TLR1- and TLR6-independent Recognition of Bacterial Lipopeptides*

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Bacterial cell walls contain lipoproteins/peptides, which are strong modulators of the innate immune system. Triacylated lipopeptides are assumed to be recognized by TLR2/TLR1, whereas diacylated lipopeptides use TLR2/TLR6 heteromers for signaling. Following our initial discovery of TLR6-independent diacylated lipopeptides, we could now characterize di- and triacylated lipopeptides (e.g. Pam3C-SK₄, Pam3C-GNNDESIFKEK), which have stimulatory activity in TLR1- and in TLR6-deficient mice. Furthermore, for the first time, we present triacylated lipopeptides with short length ester-bound fatty acids (like PamOct2C-SSNASK₄), which induce no response in TLR1-deficient cells. No differences in the phosphorylation of MAP kinases by lipopeptide analogs having different TLR2-coreceptor usage were observed. Blocking experiments indicated that different TLR2 heteromers recognize their specific lipopeptide ligands independently from each other. In summary, a triacylation pattern is necessary but not sufficient to render a lipopeptide TLR1-dependent, and a diacylation pattern is necessary but not sufficient to render a lipopeptide TLR6-dependent. Contrary to the current model, distinct lipopeptides are recognized by TLR2 in a TLR1- and TLR6-independent manner.

The innate immunity is the first barrier against foreign pathogens, including bacteria. In contrast to the adaptive immune system, which recognizes specific epitopes with highly variable immune receptors coded by rearranged genes, the innate immune system recognizes pathogen-associated molecular patterns by genetically invariant receptors, so-called pattern recognition receptors (1). Toll-like receptors (TLR), belonging to the TLR/IL-1 receptor family, are such pattern recognition receptors of the innate immune system, which are evolutionary conserved (2). So far, 11 TLR have been described. For most of them, except TLR10, natural ligands have been identified. These include various proteins, lipopeptides (LP), lipoteichoic acid, lipopolysaccharides (LPS), and oligonucleotides (double-stranded RNA, single-stranded RNA, and DNA) (2). TLR are type I transmembrane receptors having an extracellular domain of leucine-rich repeats, which are thought to be involved in ligand recognition. The intracellular domain of TLR contains regions that are highly homologous to the intracellular domain of the IL-1 receptor, the so-called Toll/IL-1 receptor domain, which is involved in the recruitment of downstream signaling molecules.

Various microbial cell wall constituents have been reported to signal through TLR2. Prominent representatives are bacterial LP and lipoteichoic acid (3–8). Among the TLR2-dependent bacterial ligands, the LP are prime candidates for analyzing the structural requirements of ligands to address TLR2. LP are structural membrane components in various bacterial species exhibiting different molecular structures. In addition, the chemical synthesis of LP analogs provides a vast variety of chemically defined molecules (9, 10). Bacterial LP possess di-O-acylated S-(2,3-dihydroxypropyl)-cysteinyl residues N-terminally coupled to distinct polypeptides. Examples include the macrophage-activating lipopeptide from Mycoplasma fermentans (MALP2) (11), the Mycoplasma salivarius lipoprotein FSL-1, and the LP from the N terminus of the cytochrome subunit of the photoreaction center of Rhodopseudomonas viridis (12). The S-(2,3-dihydroxypropyl)-cysteine may be acylated with a third amide-linked fatty acid, as is the case for the LP from the cell wall of Escherichia coli (13) and its synthetic lipopeptide analog Pam₃C-SK₄ (14). Interestingly, human and murine cells show a species-specific difference in the TLR2-dependent recognition of distinct LP structures (15), which seems to be linked to the chain length of the O-acylated fatty acids (16).

