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Mycoplasma fermentans Lipoprotein M161Ag-Induced Cell Activation Is Mediated by Toll-Like Receptor 2: Role of N-Terminal Hydrophobic Portion in its Multiple Functions

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M161Ag is a 43-kDa surface lipoprotein of Mycoplasma fermentans, serving as a potent cytokine inducer for monocytes/macrophages, maturing dendritic cells (DCs), and activating host complement on affected cells. It possesses a unique N-terminal lipopeptide, 5-diacylglyceryl cysteine. The 2-kDa macrophage-activating lipopeptide-2 (MALP-2), recently identified as a ligand for Toll-like receptor 2 (TLR2), is derived from M161Ag. In this study, we identified structural motifs sustaining the functions of M161Ag using wild-type and unlipitated rM161Ag with (SP⁺) or without signal peptides (SP⁻). Because the SP⁺ rM161Ag formed dimers via C25S, we obtained a monomeric form by mutagenesis (SP⁻C25S). Only wild type accelerated maturation of human DCs as determined by the CD83/86 criteria, suggesting the importance of the N-terminal fatty acids for this function. Wild-type and the SP⁻ form of monomer induced secretion of TNF-α and IL-12 p40 by human monocytes and DCs. Either lipid or signal peptide at the N-terminal portion of monomer was required for expression of this function. In contrast, murine macrophages produced TNF-α in response to wild type, but not to any recombinant form of M161Ag, suggesting the species-dependent response to rM161Ag. Wild-type and both monomorphic and dimeric SP⁻ forms possessed the ability to activate complement via the alternative pathway. Again, the hydrophobic portion was associated with this function. These results, together with the finding that macrophages from TLR2-deficient mice did not produce TNF-α in response to M161Ag, infer that the N-terminal hydrophobic structure of M161Ag is important for TLR2-mediated cell activation and complement activation. The Journal of Immunology, 2001, 166: 2610–2616.

The innate immune responses against infectious pathogens precede the cell-mediated immunity (1, 2). The cells of the innate immune system recognize constituents of microbes by specific receptors, which transmit signals into the cells (1, 2). Recently, mammalian Toll-like receptors (TLRs)³ were cloned and identified as signal-transducing molecules involved in innate immune defense (3–6). TLR2 and TLR4 are mainly implicated in the recognition of various bacterial components. TLR4 is involved in the recognition of Gram-negative bacterial LPS and Gram-positive bacterial lipoteichoic acids, while TLR2 recognizes Gram-positive bacterial peptidoglycans, zymosan, and several bacterial lipoproteins (7–18). Bacterial lipoproteins are characterized by a unique NH₂-terminal lipopeptide, N-acyl-5-diacylglyceryl cysteine (19), and this lipid element and peptide moieties are critical for cell activation through TLR2 (15–17).

TLRs consist of an extracellular domain with leucine-rich repeats and a C-terminal flanking region, and a cytoplasmic domain with sequence homology to the type I IL-1R termed a Toll/IL-1R domain (3). The extracellular leucine-rich repeat domains are involved in the recognition of bacterial products, and the cytoplasmic domains trigger activation of NF-kB, p38 mitogen-activated protein kinase, and Jun N-terminal kinase, leading to the induction of proinflammatory genes (20).

Mycoplasma fermentans, a human pathogen, is a potent activator of monocytes/macrophages. Several studies demonstrated the ability of this mycoplasma to enter human cells and the possibility that it acts as accessory factors in the activation of AIDS (21–23). M. fermentans DNA has been detected in the PBMCs of AIDS patients by PCR (24, 25). In addition, the products of M. fermentans affect the host immune system via B or T cell activation, monocyte/macrophage stimulation, and cytotoxic ability, which are not restricted to human cells (26–29). The ability of M. fermentans to modulate host immune responses may contribute to its pathogenic property.

M161Ag is an unglycosylated 43-kDa membrane lipoprotein of M. fermentans capable of inducing proinflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, IL-10, and IL-12) by human monocytes/macrophages and activating human complement on mycoplasma-infected cells (30–32). It possesses a unique NH₂-terminal lipopeptide, 5-diacylglyceryl cysteine, followed by 403 aa,
including five tryptophan residues encoded by TGA codons (31, 33). Biosynthetic labeling of cells revealed the palmitoylation of M161Ag (31). The N-terminal 14 aa of M161Ag were quite similar to those of macrophage-activating lipopeptide (MALP)-2 (29). Later, this molecule was demonstrated to be a proteolytic cleavage product of MALP-404, which is identical with M161Ag (34).

