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CUTTING EDGE

Cutting Edge: Preferentially the *R*-Stereoisomer of the Mycoplasmal Lipopeptide Macrophage-Activating Lipopeptide-2 Activates Immune Cells Through a Toll-Like Receptor 2- and MyD88-Dependent Signaling Pathway[1]

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Mycoplasmas and their membranes are potent activators of macrophages, the active principle being lipoproteins and lipopeptides. Two stereoisomers of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 (MALP-2) differing in the configuration of the lipid moiety were synthesized and compared in their macrophage-activating potential, the *R*-MALP being >100 times more active than the *S*-MALP in stimulating the release of cytokines, chemokines, and NO. To assess the role of the Toll-like receptor (TLR) family in mycoplasmal lipopeptide signaling, the MALP-2-mediated responses were analyzed using macrophages from wild-type, TLR2-, TLR4-, and MyD88-deficient mice. TLR2- and MyD88-deficient cells showed severely impaired cytokine productions in response to *R*- and *S*-MALP. The MALP-induced activation of intracellular signaling molecules was fully dependent on both TLR2 and MyD88. There was a strong preference for the *R*-MALP in the recognition by its functional receptor, TLR2. *The Journal of Immunology*, 2000, 164: 554–557.

Mycoplasmas are wall-less bacteria that occur as commensals or pathogens in animals and humans (1). Being wall-less, mycoplasmas lack the classical modulins such as LPS, lipoteichoic acid (LTA),[4] or murein fragments (reviewed in Ref. 2), yet they are potent activators of macrophages (3). A number of independent reports have identified this macrophage-activating material as lipoproteins (4–6) or lipopeptides (7, 8). One of these lipopeptides, the 2-kDa macrophage-activating lipopeptide-2 (MALP-2) from *Mycoplasma fermentans*, was biochemically fully characterized and has become available by synthesis (7). The lipid moiety has an asymmetric C atom at the 2 position. The formerly used synthetic MALP-2 was the *S*, *R* racemate and had a similar sp. act. as the natural compound acting at picomolar concentrations in vitro (7, 9 10).

Little is known about the signal pathways or the cell-surface receptors for MALP-2, except that MALP-2 activates the nuclear transcription factor NF-κB (11, 12). A new class of receptors of the innate immune system, the so-called Toll-like receptors (TLRs), was recently discovered (13–15), which recognize various bacterial cell-wall components such as LPS, peptidoglycan (PGN), LTA, and lipoproteins/lipopeptides (16–22). Overexpression of human TLR2 conferred responsiveness to various kinds of bacterial components (16, 17, 19–22). To investigate the in vivo roles of the TLR family in the recognition of bacterial components, we have generated TLR2-deficient and TLR4-deficient mice. A mutation in the TLR4 gene is responsible for the LPS hyporesponsiveness of C3H/HeJ mouse strain (18), and the deficiency results in lack of responsiveness to LPS and LTA (23, 24). In contrast, TLR2-deficient mice show impaired responsiveness to PGN, but normal responses to LPS (24). These observations indicate different respective specificities of TLR2 and TLR4 in the recognition of bacterial components. The TLR family, whose cytoplasmic domain is homologous to that of IL-1R, has been shown to interact with an adapter molecule, MyD88, for the activation of IL-1R-associated kinase (IRAK) (25). Ultimately, NF-κB translocates from the cytoplasm to the nucleus and activates genes with NF-κB binding sites in their promoters. We have previously shown that

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[4] Abbreviations used in this paper: LTA, lipoteichoic acid; TLR, Toll-like receptor; MALP, macrophage-activating lipopeptide; PGN, peptidoglycan; IRAK, IL-1R-associated kinase; JNK, c-Jun N-terminal kinase

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MyD88-deficient mice are unresponsive to LPS, IL-1, and IL-18 (26, 27).

To assess the role of TLR family and MyD88 in mycoplasmal lipopeptide signaling, we analyzed MALP-2-mediated responses using two steroisomers of MALP-2 and macrophages from TLR2-, TLR4-, and MyD88-deficient mice. We will show that there is a stringent requirement of the correct stereochemistry in the lipid moiety for the recognition of MALP by its functional receptor. We will further show that this receptor is TLR2, which transfers its signal via MyD88.