Information on the structural factors determining the TLR2/TLR1 versus TLR2/TLR6 heteromer usage of a given ligand was obtained from experiments using cells of TLR2-, TLR6-, and TLR1-deficient mice. The diacylated MALP2 requires TLR2 and TLR6 for signaling, whereas the triacylated synthetic lipopeptide Pam₃C-SK₄ is able to induce activation of innate immune cells independently of TLR6 (17–20). These findings and additional investigations using various di- and triacylated LP finally led to the model according to which triacylated LP signal through TLR2/TLR1 heteromers, whereas diacylated LP signal through TLR2/TLR6 heteromers (2). However, we have found recently that not only triacylated LP, but also diacylated LP, like Pam₃C-SK₄ and the C-terminally elongated MALP2 derivative Pam₃C-GNNDESIFKEK-SK₄ (MALP2-SK₄), induce cell activation in a TLR6-independent manner. The absence of a long chain amide-bound fatty acid, therefore, is necessary but not sufficient to characterize a TLR6-dependent LP. The amino acid sequence of the peptide part of LP also takes part in the specificity of recognition by TLR2/TLR6 heteromers (21).

So far, for triacylated LP, no evidence for signaling through TLR2/TLR6 heteromers has been obtained, leading to the assumption that...
they signal through TLR2/TLR1 heteromers. However, the usage of TLR2/1 heteromers by LP has not been characterized thoroughly. We have now investigated in detail the structure-activity relationships of synthetic LP during signaling through TLR2/TLR1 and TLR2/TLR6 heteromers using cells from TLR1- and TLR6-deficient mice. For the interaction of LP, both the acyl residues as well as the N-terminal amino acid moiety of LP contribute to the specificity of recognition by TLR2/TLR1 and TLR2/TLR6 heteromers. For the first time, our investigations led to LP with ester-bound short chain length fatty acids, which do not induce signaling in TLR1-deficient mice. Interestingly, there are LP, which stimulate cells from TLR1- as well as TLR6-deficient mice, indicating that in native cells, TLR2 might be able to signal as homomers.

EXPERIMENTAL PROCEDURES

Reagents—Dulbecco’s modified Eagle’s medium, RPMI 1640, penicillin-streptomycin, L-glutamine, sodium pyruvate, and Hepes buffer were obtained from Invitrogen (Karlsruhe, Germany). Fetal calf serum (Linaris, Wertheim-Bettingen, Germany) was heat-inactivated before use. LPS from Salmonella enterica serovar Friedenau was a kind gift from Prof. Dr. H. Brade (Department of Immunology and Biochemical Microbiology, Research Center Borstel, Germany). This LPS preparation showed no TLR2-mediated activity. All synthetic LP were synthesized and analyzed by EMC microcollections GmbH (Tübingen, Germany). All synthetic LP used in this study are strictly TLR2-dependent as determined by the use of TLR2-deficient mice and HEK-TLR2 cells (data not shown). The chemical structures, denotations, names, and abbreviations of fatty acids and some synthetic LP analogs used in this study are summarized in Fig. 1.

TLR1- and TLR6-deficient Mice—TLR1- and TLR6-deficient mice and the corresponding wild-type mice were F2 interbred from the 129/C57BL/6 F1 strain and had been generated by gene targeting as described previously (19, 20). Mice at the age of 8–12 weeks were killed after CO2 anesthesia, and single cell suspensions were prepared from spleen and bone marrow. All animal experiments were approved by the “Ministerium für Umwelt, Naturschutz und Landwirtschaft, Schleswig Holstein.”

Cell Culture—Polyclonal activation of B-lymphocytes from murine spleen cells was performed as described (22). In brief, 4 × 10^6 spleen cells/200 μl in 96-well flat bottom cell culture dishes were stimulated for a total culture time of 48 h and incubated with [3H]thymidine for the final 24 h of culture. BMDM were differentiated from bone marrow cells through incubation with 100 ng/ml human macrophage colony-stimulating factor as described elsewhere (23). For stimulation with LP, BMDM were seeded at 3 × 10^6 cells/400 μl/well in 48-well cell culture dishes for 2 h and afterward stimulated with the indicated synthetic LP. After 18 h of culture, the concentration of IL-6 and/or tumor necrosis factor in the culture supernatants was determined by enzyme-linked immunosorbent assay (BIOSOURCE, Camarillo, CA).