Interestingly, Lien et al. (35) reported that a synthetic dipalmitoyl lipopeptide based upon the full-length MALP-2 (sMALP-2) was dimerized across the cysteine residue during purification with a Ni2+ column (see Fig. 1). The rM161Ag contained <1 endotoxin U/μg, as determined by modified Limulus amebocyte assay (Seikagaku, Tokyo, Japan). Native and rM161Ag were treated with polymyxin B (5 μg/ml) for 1 h at 37°C before stimulation of the cells.

DNA cloning and NF-κB reporter assay

The coding region of human TLR2 minus the respective NH2-terminal signal sequence was amplified by RT-PCR from total RNA isolated from human monocyte-derived iDCs. The predicted amino acid sequence matched that previously published (5). The Flag-TLR2 expression vector was constructed by inserting the cDNA fragment into the mammalian expression vector pFlag-CMV-1 (Sigma) at HindIII and KpnI sites, in which the preprotrypsin leader sequence precedes an NH2-terminal Flag epitope. The dominant-negative version of human TLR2 plasmid (pFlag-TLR2-DN), which lacks C-terminal 155 aa of human TLR2, was provided by Dr. R. Medzhitov (Yale University School of Medicine, New Haven, CT). The plasmids were prepared with an endotoxin-free Plasmid Maxi kit (Qiagen, Valencia, CA).

THP-1 cells (2.5 × 104) were transfected with a mixture of DNA (1.5 μg) using Effectene (50 μl; Qiagen) in a 60-mm dish. The DNA mix consisted of NF-κB reporter plasmid encoding luciferase (0.5 μg; Stratagene) and control pFlag-CMV-1 vector (1 μg) or pFlag-TLR2-DN vector (1 μg). Twenty-four hours after transfection, cells were harvested, seeded into 24-well plate (5 × 104/well), and stimulated with medium alone, polymyxin B-treated native M161Ag (5 ng/ml), and rM161Ag (SP’ C25S) (1 μg/ml) for 6 h. The cells were lysed using reporter lysis buffer (PicaGene; Toyo Ink, Tokyo, Japan), and 20 μl of lysate was assayed for luciferase activity according to the manufacturer’s instructions.

Cytokine assay

Human monocytes or iDCs (1 × 106/ml) were stimulated with polymyxin B-treated native (5 ng/ml) and unlipidated rM161Ag (10, 100, and 1000 ng/ml) for 24 h. For control stimulation, polymyxin B-treated and untreated LPS (10 ng/ml) were used. Concentrations of TNF-α in culture supernatants were measured by ELISA (Amersham Pharmacia Biotech AB). Production of IL-12 p40 was also measured by ELISA (Genzyme, Cambridge, MA). In the case of THP-1 cells, cells (5 × 104) were stimulated with medium alone, polymyxin B-treated native M161Ag (5 ng/ml), and rM161Ag (SP’ C25S) (1 μg/ml) for 24 h. Concentration of IL-8 in culture supernatants was measured by ELISA (Amersham Pharmacia Biotech AB).

DC maturation

iDCs (1 × 106/ml) were stimulated with polymyxin B-treated native M161Ag (5 ng/ml) and unlipidated rM161Ag (1 μg/ml), or LPS (10 ng/ml) for 24 h. After 24 h, cells were harvested and the expression levels of CD83 and CD86 were analyzed by flow cytometry using FACS Calibur (Becton Dickinson, San Jose, CA). Production of IL-12 p40 and TNF-α (data not shown) was measured by ELISA.

MALDI-MS and circular dichroism (CD) measurements

Mass-spectrometric measurements were performed using a Voyager Elite XL time of flight mass spectrometer equipped with a delayed extraction system (PE Biosystems, Foster, CA) with flight paths of 4.2 and 6.5 m for...
the linear mode and reflector mode, respectively. Protein or peptide solutions (about 1 µl, containing 1–5 pmol) were mixed with the matrix solution, the supernatant of a 50% or a 33% acetonitrile/water solution saturated with α-cyano-4-hydroxycinnamic acid or sinapinic acid, respectively, and then air dried on the flat surface of a stainless steel plate. Other measurement conditions were as described previously (40).