Materials and Methods

Stereospecific synthesis and HPLC purification of R- *and* S-*MALP-2*

The stereoisomers of *S*-(2,3-dihydroxypropyl)-L-cystein were synthesized as outlined by Metzger et al. (28) using (*S*)-(-)-glycidol and (*R*)-(+)-glycidol, respectively, obtained from Sigma-Aldrich (St. Louis, MO), as starting materials. According to the supplier, these reagents contained >99% of the respective pure enantiomers. The N_α-fluorenylmethoxycarbonyl-protected *S*-(2(*S*),3 bis(palmitoyloxy)propyl]-L-cystein or *S*-(2(*R*),3-bis(palmitoyloxy)propyl]-L-cystein isomers, respectively, were synthesized and coupled to the carrier-bound fluorenylmethoxycarbonyl-protected peptide as described (28). It is important to point out that, although the configuration of the asymmetric carbon atom of the glycidol remains the same during this procedure, its designation changes from *S* to *R* and vice versa because of the Cahn Ingold-Prelog rules of assigning priorities to substituents according to their atomic mass. Crude MALP-2 was further purified in 10-mg batches by reversed phase HPLC on a SP 250/10 Nucleosil 300-7 C8 column (Macherey & Nagel, Düren, Germany) and was eluted at 40°C with a linear water/2-propanol gradient containing 0.1% TFA. Elution of active material was monitored by the NO release assay (7). The final product was characterized by mass spectroscopy and amino acid analysis by which also the exact peptide content was determined. MALP-2 was kept as a stock solution of 1 mg/ml in water/2-propanol 1/1 (v/v) at 4°C. For in vitro use, stock solutions were first diluted with 25 mM octyl glucoside in saline to provide a carrier and optimal solubilization and were then further diluted with culture medium. The maximal final detergent concentration in these studies was adjusted to 25 μM in all cultures and had no effects on the cells.

Mice

C3H/HeJ endotoxin low-responder mice were from The Jackson Laboratory (Bar Harbor, ME). The mutant mouse (F_2 interbred from 129/Ola × C57BL/6) strains deficient in TLR2, TLR4, or MyD88 were generated by gene targeting as described previously (23, 24, 26). Age-matched groups of wild-type, TLR2-, TLR4-, and MyD88-deficient mice were used for the experiments.

Cell culture and macrophage/monocyte stimulation assays

Adherent cells from either resident peritoneal exudate cell (PEC) from C3H/HeJ endotoxin low-responder mice or from thioglycollate-elicited PEC from the 129/Ola × C57BL/6 wild-type or mutant strains were used as source of murine macrophages. Nonadherent cells were removed, and fresh medium, DMEM, 5% FCS, 2.5×10^{-5} M 2-ME, with or without stimulants were added. Human monocytes from healthy volunteers were prepared by elutriation and stimulated as described (10). Samples for assaying cytokines, chemokines, or NO were removed after the indicated times. TNF-α was tested in a cytotoxicity assay as described (9) or by ELISA (Genzyme, Cambridge, MA), IL-6 was determined in a capture ELISA (9). NO release was assayed as described (7) or using an NO_2/NO_3 assay Kit-C (Dojindo, Kumamoto, Japan). Concentrations of IL-8 and monocyte chemoattractant protein-1 were measured by ELISA as described previously (10).

In vitro kinase assay and Western blotting

Peritoneal macrophages (1×10^6) were stimulated with 0.3 ng/ml of *R*-MALP for 10 min. The cells were lysed with lysis buffer and immunoprecipitated with anti-IRAK Ab. The IRAK activity was measured by in vitro kinase assay as described previously (27). Anti-IRAK Ab was kindly provided by Hayashibara Biochemical Laboratories (Okayama, Japan). For determination of c-Jun N-terminal kinase (JNK) activity, cell lysates were immunoprecipitated with anti-JNK1 Ab, and the in vitro kinase assay was performed using GST-c-Jun as substrate as described previously (27). The cell lysates were applied to SDS-PAGE and transferred to a nitrocellulose membrane. IRAK and JNK were detected with anti-IRAK (Transduction Laboratories, Lexington, KY) or anti-JNK1 Ab.