Cell Lysis and Western Blot Analysis—Lysates of BMDM were obtained by lysing the cells in 50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1.5% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM NaVO4, 1 mM

![FIGURE 1. Structures, denotations, name, and abbreviations of fatty acid residues and some synthetic LP analogs used in this study. A, abbreviations, structures, and names of fatty acid residues used in this study. B, to illustrate the structure denotation of LP analogs used in this study, the structures of three synthetic LP analogs are given. The chemical structure of the scaffold with the four reactive groups is shown. R’ and R” represent the amide-bound fatty acid residues, R’ and R” represent the ester-bound fatty acid residues, and R’ represents the peptide moiety of the LP analogs.](image)

![FIGURE 2. Stimulation of murine spleen cells from TLR1- or TLR6-deficient mice by various TLR6-independent lipopeptide analogs. Murine spleen cells from wild-type, TLR1-, or TLR6-deficient mice were stimulated with synthetic LP at various concentrations, and the [3H]thymidine incorporation into the B-lymphocytes was determined after 48 h of culture. The results are expressed as mean ± S.D., n = 3. A, wild-type cells; ○, TLR1-deficient cells.](image)
FIGURE 3. Stimulation of murine spleen cells from TRL1- or TLR6-deficient mice by various MALP2-analogs. Murine spleen cells from wild-type, TLR1-, or TLR6-deficient mice were stimulated with synthetic MALP2-analogs at various concentrations, and the [3H]Tdr incorporation into B-lymphocytes was determined after 48 h of culture. The results are expressed as mean ± S.D., n = 3. A, wild-type cells; ○, TLR6-deficient cells. △, wild-type cells; ●, TLR1-deficient cells.

FIGURE 4. Stimulation of cells from TLR1-deficient and wild-type mice with various Pam3C-SSNASK4 analogs. Murine spleen cells and BMDM were stimulated with synthetic LP at various concentrations. [3H]Tdr incorporation into the B-lymphocytes was determined after 48 h of culture. The IL-6 and tumor necrosis factor (TNF) release into the culture supernatant by BMDM was measured by enzyme-linked immunosorbent assay after a culture period of 18 h. The results are expressed as mean ± S.D., n = 3. ●, wild-type cells; ○, TLR1-deficient cells.
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**RESULTS**

**TLR6- and TLR1-independent Diacylated LP**—Recently, we have found that several diacylated lipopeptides including Pam\(_{3}\)C-SK\(_4\) and MALP2-SK\(_4\) stimulate murine macrophages and B-lymphocytes in a TLR6-independent manner (21). Based on the assumption that TLR2 signals only as a heteromer in association with either TLR6 or TLR1, we hypothesized that these lipopeptides instead utilize TLR1 as the coreceptor and should therefore not activate cells derived from TLR1-deficient mice. To test this hypothesis, spleen cells from wild-type mice and TLR1- or TLR6-deficient mice were stimulated with these lipopeptides. Consistent with our previous observations, stimulation of spleen cells from wild-type mice and TLR6-deficient mice with Pam\(_{3}\)C-SK\(_4\) and MALP2-SK\(_4\) resulted in proliferation of B-lymphocytes (Fig. 2). Control stimulation with MALP2 confirmed the unresponsiveness of TLR6-deficient cells to MALP2, whereas the response to Pam\(_{3}\)C-SK\(_4\) was not affected by the absence of TLR6. However, surprisingly, stimulation of spleen cells of TLR1-deficient mice by Pam\(_{3}\)C-SK\(_4\) and MALP2-SK\(_4\) also induced a strong proliferation of B-lymphocytes despite the lack of TLR1. MALP2 led to equal responses in both TLR1-containing as well as TLR1-deficient cells, whereas the response to Pam\(_{3}\)C-SK\(_4\) was diminished to about 50% in TLR1-deficient B-lymphocytes (Fig. 2).

