REVIEW (JAOB/Rising Members Award)

Possible Roles of Toll-like Receptor 2/6 and Extracellular ATP in Mycoplasmal Membrane Lipoprotein-induced Cell Activation and Death

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Introduction

Mycoplasmas, classified into a class called Mollicutes, form a group of prokaryotic microorganisms and are different from other bacteria in many aspects. For example, i) they have small diameter that allows them to pass through bacterial-retaining filters, ii) they have no cell wall, which usually retains bacterial rigid forms, iii) they require cholesterol for their survival and growth, iv) they have small genomes (580–2,200 kb compared with the 4,640 kb of Escherichia coli) with high A + T content (67–76%), and v) they use the universal stop codon UGA for tryptophan³⁴. They are also known as the smallest self-replicable microorganisms and are widely distributed in humans, animals and plants, but most mycoplasmas are parasites exhibiting strict host and tissue specificities owing to their limited biosynthetic capabilities for replication and survival.

Some mycoplasmas serve as real pathogens of humans. Indeed, two well-known mycoplasma species, Mycoplasma pneumoniae and Ureaplasma urealyticum, have fulfilled Koch's postulate for pathogens in pneumonia and nongonococcal urethritis, respectively⁴⁵. In addition, other mycoplasma species including M. genitalium, M. fermentans, M. penetrans, M. hominis and M. salivarium have been implicated in infectious diseases such as arthritis, pneumonia, urethritis and gingivitis in humans⁶–¹⁰. These mycoplasmas have long been known to possess immunomodulatory or toxic effects on host cells¹¹,¹². Currently, in fact, one of the well-documented actions of mycoplasmas is their capacity to interact with immunocytes, leading to production of proinflammatory mediators or cell death. Attempts have been made to determine which components of mycoplasmas are responsible for these activities. As mycoplasmas, unlike other bacteria, have no cell wall, their membrane components, such as glycolipids, phospholipids and membrane proteins, are the only structures that are considered to regulate the interaction with the external environment. Membrane proteins are classified into two categories, integral proteins and peripheral proteins¹³. Outer peripheral proteins, also termed membrane lipoproteins (LP), are thought to have immunostimulatory effects and to be directly involved in the interaction with host cells¹⁴. In addition, mycoplasmas have much larger genomes encoding LP than other bacteria, enabling the modification of the antigenicity of the cell surface, so-called phase variation, for evading the host defense system¹⁵. Thus, mycoplasmal LP have been considered the most potent initiator of inflammatory reactions in mycoplasmal infections.

We have studied the biological activities of myco-
plasmal LP, and our recent findings have demonstrated that mycoplasmal LP not only initiate proinflammatory reactions, but also induce cell death in host cells. In the present review, we focused on these activities of mycoplasmal LP and how the host cells recognize them to initiate these responses. In addition, we offer one possible mechanism by which these biological activities of mycoplasmal LP in host cells may be regulated and/or modulated.

**Mycoplasmal LP-induced Cell Activation and Death**

Triton X114 phase separation, a method introduced by Bricker, et al.\(^{10}\), is useful for the preparation of native mycoplasmal LP from cultured mycoplasmal cells, because mycoplasmas lack a cell wall and are enriched with LP in their cell membrane. It has been demonstrated that LP prepared from various mycoplasmal species are immunocompetent. For example, mycoplasmal LP induce production of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 by monocytes/macrophages, which in turn serve as endogenous inflammatory mediators.\(^4\)\(^8\)\(^9\) Rawadi, et al.\(^{11,12}\) have shown that LP derived from *M. fermentans* cells activate the transcription factors nuclear factor (NF)-\(\kappa\)B and activated protein (AP)-1, which accelerate the transcription of various proinflammatory mediators including TNF and IL-1 in monocytes/macrophages. Our recent study also demonstrated that LP prepared from an oral mycoplasma, *M. salivarium*, activate NF-\(\kappa\)B and AP-1, inducing production of IL-6 and IL-8 by human gingival fibroblasts.\(^19\) Thus, numerous lines of evidence have already been accumulated that mycoplasmal LP possess potent immunomodulatory properties. It is now thought that LP are comparable in activity to and equally wide spread in Mollicutes as lipopolysaccharides (LPS) in Gram-negative bacteria.