EMSA

Peritoneal macrophages (2×10^6) were stimulated with 0.3 ng/ml of *R*-MALP for the indicated periods. Nuclear extracts were prepared from these cells and incubated with a ^{32}P-labeled specific probe for NF-κB DNA binding site. Samples were electrophoresed and visualized by autoradiography as described previously (26).

Results and Discussion

Biological activity of MALP-2 depends on the stereochemistry of the lipid moiety

Natural MALP-2 isolated from a high-producer clone of *M. fermentans* shows a similar sp. act. as that of racemic synthetic MALP-2 (7). If we assume that the biosynthesis of mycoplasmal lipoproteins in principle proceeds like in *Escherichia coli* (29), the entire lipid moiety from phosphatidylglycerol is transferred to the prolipoprotein. Natural phosphatidylglycerol has the *S* configuration at the 2 position of the fatty acid-substituted glycerol. Because according to the Cahn-Ingold-Prelog rules the assignment of substituents of an asymmetric atom depends on the atomic mass of the neighboring substituents, the designation changes from *S* to *R* when the lipid moiety is transferred to the lipoprotein. Therefore,

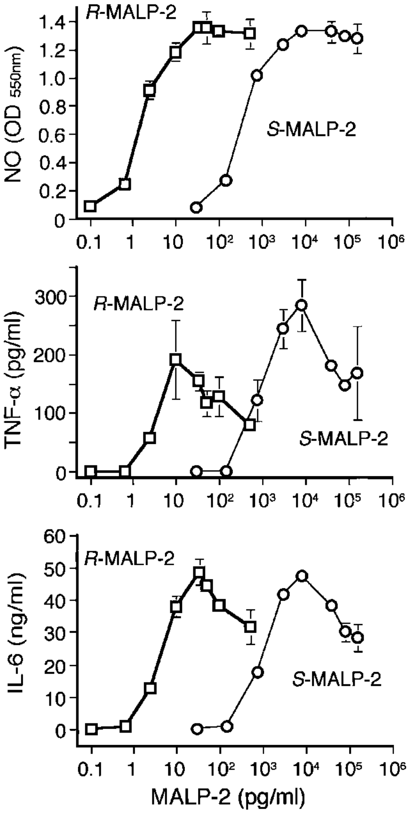

FIGURE 1. C3H/HeJ macrophages show a different dose response to *R*- and *S*-MALP stereoisomers. A total of 6×10^5 PEC were seeded in 1.25 ml DMEM, 5% FCS, 2.5×10^{-5} M 2-ME into 24-well cell culture plates, and macrophages were allowed to adhere during an overnight incubation. Nonadherent cells were removed and fresh medium with or without stimulants were added. The cultures were simultaneously stimulated with rIFN-γ to determine the release of TNF-α, IL-6, and NO from identical cultures. Samples for assaying cytokines, chemokines, or NO were removed after 3, 21, or 46 h, respectively. Results represent values from duplicate cultures ± SD.

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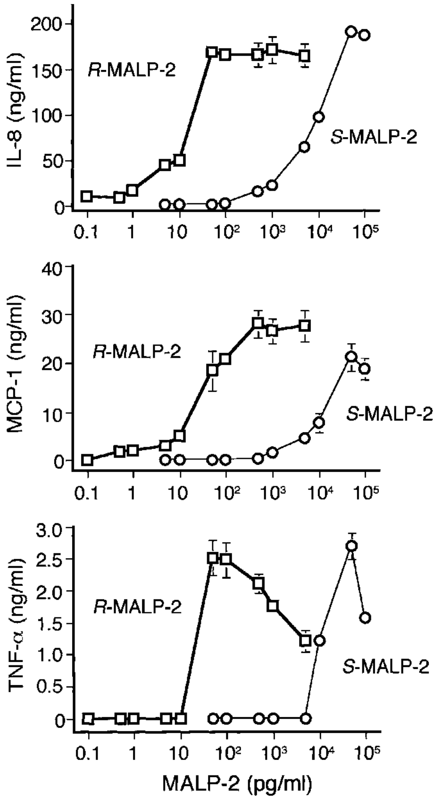

FIGURE 2. Human monocytes show a different dose response to *R*- and *S*-MALP stereoisomers. A total of 7.5 × 10^5 elutriated human monocytes were stimulated for 20 h with the indicated concentrations of the MALP stereoisomers. Cytokine and chemokine levels were determined by specific ELISAs. Results represent values from duplicate cultures ± SD.