CD measurements were performed with a Jasco spectropolarimeter, model J-720. The temperature was controlled at 20°C with a thermostatically controlled cell holder. Spectra were measured with a 1-mm cell at a protein concentration of 0.2 mg/ml (43). The instrument was calibrated with ammonium d-10-camphorsulfonic acid. The results were expressed as the mean residue ellipticity, [θ], which was defined as [θ] = 1000 θd/λc, in which θd is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters.

Results

Three kinds of unlipidated rM161Ag with or without SP are shown in Fig. 1. Comparative analysis between native and recombinant M161Ag was performed using murine macrophages lacking either TLR2 or TLR4. Peritoneal macrophages from wild-type, TLR2-deficient, and TLR4-deficient mice were cultured with various polymyxin B-treated rM161Ag and native M161Ag, or LPS for 24 h, and the production of TNF-α was measured. Macrophages from wild-type and TLR4-deficient mice secreted TNF-α in response to native M161Ag (Fig. 2). In contrast, TLR2-deficient macrophages did not produce TNF-α, indicating that M161Ag mediates cell activation through TLR2 similarly to MALP-2 (35, 36). The lipid moiety of M161Ag participates in cell activation, because rM161Ag lacking fatty acids could not induce TNF-α production by macrophages isolated from wild-type or TLR4-deficient mice (Fig. 2).

In contrast, human monocytes secreted TNF-α and IL-12 p40 in response to monomeric form of rM161Ag with signal peptide (SP+ C25S), when its concentration was sufficiently high (Fig. 3). The sample of the SP+ C25S contained no lipid-attached form based on the mass-spectrometric analysis, excluding the possibility that a lipid-attached form contributes to the cytokine-inducing activity. In contrast, dimeric form of rM161Ag (SP+) and rM161Ag without signal peptide (SP-) did not induce release of TNF-α or IL-12 p40 by human monocytes. Because these activities were not abolished by treatment with polymyxin B sulfate, which completely destroyed LPS activity, cytokine production was induced by the unlipidated monomeric form of rM161Ag and not by contaminating endotoxin.

These results on human and murine cells suggested the species-dependent recognition of rM161Ag by TLR2. We then performed NF-κB reporter gene assay using THP-1 cells to confirm TLR2-mediated cell activation by rM161Ag (SP+ C25S). Human monocytic THP-1 cells endogenously express TLR2 (44) and secreted IL-8 in response to native M161Ag and rM161Ag(SP+ C25S), similar to human monocytes (Fig. 4A). As shown in Fig. 4B, activation of NF-κB induced by native and rM161Ag was decreased
in THP-1 cells transiently transfected with the DN version of human TLR2. These results indicate that rM161Ag (SP\(^{1}C25S\)) induces cell activation through TLR2. Thus, human and murine TLR2 differentially recognize unlipidated rM161Ag.

We next examined the responses of human monocyte-derived iDCs to native and unlipidated rM161Ag, because bacterial products such as LPS induce iDC maturation as well as IL-1\(\beta\) or TNF-\(\alpha\) (37, 45–47). Native M161Ag up-regulated the expression of CD83 and CD86 and IL-12 p40 production in iDCs, suggesting that M161Ag is a DC maturation inducer similarly to LPS (Figs. 5 and 6). In contrast, the monomeric form of rM161Ag with SP did not affect CD83 expression in iDCs, but induced IL-12 P40 production. Again, rM161Ag without SP and dimeric form of rM161Ag with SP had no effect on iDCs. When stimulated with LPS, iDCs produced large amounts of IL-12 p40 (Fig. 5) and IL-12 p70 (300–400 pg/ml, data not shown), while native and monomeric forms of rM161Ag could not induce IL-12 p70 production (data not shown). These results suggested that the lipid moiety of M161Ag is critical for TLR2-mediated cytokine production by murine macrophages, but not by human monocytes/iDCs. They also showed that, in human iDCs, cytokine production and CD83/CD86 expression were independently induced by M161Ag through TLR2.