**Identification of Triacylated MALP2 Analogs That Are Neither TLR1- nor TLR6-dependent**—The diacylated MALP2 analog was converted into a TLR6-independent ligand by elongation of the peptide moiety. Recently, it has been described that a tripalmitoylated MALP2 analog, in contrast to the dipalmitoylated MALP2, also induces signaling in a TLR6-independent manner (18). Given the unexpected TLR1 independence of the diacylated MALP2-SK\(_4\), we therefore intended to investigate whether attachment of the third acyl chain rendered this TLR6-independent ligand TLR1-dependent, in accordance with the original concept of coreceptor usage of LP. To investigate in detail the role of the chain length of the amide-bound fatty acid for the TLR6 independence of MALP2 analogs, MALP2 analogs with a third, amide-bound acyl moiety ranging from acetic acid up to palmitic acid were synthesized. When testing these MALP2 analogs using B-lymphocytes from wild-type mice and TLR6-deficient mice, we found that already the attachment of acetic acid resulted in a weak response of TLR6-deficient cells, although only at a concentration of 1 \(\mu\)M (Fig. 3). Elongation of the amide-bound fatty acid to up to 10 carbon atoms (decanoic acid) increased the stimulatory activity only slightly. All the MALP2 analogs with a short chain length of the amide-bound fatty acid were strongly TLR6-dependent at a concentration of 100 \(nM\), which is sufficient to induce substantial signaling in B-lymphocytes of wild-type mice. However, stimulation of TLR6-deficient B-lymphocytes with MALP2 analogs having amide-bound fatty acids of 12, 14, or 16 carbon atoms at a concentration of 100 \(nM\) induced strong responses comparable with the response of 100 \(nM\) of MALP2 in wild-type cells (Fig. 3). This result underscores the influence of the amide-bound fatty acid of LP during recognition by TLR2/TLR6 heteromers. It is the current concept that LP are recognized either by TLR2/TLR6 or by TLR2/TLR1. To probe this concept, we also tested these MALP2 analogs in TLR1-deficient B-lymphocytes. Although triacylated MALP2 analogs are at least partially TLR6-independent, the stimulatory activity of these MALP2 analogs was comparable in TLR1-deficient and in wild-type cells (except for the N-decanoyl MALP2 analog exhibiting a somewhat reduced response in cells from TLR1-deficient mice) (Fig. 3). Therefore, we conclude that there are not only diacylated but also triacylated lipopeptides, which induce signaling in a TLR1- as well as TLR6-independent manner.

**The Chain Length of the Ester-bound Fatty Acids Determines the TLR1 Dependence of Signaling**—Although Pam\(_{3}\)C-SK\(_4\) is generally considered to signal through TLR2/TLR1 heteromers, this LP was also shown to have TLR1-independent properties (20). However, so far, no detailed analysis has been performed to determine the structural
requirements for triacylated LPs to signal in a TLR1-independent or -dependent manner. Having shown that for the MALP2 analogs, the chain length of the amide-bound fatty acid has great influence on the coreceptor usage, we next decided to systematically vary the chain length of the ester-bound fatty acids of Pam3C-SK4. In the absence of ester-bound fatty acids, LP were unable to signal through TLR2 (data not shown). Stimulation of B-lymphocytes of wild-type mice with N-palmitoyl LP analogs carrying two ester-bound fatty acids with chain lengths of 6 (Hex) to 16 carbon atoms (Pam) confirmed that ester-bound long-chain fatty acids are necessary to induce considerable responses in these cells (Fig. 4). The LP analog PamHex2C-SSNASK4 exerted only low stimulatory activity and PamOct2C-SSNASK4 and PamPel2C-SSNASK4 have moderate bioactivity, whereas PamDec2C-SSNASK4 up to PamPam2C-SSNASK4 were strong stimulators of B-lymphocyte proliferation. When testing these LP analogs in TLR1-deficient B-lymphocytes, no responses of the cells were observed after stimulation with LP analogs, having short and moderate length (6 up to 10 carbon atoms), of the ester-bound fatty acids (Fig. 4). PamDod2C-SSNASK4, PamMyr2C-SSNASK4, and PamPam2C-SSNASK4 stimulated TLR1-deficient B-lymphocytes, but just as for Pam3C-SK4, the response was reduced by about 50% in comparison with the responses in wild-type cells. A similar structure-activity relationship of these LP analogs was found for stimulation of IL-6 and tumor necrosis factor release in bone marrow-derived macrophages. No differences in the response of wild-type- and TLR6-deficient cells were observed after stimulation with these LP analogs (data not shown).