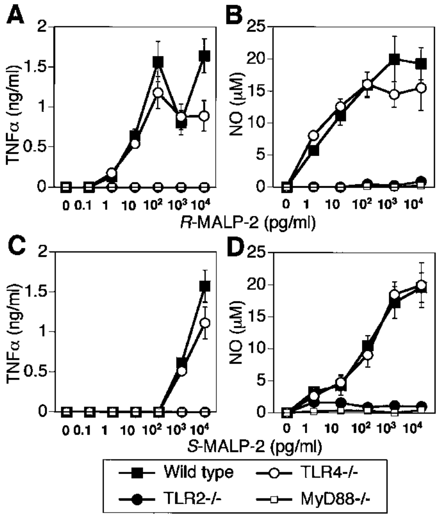

FIGURE 3. TLR2- and MyD88-deficient mice are unresponsive to both *R*- and *S*-MALP. Peritoneal macrophages from wild-type, TLR2-, TLR4-, and MyD88-deficient mice were cultured with the indicated amount of *R*-MALP (*A* and *B*) or *S*-MALP (*C* and *D*) in presence (*B* and *D*) or absence (*A* and *B*) of IFN-γ (30 U/ml) for 24 h. Concentrations of TNF-α (*A* and *C*) and NO (*B* and *D*) in the culture supernatants were measured by ELISA.

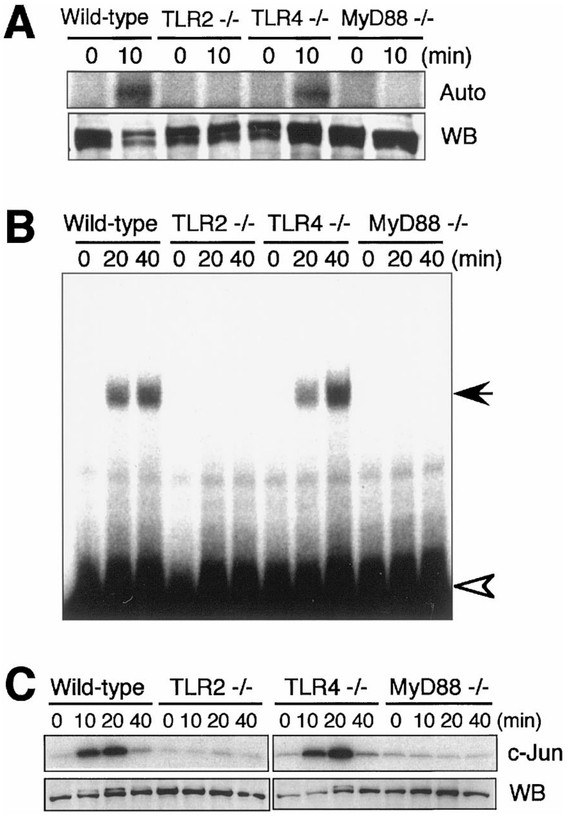

FIGURE 4. R-MALP-induced activation of IRAK, NF-κB, and JNK is abrogated in TLR2- and MyD88-deficient macrophages. *A,* Peritoneal macrophages were stimulated with 0.3 ng/ml of *R*-MALP for 10 min. Then cells were lysed and immunoprecipitated with anti-IRAK-1 Ab. IRAK activity was determined by in vitro kinase assay (*upper panel*). The same lysates were immunoblotted with anti-IRAK Ab (*lower panel*). *B,* Peritoneal macrophages were exposed with 0.3 ng/ml of *R*-MALP for indicated periods. The nuclear extracts were prepared and incubated with a radiolabeled specific probe containing NF-κB binding site. NF-κB activation was analyzed by EMSA. An arrow indicates induced NF-κB complex. Free probes are indicated by an arrowhead. *C,* Peritoneal macrophages were stimulated with 0.3 ng/ml of *R*-MALP for indicated periods. Then cell lysates were prepared and immunoprecipitated with anti-JNK1 Ab. The kinase activity of JNK was measured by in vitro kinase assay using GST-c-Jun as the substrate. Auto, Autophosphorylation. WB, Western blotting.