Interestingly, complement-activating ability was reserved in rM161Ag with SP, but not in rM161Ag without SP (Fig. 7). When blotted onto nitrocellulose sheets, native M161Ag and both monomeric and dimeric forms of rM161Ag with SP activated complement via the alternative pathway, followed by deposition of C3 fragments after incubation with Mg\(^{2+}\)-EGTA-NHS. In contrast, rM161Ag without SP did not. Again, higher doses of rM161Ag with SP (>0.5 \(\mu\)g) were required for complement activation than those of native form (Fig. 7). In contrast, sMALP-2 and LPS did not induce C3 deposition on itself in the range of 1–100 ng (Fig. 7, data not shown). No C3 fragments bound to native or rM161Ag were detected after treatment with EDTA-NHS (data not shown).

Major structural difference between bioactive (SP\(^{1}C25S\)) and inactive (SP\(^{2}\)) rM161Ag relied on the presence or absence of SP (Fig. 1). To test the possibility that the presence of N-terminal hydrophobic SP affects the tertiary structure of M161Ag, CD spectra of these rM161Ag were measured. As shown in Fig. 8, there were no differences in CD spectra between bioactive and inactive rM161Ag, suggesting that bioactivity of rM161Ag is dependent on the N-terminal hydrophobic SP and not the tertiary structure of rM161Ag.

**Discussion**

In this study, we focused on the structural motifs sustaining the functions of mycoplasma lipoprotein M161Ag and its signaling through TLR. The findings were as follows: 1) The lipid moiety of M161Ag is critical for TLR2-mediated cytokine production by murine macrophages; 2) in contrast, monomeric form of rM161Ag with an N-terminal hydrophobic portion, either lipid or SP, is required for induction of TNF-\(\alpha\) and IL-12 p40 by human monocytes.

**FIGURE 4.** Unlipidated monomeric form of rM161Ag with SP induces cell activation through TLR2. A. THP-1 cells secrete IL-8 in response to M161Ag and unlipidated rM161Ag. Cells (5 \(\times\) 10\(^5\)) were stimulated with medium alone, polymyxin B-treated native M161Ag (5 ng/ml), and rM161Ag (SP\(^{1}C25S\)) (1 \(\mu\)g/ml) for 24 h. The concentrations of IL-8 in culture supernatants were measured by ELISA. B. NF-\(\kappa\)B activation induced by native and rM161Ag was reduced in THP-1 cells expressing TLR2-DN. THP-1 cells were transiently cotransfected with NF-\(\kappa\)B reporter plasmid encoding luciferase (0.5 \(\mu\)g) and either pFlag-CMV-1 empty vector (pFlag; 1 \(\mu\)g) or pFlag-TLR2-DN vector (TLR2-DN; 1 \(\mu\)g). After 24 h, the cells were stimulated with medium alone, polymyxin B-treated native M161Ag (5 ng/ml), and rM161Ag (SP\(^{1}C25S\)) (1 \(\mu\)g/ml) for 6 h, and NF-\(\kappa\)B activation was measured by luciferase activity in cell lysates and normalized to protein content. Determinations were performed in duplicate, and results are expressed as means ± SD. Results are representative of three separate experiments.

**FIGURE 5.** Unlipidated monomeric form of rM161Ag with SP induces IL-12 p40 production by human iDCs. Monocyte-derived iDCs (1 \(\times\) 10\(^6\)/ml) were stimulated with polymyxin B-treated native M161Ag (5 ng/ml), polymyxin B-treated various rM161Ag (1 \(\mu\)g/ml), LPS (10 ng/ml), and polymyxin B-treated LPS (10 ng/ml). After 24 h, the concentrations of IL-12 p40 in the supernatants were measured by ELISA. Determinations were performed in duplicate, and results are expressed as means ± SD. Results are representative of three separate experiments.
and iDCs, suggesting the species-dependent recognition of rM161Ag by TLR2; 3) M161Ag-induced DC maturation (up-regulation of CD83 and CD86) is dependent on the lipid moiety; 4) the N-terminal hydrophobic portion is associated with complement-activating function.

Several microbial lipoproteins/lipopeptides stimulate NF-κB signaling and trigger activation of the host defense system through TLR2 (15, 16, 35, 36). MALP-2 also induces AP-1 and NF-κB activity and cytokine secretion in murine macrophages via activation of the mitogen-activated protein kinase pathway (48). Interestingly, nonacylated forms of lipopeptide completely lacked stimulatory activity (15, 35), and the synthetic lipo-amino PamCys and the monoacylated synthetic lipopeptide, PamCysSerLys, did not activate NF-κB luciferase reporter gene in 293 cells expressing human TLR2 (16). These results suggested that the di- or triacyl groups and peptide moieties might be critical for TLR2-dependent cell activation, although activating efficiency was different among lipopeptides (35, 36).