For Triacylated LP, the Structure of Both the Peptide Moiety and the Ester-bound Fatty Acids Determines the TLR1 Dependence—Our previous results indicated that the engagement of TLR6 for signaling by LP is not only determined by the acylation pattern but also by the amino acid sequence of the peptide moiety (21). We, therefore, investigated whether a modification of the peptide sequence of our TLR1-dependent LP collection, as described above, resulted in a loss of TLR1 dependence. First, we synthesized a diacylated PamOct2C LP analog with the

![Graph A](image1.png)

![Graph B](image2.png)

**FIGURE 6.** Stimulation of spleen cells from TLR1-deficient and wild-type mice with PamPel2C-lipopeptide analogs. Murine spleen cells were stimulated with MALP2 (100 nM) or with synthetic PamPel2C-lipopeptide analogs (1000 nM), and the [³H]Tdr incorporation into B-lymphocytes was determined after 48 h of culture. The results are expressed as mean ± S.D., n = 3. A, a comparison of PamPel2C-SSNASK4 analogs with PamPel2C-SK3 analogs. B, a comparison of PamPel2C-SSNASK4 analogs with PamPel2C-(VPGVG)₄VPGKG-NH₂ analogs. Black bars, wild-type cells; gray bars, TLR1-deficient cells.
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sequence SK₄ instead of SSNASK₄ of the peptide chain. Both PamOct₃C-SK₄ and PamOct₃SSNASK₄ stimulated wild-type cells equally well, and both analogs showed nearly no stimulation of TLR1-deficient B-lymphocytes (Fig. 5). Next, we compared a series of triacylated LP with an amide-bound Pam and ester-bound fatty acids of various chain lengths carrying peptide tails with the amino acid sequences SSNASK₄ or SK₄, or the (VPGVG)$_4$VPGKG-NH₂ peptide moieties. The latter 25-amino acid peptide is characterized by a high proportion of uncharged amino acids but has, nevertheless, a good solubility in water. All LP from these series showed a similar response pattern, independent of the amino acid sequence of the peptide tail. In particular, no response was detected in TLR1-deficient B-lymphocytes after stimulation with PamPelC-(VPGVG)$_4$VPGKG-NH₂, PamOct₃C-(VPGVG)$_4$VPGKG-NH₂, and PamDecC-(VPGVG)$_4$VPGKG-NH₂ (Fig. 6).

LP Using Different TLR2 Heteromers Induce the Same Downstream Signaling Events—At this stage, we have now defined synthetic LP that are entirely TLR1-dependent (e.g. PamOct₃C-(VPGVG)$_4$VPGKG-NH₂ and PamOct₃C-SSNASK₄) that are entirely TLR6-dependent (e.g. MALP2), and finally also LP analogs that are neither TLR1-nor TLR6-dependent (e.g. Pam₃C-SK₄) or only partially TLR1-dependent (e.g. Pam₃C-SK₄). These differences on the level of coreceptor usage raised the question of whether the different LP analogs also activate different signaling pathways. Therefore, we investigated the phosphorylation of the MAP kinases, p38, Erk, and JnK, in BMDM, which are involved in TLR-mediated signaling (24). For all four different LP analogs, all three MAP kinases were phosphorylated as early as 7.5 min after stimulation (Fig. 7). In contrast, 30 min after stimulation, nearly no phosphorylated MAP kinases were detected. Although there were some quantitative differences in the phosphorylation of the individual MAP kinases, there were no qualitative differences for the LP analogs. We also investigated whether one of these LP analogs may signal in a MyD88-independent manner. Consistent with the present knowledge on the MyD88 dependence of TLR2 signaling, p38 was not phosphorylated in BMDM of MyD88-deficient mice after stimulation with the LP analogs. In contrast, phosphorylation was detected after stimulation with LPs (Fig. 8).