natural MALP-2 is expected to have the *R* configuration. A comparison of the biological activities of *R*- and *S*-MALP-2 indeed shows that *R*-MALP exhibits a much higher sp. act. than its *S* counterpart. This appears to be valid for murine as well as human MALP-2-reactive cells (Figs. 1 and 2). In fact, a contamination of *S*-MALP with the *R* isomer resulting from <1% impurity of the starting material would explain the remaining activity of the *S*-MALP-2, which in its pure form may be quite inactive. The data suggest that a putative MALP-2 receptor is capable to specifically recognize the configuration of the lipid moiety and to discriminate between the *R* and *S* stereoisomers. This is in keeping with previous observations that the peptide moiety, as long as solubility is ensured, is of little if any consequence for the macrophage stimulatory activity of lipopeptides (see also Ref. 8).

Identification of the MALP receptor

To identify a putative cell-surface receptor responsible for the signaling of MALP-2, we examined the responsiveness of peritoneal macrophages from wild-type, TLR2-, TLR4-, and MyD88-deficient mice to *R*-MALP and *S*-MALP. Peritoneal macrophages from wild-type and TLR4-deficient mice produced comparable amount of TNF-α or NO in a dose-dependent manner. In contrast, macrophages from TLR2- and MyD88-deficient mice produced neither TNF-α nor NO (Fig. 3, *A* and *B*). Similar results were

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obtained using *S*-MALP as the stimulants, although much higher doses were required (Fig. 3, *C* and *D*). IL-6 production in response to *R*- and *S*-MALP was also abrogated in TLR2- and MyD88-deficient macrophages (data not shown). These results clearly demonstrate that a TLR2-dependent signaling pathway is essential for the cellular responses to mycoplasmal lipoproteins/lipopeptides, exemplified by *R*-MALP-2.

Signaling pathway of MALP-2

We next examined the downstream signaling pathway of MALP-2. Triggering of the TLR/IL-1R family signaling cascade requires the recruitment of MyD88 to the receptor complex, which then activates NF-κB and JNK, via IRAK (25, 27). As shown in Fig. 3, *A* and *B*, cytokine productions from MyD88-deficient macrophages were severely impaired compared with those from wild-type macrophages. We then analyzed the kinase activity of IRAK by an in vitro kinase assay using these mutant macrophages. Autophosphorylation of IRAK was observed in wild-type and TLR4-deficient macrophages after the treatment with *R*-MALP. In contrast, IRAK activation was abrogated in TLR2- and MyD88-deficient macrophages (Fig. 4*A*). We further examined the MALP-2-induced NF-κB activation. In wild-type and TLR4-deficient macrophages, NF-κB was activated within 20 min of MALP treatment. In contrast, no NF-κB activation was detected in TLR2- or MyD88-deficient macrophages (Fig. 4*B*). Finally, we determined whether or not MALP-induced JNK activation was also TLR2- and MyD88-dependent. As shown in Fig. 4*C*, JNK was activated in wild-type and TLR4-deficient macrophages. Again in TLR2- or MyD88-deficient macrophages no JNK activation was observed.

It is interesting to compare the intracellular signaling pathways of LPS on the one hand and of MALP on the other. Whereas in TLR4-deficient macrophages neither NF-κB activation nor cellular responses were observed in response to LPS (23, 24), MyD88-deficient macrophages displayed a significant activation of both NF-κB and JNK in response to LPS, although the LPS-mediated cellular responses were also completely abrogated (27). In contrast, the MALP-induced intracellular signaling pathway was fully dependent on both TLR2 and MyD88. These results suggest that the signaling pathways of TLR2 and TLR4 diverge, in spite of great similarity.

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