Several researchers have focused on the cytoidal activity of the mycoplasmal membrane toward human cancerous cells or immunocytes.\(^4\)\(^7\)\(^8\)\(^9\) However, it has not been fully elucidated whether mycoplasmal LP have such activity. Our recent study reported that LP prepared from *M. fermentans* using the Triton X114 method (LPfer) induced cell death in several human lymphocytic and monocytic cell lines.\(^14\) Flow cytometric and electron microscopic analyses (Fig. 1) revealed that LPfer preferentially induced necrotic cell death in lymphocytic cell lines and apoptotic cell death in monocytic cell lines.\(^14\) This result was further supported by the analysis of genomic DNA electrophoresis.\(^14\) Therefore, we hypothesized that mycoplasmal LP might have the ability to induce distinct cell death features, apoptosis and necrosis, which are dependent on the different cell types used. To verify this hypothesis, we investigated the intracellular events of mycoplasmal LP-treated cells. Since the best characterized intracellular key effectors of apoptosis are the caspase family members participating in a tightly regulated proteolytic cascade,\(^13\)\(^16\) the involvement of caspases in LPfer-induced cell death were examined using several caspase inhibitors. Both cell death features were attenuated by several inhibitors of caspases, especially caspase-3, -8 and -9, but not caspase-1.\(^14\) Furthermore, the pan-caspase inhibitor z-VAD-fmk completely diminished the activity of mycoplasmal LP.\(^14\) Thus, mycoplasmal LP-induced necrotic and apoptotic cell death are largely dependent on the proteolytic activities of some caspase members. In fact, the activation of caspase-3 assessed by cleavage of poly (ADP-ribose) polymerase (PARP), a substrate of effector caspases, was observed in LPfer-treated lymphocytes and monocytes.\(^14\) Furthermore, this observation raises the possibility that distinct cell death features are dependent on the cellular sensibility toward mycoplasmal LP, which might be attributed to the expression levels of caspases and/or cell surface receptor(s) for LP. Next, we were very interested in whether the magnitude of cytoidal activity of LP was dependent on the pathogenicity of each mycoplasmal species. LP prepared from fermentative mycoplasmas such as *M. pneumoniæ*, *M. fermentans* and *M. penetrans* had stronger activity than those from non-fermentative mycoplasmas such as *M. salivarium* and *M. hominis* (Fig. 2).\(^14\) These differences in the cytoidal activity may be dependent on those in the structures of LP existing in the cell membrane of fermentative and
Thus, mycoplasmal LP are recognized by the innate immune system of host cells as extraordinary "non-self" components, initiating cell death as well as proinflammatory reactions.

**The Biological Active Entity of Mycoplasmal LP**

All characterized mycoplasmal LP have a signal peptide similar to those found in other prokaryotes. Cleavage of this signal peptide, including specific

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Fig. 1 Cell death in MOLT-4 and HL-60 cells induced by LPfer. Scanning (upper) and transmission (lower) electron microscopy of MOLT-4 (A) and HL-60 (B) cells treated with LPfer (10 µg/ml) for 22 h (modified from Into, et al. 19). Pictures on the left side show the control cells.
amino acid sequences, is associated with translocation across the membrane, maturation of the protein and anchorage to the membrane. The proteins are anchored to the membrane lipids via a cysteine residue just downstream of the signal peptide cleavage site. This cysteine residue with lipolyated modification is characteristic of mycoplasmal LP.

Since LP from *M. kyorinis*, *M. fermentans* and *M. salivarum* lack the N-terminal acyl-modification in the cysteine residue17–19, this type of LP with only two ester-bound fatty acids had previously only been discovered in *Rhodopseudomonas viridis*8. The lack of the amide–bound fatty acid at the N-terminus is an unusual feature because general bacterial LP are commonly substituted by two ester-linked and a third amide–bound fatty acid. It is now considered that the absence of the third fatty acid at the N-terminus may be due to the lack of a specific enzyme, N-acyltransferase, which is required to transfer the third fatty acid to the free N-terminus. This lack has great consequences for the biological activity of the LP, because recent studies have suggested that the biological active entity of mycoplasmal LP is the N-terminal lipopeptide moiety, by demonstrating that the biological activity of an *M. fermentans* lipoprotein called MALP–404 can be substituted by the lipopeptide termed macrophage-activating lipopeptide 2–kDa (MALP–2) (Fig. 3), synthesized on the basis of the N-terminal structure8,20,21. We have also determined the structure of the N-terminal lipopeptide moiety of LP44 derived from *M. salivarum*, and demonstrated that the lipopeptide synthesized on the basis of the structure (fibroblast-stimulating lipopeptide–1, FSL–1) (Fig. 3) causes the activation of human gingival fibroblasts and monocytes/macrophages19.