For TLR1- and TLR6-dependent LP, TLR2 Alone Is Not a Functional Recognition Molecule—PamOct₃C-(VPGVG)$_4$VPGKG-NH₂ is a strong TLR2- and TLR1-dependent ligand, whereas MALP2 signals through TLR2 in a TLR6-dependent manner (19). In addition, Pam₃C-SK₄, which is a TLR1- and TLR6-independent ligand (21), stimulates cells of TLR1- as well as TLR6-deficient mice. Given the fact that for many heteromeric receptors, each receptor unit contributes a binding site for the ligand, we tested whether PamOct₃C-(VPGVG)$_4$VPGKG-NH₂ is able to interact with TLR2 in the absence of TLR1, which may lead to a blocking of the TLR2 receptor and subsequent inhibition of the activation of TLR1-deficient cells by MALP2 or Pam₃C-SK₄. BMDM from TLR1-deficient mice were first preincubated by PamOct₃C-(VPGVG)$_4$VPGKG-NH₂ and then stimulated with MALP2 or Pam₃C-SK₄. Preincubation with a TLR2/TLR1 ligand had neither an inhibitory nor a synergistic effect on the activation of BMDM with MALP2 or Pam₃C-SK₄ (Fig. 9). Vice versa, TLR6-deficient BMDM were preincubated with MALP2 and then stimulated with PamOct₃C-(VPGVG)$_4$VPGKG-NH₂ or Pam₃C-SK₄. Again, no inhibitory or synergistic effect was observed (Fig. 9).

DISCUSSION

TLR2 is a prominent receptor of the innate immune system that recognizes a variety of different molecules from bacterial and non-bacterial origin. So far, TLR2 among all other TLRs has been considered to possess a unique characteristic. It has been assumed that this receptor does not signal as a homomer but only as a heteromer in collaboration with either TLR1 or TLR6 (25). The original concept, according to which triacylated LP recognize TLR2/TLR1 heteromers, whereas diacylated LP recognize TLR2/TLR6 heteromers, has recently been confronted by our finding that diacylated LP, like Pam₃C-SK₄ and MALP2-SK₄, are able to stimulate cells from TLR6-deficient cells (21). Given the

FIGURE 8. Phosphorylation of p38 MAP kinase induced by various TLR1- and/or TLR6-independent lipopeptide analogs in macrophages from wild-type and MyD88-deficient mice. BMDM from wild-type and MyD88-deficient mice were stimulated with MALP2, Pam₃C-SK₄, PamOct₃C-SSNASK₄, and Pam₃C-SK₄ at 100 nM and with LPS (10 ng/ml). After various culture times, cell lysates were fractionated using SDS-PAGE and immunoblotted for pJNK, pERK, and pp38. The levels of total p38 were used for normalization. C, control.
should emphasize that Pam3C-SK4, which is entirely TLR6-independent, was not compensated by a TLR1 dependence. In addition, we found that triacylated MALP2 analog. Unexpectedly, for these analogs, the TLR6 independence was only partial. Our experiments revealed that the amide-bound fatty acid of various chain lengths, we found that an amide-bound fatty acid of 6, 8, 9, or 10 carbon atoms (Hex, Oct, Pel, or Dec) in combination with an amide-bound Pam (16 carbon atoms) were nearly unable to stimulate TLR1-deficient cells. It should be noted that PamC-SSNASK4, an LP with only one amide-bound Pam on the S-glyceryl-cysteine, is unable to exert any agonistic activity even in wild-type cells (data not shown). The peptide chain of these LP analogs seems to have only a marginal influence on the recognition by TLR2/TLR1. The triacylated LP collection with -SSNASK4 had a dose-response relationship similar to the one of the triacylated LP collections with -SK4, -SK5, or even a -(VPGVG)4VPGKG-NH2-peptide moiety. The (VPGVG)4VPGKG-NH2-peptide consists of mostly uncharged amino acids and, besides, shows only weak agonistic activity even in wild-type cells (data not shown). The peptide chain of these LP analogs seems to have only a marginal influence on the recognition by TLR2/TLR1. The triacylated LP collection with -SSNASK4 had a dose-response relationship similar to the one of the triacylated LP collections with -SK4, -SK5, or even a -(VPGVG)4VPGKG-NH2-peptide moiety. The (VPGVG)4VPGKG-NH2-peptide consists of mostly uncharged amino acids, and these LP analogs showed the lowest stimulatory activity in TLR1-deficient cells.

