $p < 0.05$ and highly significant for $p < 0.01$.

■ ASSOCIATED CONTENT

Supporting Information

HPLC data of tested compounds and transcriptional activity of desmuramyldipeptides in Ramos Blue cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CSF, colony stimulating factor; HEK293, human embryonic kidney; iE-DAP, D-glutamyl-*meso*-DAP; IL, interleukin; MAPK, mitogen-activated protein kinase; MB, murabutide; MDP, muramyldipeptide; MTS, ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium); NF-κB, nuclear factor κB; NLR, nucleotide-oligomerization-domain-like receptor; Nod, nucleotide oligomerization domain; PAMP, pathogen-associated molecular pattern; PBMC, peripheral blood mononuclear cells; PGN, peptidoglycan; PMA, phorbol 12-myristate 13-acetate; PRR, pattern recognition receptor; RICK, receptor-interacting serine−threonine kinase; RIG, retinoic acid inducible gene; SAR, structure−activity relationship; SEAP, secreted embryonic alkaline phosphatase; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α

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