**Recognition of Mycoplasmal LP/lipopeptides by Toll-like Receptors**

Microbes are covered with and contain components

![Fig. 2 Cytocidal activity of LP prepared from several human pathogenic mycoplasmas. Mycoplasmal LP were prepared by the Triton X114 method and used for cell stimulation. HL-60 cells were stimulated for 15 h with 10 μg/ml of mycoplasmal LP. Cytocidal activities of LP were determined by LDH release assay (modified from Into, et al.18).](image)

**FSL-1**

\[
\text{CH}_3\text{(CH}_2\text{)}_{14}\text{O} \quad \text{O} \quad \text{O} \quad \text{NH}_2 + \text{Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe}
\]

**MALP-2**

\[
\text{CH}_3\text{(CH}_2\text{)}_{14}\text{O} \quad \text{O} \quad \text{NH}_2 + \text{Gly-Asn-Asn-Glu-Ser-Asn-Ile-Ser-Phe-Lys-Glu-Lys}
\]

**Fig. 3** Structures of synthetic mycoplasmal diacylated lipopeptides, FSL–1 and MALP–2
commonly produced by a broad group of microbial species, so-called pathogen-associated molecular patterns (PAMPs). In microbes, PAMPs are usually the cell wall compounds such as LPS, peptidoglycans (PGN) and lipoteichoic acid. PAMPs have been generally considered to have immunostimulatory effects by recognition through binding to soluble and cellular receptors constitutively expressed by host cells. As mycoplasmas have no cell wall, as described above, LP can act as a major PAMP in mycoplasmal species. The recognition mechanisms of mycoplasmal LP/lipopeptides by certain cellular receptors are becoming clear.

Toll-like receptors (TLRs), a set of germ-line-encoded receptors, have been revealed to play crucial roles in the innate recognition of various microbial PAMPs. Eleven mammalian TLRs have been identified so far, and all of them share extracellular leucine-rich repeat motifs and an intracellular Toll/IL-1 receptor homology (TIR) domain. Among the TLR family members, TLR2 has a broader role as a pattern-recognition receptor for LP/lipopeptides from several microbes, PGN from Gram-positive bacteria such as Staphylococcus aureus, GPI anchors from Trypanosoma cruzi, lipoparabionomannans from Mycobacterium tuberculosis, porins from Neisseria meningitidis and the yeast cell-wall component zymosan. TLR2 transmits intracellular signaling using several molecules such as myeloid differentiation factor-88 (MyD88), IL-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor (TRAF) 6 and mitogen-activated protein kinases (MAPKs), leading to activation of NF-κB and AP-1 which initiates innate proinflammatory responses after the recognition of cognate ligands. Although Ozinskiy, et al. first suggested that TLR2 recognizes cognate PAMPs by heterodimerization with other TLRs, Takeuchi, et al. have demonstrated the mechanisms of circumstantial recognition of microbial LP/lipopeptides by TLR2. As stated above, mycoplasmal LP/lipopeptides contain a lipolyated N-terminal diacylated cysteine residue, whereas bacterial LP/lipopeptides contain a triacylated one. This difference in the N-terminal structure in microbial LP/lipopeptides plays crucial roles in the recognition by TLR2. That is, mycoplasmal diacylated LP/lipopeptides are recognized by TLR2 and TLR6, whereas bacterial triacylated ones are recognized by TLR2 and TLR1. The schematic model of the recognition of microbial LP by these TLRs is shown in Fig. 4.

Roles of TLR2/6 in Bifurcate Cell Reactions Induced by Mycoplasmal LP

Although mycoplasmal LP stimulate host cells to induce proinflammatory reactions via the activation of transcriptional factors after recognition by cognate TLRs, it remained unknown how mycoplasmal LP express cytocidal activity toward host cells. Therefore, we first tried to determine whether TLR2 mediated the cytoidal activity of LP, which is not attenuated by blocking antibodies to FasL, TNF and IL-1, because Aliprantis, et al. have already reported that TLR2 stimulation with a triacylated bacterial lipopeptide directly initiates apoptotic cell death. Expectedly, LP-induced cell death in PMA-treated THP-1 cells was significantly suppressed by a specific monoclonal antibody to TLR2. Furthermore, transfection of HEK293 cells, a cell line lacking TLR2 expression, with TLR2-encoding plasmid, sensitized the cells toward stimuli of LP and MALP-2, inducing cell death. These observations indicate that mycoplasmal LP show cytoidal activity through TLR2-mediated mechanisms. In addition, co-transfection of TLR6 synergically accelerated the TLR2-mediated cytoidal activity of LP and MALP-2 in HEK293 cells, while TLR6 itself did not mediate the activity. This phenomenon was not observed when other TLRs were used for co-transfection with TLR2. The expression of the dominant negative form (DN) of TLR6 reduced, but did not abrogate, the TLR2-mediated activity. Thus, stimulation of TLR2 with mycoplasmal LP/lipopeptides was found to lead to cell death, which was upregulated by coexpression of TLR6.