It would be of interest to confirm our results obtained with mouse knock-out cells in human cells, too. Human HEK293 cells are deficient for various TLRs, including TLR2, and commonly used for investigations of TLR-ligand recognition after transfection with the respective TLR. However, HEK293 cells are only deficient for TLR2 but express low but considerable amounts of native TLR1 and TLR6 and respond to Pam3C-SK4 and MALP2 after transfection with only TLR2 (Refs. 26–28 and our own observations). Therefore, this cell system is not suitable as a knock-out system for TLR1 nor for TLR6. Similar to native TLR1 and native TLR6 (data not shown). In addition, we and others recently found species-specific recognition of LP recognition by mice and human TLR2 (15, 16). In particular, PamOct2C-derivatives signal through TLR2/1 and, at least in part, through TLR2/6 in human SW620 cells. However, the results may also be inter-

### TABLE 1

| Lipopeptide analog | Response of cells from gene-deficient mice |
|--------------------|-------------------------------------------|
| PamC-SK4           | TLR1= +; TLR2= -; TLR6= +                 |
| PamC-SK5           | TLR1= +; TLR2= -; TLR6= +                 |
| PamC-SK6           | TLR1= +; TLR2= -; TLR6= +                 |
| PamOct2C-SK4       | TLR1= +; TLR2= -; TLR6= +                 |
| MALP2              | TLR1= +; TLR2= -; TLR6= +                 |
| MALP2-SK4          | TLR1= +; TLR2= -; TLR6= +                 |
| PamC-MALP2         | TLR1= +; TLR2= -; TLR6= +                 |
| PamC-SSNASK4       | TLR1= +; TLR2= -; TLR6= +                 |
| PamOct2C-SSNASK4   | TLR1= +; TLR2= -; TLR6= +                 |
| PamC-MALP2-(VPGVG)4VPGKG-NH2 | TLR1= +; TLR2= -; TLR6= + |

a +, response.  
b –, no response.  
c +-, strong response.
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pertrated such as these ligands may be, at least in part, TLR1- and TLR6-independent.

The different receptor requirements of LP led us to investigate whether equal signaling pathways were used by the various LP. It is the current opinion that after ligand binding, TLRs dimerize. This process results in a recruitment of downstream signaling molecules. Finally, MAP kinases are phosphorylated followed by a phosphorylation and ubiquitination of IKK and activation of the transcription factor NFκB (30). For TLR4 and TLR3, a MyD88-independent pathway for signaling has been described that results in the induction of expression of interferon-α/β (31, 32). To obtain first insights of whether signaling through different TLR2 heteromers and signaling in the absence of TLR1 and TLR6 led to the activation of different signaling pathways, we determined the phosphorylation of MAP kinases in response to various LP in wild-type and MyD88-deficient cells. First, our results provide evidence that all tested LP are MyD88-dependent. With respect to MAP kinases, no qualitative differences in the phosphorylation of these MAP kinases were detected. These results indicated that all of these LP, although addressing different TLR2 heteromers, use the same signal pathway during stimulation of cells. Recently, however, it has been reported that different synthetic LP, including Pam\(\text{C}-\text{SK}_{4}\) and Pam\(\text{C}-\text{SK}_{4}\), carry diverse adjuvant properties, which had been demonstrated by the induction of distinct patterns of cytokine and chemokine production and expression of the costimulatory surface molecule CD86 on dendritic cells (33). Furthermore, CD36, which is expressed on monocytes, macrophages, endothelial cells, and on some dendritic cells, has been found to facilitate the response to lipoteichoic acid and the \(\beta\)-enantiomer of MALP2 (34). These results indicate that in addition to TLR2 and its heteromers, additional accessory proteins may be involved in LP-induced signal transduction that affects the outcome of the signaling. Using TLR2/CD4 recombinant fusion proteins, it has been shown that all TLRs but not TLR2 are able to signal as homomers, whereas TLR2 forms heteromers with either TLR1 or TLR6 to attain specificity for a given stimulus (25). It remains to be shown whether the proteins mentioned above act as alternative coreceptors for those LP for which neither an involvement of TLR1 nor an involvement of TLR6 was shown. Moreover, it has been speculated that TLR2 may also form heteromers with TLR10 (35). However, this assumption has not been verified so far, and TLR10 is absent in the murine genome. Therefore, heteromerization of TLR2 with TLR10 is not an explanation of our results. We, therefore, like to speculate that native TLR2 is able to signal as homomer.