However, our experiments demonstrated that the unlipidated rM161Ag could induce TLR2-mediated cytokine production by human monocytes and iDCs, if a hydrophobic SP was present at the N terminus. There were no differences in CD spectra between bioactive and inactive rM161Ag. Hence, an N-terminal hydrophobic element, either lipid or SP, and a subsequent hydrophilic portion may represent a molecular pattern of lipoproteins recognized by human TLR2 (49).

Recently, Takeuchi et al. (36) showed that the configuration of the lipid moiety in MALP-2 affected the TLR2-mediated cell responses (release of cytokines, chemokines, and NO); R-stereoisomer of the MALP-2 was 100 times more active than S-MALP in both human and murine cells. Taken together with our results that the lipidated form of M161Ag was 200-fold more active than the SP form in human cells, preferential recognition of M161Ag by TLR2 may be dependent on the N-terminal hydrophobic structure. In addition, the differences in responsiveness of murine and human macrophages to rM161Ag suggested the species-dependent recognition of rM161Ag by TLR2. Similar species-dependent discrimination has been reported on TLR4 in terms of intact LPS vs tetraacyl LPS (50).

The native M161Ag is a maturation inducer of iDC because of the up-regulation of a maturation marker, CD83 and CD86, and induction of IL-12 p40 production (51). Monocyte-derived iDCs express TLR2, TLR4, and MD-2, but not CD14 (M. Matsumoto et al., manuscript in preparation.)
and T. Seya, unpublished data), suggesting that LPS and M161Ag stimulate iDCs through TLR4-MD-2 complex and TLR2, respectively. The monomeric form of rM161Ag with SP could not up-regulate CD83 expression in iDCs, but induced IL-12 p40 production. There may be some differences in the TLR2-mediated signaling pathway between cytokine production and CD83/CD86 expression. These studies demonstrated that the microbial constituents act as inducers of DC maturation as well as cytokine production by monocytes/macrophages, at least in humans, and the cell responses may be dependent on the ligand structure.

The complement-activating ability of M161Ag is associated with the N-terminal hydrophobic portion, although the lipid form is more active than the SP form. Complement is a pivotal factor in the innate immune system. C3 fragment deposition onto microbial components allows the components to provide the second ligand for host immune receptors. Complement receptors, CR1, CR2, CR3, and CR4, can be activated by the postformed C3 ligands. Many microbial products, such as high doses of LPS, peptidoglycan, and zymosan, all stimulating TLRs, serve as targets for complement. C3 and C4 fragments are deposited on them via the alternative pathway (52–54). It is still unknown whether the consensus sequence or motif is required for stable C3 convertase formation on the complement-activating molecules. In M161Ag, the N-terminal fatty acids and following stretch of amino acids are required for complement activation, because sMALP-2 cannot activate complement at concentrations capable of stimulating the cells. A homology search of the nucleotide databases revealed that there is an M161Ag-like consensus motif conserved in lipoproteins of several bacteria (Mycoplasma arginiti, B. burgdorferi, T. pallidum, and Listeria monocytogenes) (32, 34). It will be of interest to determine in future studies whether the immune modulatory functions observed in M161Ag are conserved in these proteins.

An intriguing implication of these observations is an application of M161Ag to innate immune therapy for cancer. Bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) induces DC maturation, which contributes to the induction of tumor immunity (38, 55, 56). In murine macrophages, both TLR2 and TLR4 participate in BCG-CWS-mediated cell activation (38, 57). BCG-CWS and M161Ag activate the innate immune system in different ways. Identification of the ligand structure and their signaling events through TLR2 and TLR4 in macrophages/DCs may be important for advanced tumor immune therapy.

In summary, M161Ag is a pathogen-associated molecular pattern (PAMP) affecting at least two receptors, TLR2 and complement receptors. In our recent studies, another representative PAMP, BCG-CWS, was shown to use two receptors, TLR and a novel lectin receptor (S. Tsuji, J. Vehori, and T. Seya, unpublished data). An attractive hypothesis is a two-receptor theory, in which PAMP simultaneously stimulates two sorts of receptors, TLR and others. TLR mainly participates in intracellular signaling for cell maturation and survival, while the partner receptors act in phagocytosis. Further studies of PAMP and their receptors are needed to consolidate this hypothesis.

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