MALP2, Pam\(\text{Oct}_{2}\)-C-(VPGVG)\(_{4}\)VPKKG-NH\(_{2}\), and Pam\(\text{C}-\text{SK}_{4}\) all are TLR2-dependent LP. However, MALP2 is a TLR2/TLR6-dependent LP (19), Pam\(\text{Oct}_{2}\)-C-(VPGVG)\(_{4}\)VPKKG-NH\(_{2}\) is a TLR2/TLR1-dependent LP, whereas Pam\(\text{C}-\text{SK}_{4}\) is a TLR2-dependent but TLR1- and TLR6-independent LP. We addressed the question of whether MALP2 is able to interact with TLR2 in the absence of TLR6 and blocks stimulation of cells by TLR6-independent LP in this way. We found that MALP2 has no antagonistic activity in TLR6-deficient BMDM during stimulation with Pam\(\text{Oct}_{2}\)-C-(VPGVG)\(_{4}\)VPKKG-NH\(_{2}\) and Pam\(\text{C}-\text{SK}_{4}\) (Fig. 9). Vice versa, Pam\(\text{Oct}_{2}\)-C-(VPGVG)\(_{4}\)VPKKG-NH\(_{2}\) is unable to block the stimulation of TLR1-deficient BMDM with MALP2 and Pam\(\text{C}-\text{SK}_{4}\). This indicates that TLR6 is not only necessary to mediate MALP2-induced signaling but is also essential for the physical interaction of MALP2 with TLR2; vice versa, Pam\(\text{Oct}_{2}\)-C-(VPGVG)\(_{4}\)VPKKG-NH\(_{2}\) binds to TLR2 only in the presence of TLR1. We assume that the recognition by TLR2/TLR1, TLR2/TLR6, and TLR2 alone represents separate recognition pathways independent of each other.

Our results show the substantial importance of the amide-bound acyl residue, the two ester-bound acyl residues, and the amino acid sequence of the peptide chain within LP for the induction of signaling through TLR2 hetero- or homomers (Table 1). LP are amphiphilic molecules, and they are able to form, like LPS, supramolecular structures in aqueous media, at air-water interfaces, and in lipid bilayer membranes (36–40). We presented strong evidence that the molecular shape and the supramolecular conformation of LP, which is responsible for recognition by TLR2 polymers, is determined not only by the fatty acids but also by the amino acid sequence. Further experiments, however, will be required to resolve the relationship between structure and activity for the interaction of LP with TLR2/TLR6-polymers in detail.

LP with the \(N\)-acyl-S-diacylglyceryl cysteine backbone has been found in all bacteria. During bacterial infection, these LP may be involved in the initiation of the response of the innate immune system and activation/modulation of antigen-presenting cells including dendritic cells, thereby modulating the pattern of the response of the adaptive immune system. Therefore, it is reasonable to conclude that the structure of bacterial LP may play a role for the outcome of a bacterial infection. Because there seems to be no interplay between the different LP at the level of recognition by different TLR2 heteromers, a mixed infection with various bacteria or different LP from one bacteria may induce diverse patterns of response of the innate immune system.

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