LP induced NF-κB activation, but not cell death, in HEK293 cells under the condition of coexpression of TLR2 and TLR6 after 6 h of stimulation. The apparent cell death features, including morphological changes, DNA laddering and lactate dehydrogenase...
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demonstrated indicating changes, a cules.
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stimulation'n'. (LDII)

We demonstrated that MyD88, one of the major intracellular adaptors that transduce cellular signaling via TLRs, IL-1 receptor and IL-18 receptor[22], was considered to be involved in the responses. In fact, MyD88 is known to play essential roles in TLR2-mediated NF-κB activation induced by MALP-2[23,31], and apoptosis induced by a triacylated bacterial lipopeptide[32]. MyD88 contains a highly conserved death domain (DD) in its N-terminus that facilitates interaction with other DD-containing molecules. We demonstrated that MyD88-DN, which lacks a DD, reduced NF-κB activation, caspase-8 activation, cleavage of PARP, apoptotic morphological changes, LDH release, and genomic DNA degradation into oligonucleosomal fragments induced by LPfer[20], indicating that mycoplasmal LP-induced NF-κB activation and cell death require DD-DD interaction between MyD88 and other molecules. We further demonstrated that overexpression of wildtype MyD88 potentiates the cytocidal activity of LPfer[30], indicating that the expression level of MyD88 has a great effect on the triggering of TLR2/6-mediated apoptotic cell death. Thus, the functions of MyD88 are crucial for the regulation of cellular responses induced by mycoplasmal LP/lipopeptides, which are mediated by the subsequent binding to DD-containing molecules. We next investigated the involvement of Fas-associated death domain protein (FADD), consisting of an N-terminal death effector domain (DED) and a C-terminal DD that interacts with MyD88 by DD-DD interaction[22]. Generally, in the initiation of the several death receptors-induced apoptotic pathway, FADD recruits the DED-containing procaspase-8 and procaspase-10 via homophilic DED-DED interactions and initiates the processes leading to the subsequent cleavage of downstream effector caspases[15,16]. We demonstrated that FADD-DN, which lacks a DED, downregulated LPfer-induced apoptotic responses, including caspase-8 activation, PARP cleavage, DNA laddering and LDH release, via the recognition by TLR2/6[20]. Furthermore, FADD-DN reduced LPfer-induced NF-κB activation[30]. Thus, FADD downstream of MyD88 is also a key mediator for the regulation of the sequential bifurcate cellular events induced by mycoplasmal LP. The DD-containing kinase IRAK-4, the newest member of the IRAKs, has.

![Diagram of TLRs and lipopeptides](attachment:diagram.png)

**Fig. 4** The recognition of microbial LP by TLRs. Mycoplasmal diacylated LP/lipopeptides are recognized by TLR2 and TLR6, whereas bacterial triacylated ones are recognized by TLR2 and TLR1 (modified from Takeuchi, et al.[27,28]).

(LDH) release, did not emerge until at least 18 h after stimulation[20]. Thus, stimulation of TLR2/6 with mycoplasmal LP kinetically induces sequential bifurcate cellular responses: NF-κB activation at an early stage and apoptosis at a later stage. In an attempt to discriminate these bifurcate responses, experiments were performed to determine whether several intracellular signaling molecules downstream of TLR2/6 are involved in these responses. MyD88, one of the major intracellular adaptors that transduce cellular signaling via TLRs, IL-1 receptor and IL-18 receptor[22], was considered to be involved in the responses. In fact, MyD88 is known to play essential roles in TLR2-mediated NF-κB activation induced by MALP-2[23,31], and apoptosis induced by a triacylated bacterial lipopeptide[32]. MyD88 contains a highly conserved death domain (DD) in its N-terminus that facilitates interaction with other DD-containing molecules. We demonstrated that MyD88-DN, which lacks a DD, reduced NF-κB activation, caspase-8 activation, cleavage of PARP, apoptotic morphological changes, LDH release, and genomic DNA degradation into oligonucleosomal fragments induced by LPfer[20], indicating that mycoplasmal LP-induced NF-κB activation and cell death require DD-DD interaction between MyD88 and other molecules. We further demonstrated that overexpression of wildtype MyD88
been shown to participate in a MyD88-dependent signaling pathway as a central signaling mediator downstream of TLRs and IL-1 receptor. We showed that overexpression of IRAK-4 slightly attenuated LPfer-induced cell death under the condition of coexpression with TLR2 and TLR6. In addition, IRAK-4-DN, which lacks a kinase domain, reduced LPfer-induced NF-κB activation but not induction of cell death. Therefore, it is speculated that kinase activity of IRAK-4 contributes to mycoplasmal LP-induced proinflammatory responses via activation of NF-κB, but not to cell death. The involvement of MAPKs in mycoplasmal LP-induced cell death was further investigated because it is not known whether MAPKs are involved in the TLR-mediated apoptotic pathway. MAPK cascades are evolutionarily conserved phosphorylation-regulated protein kinase cascades and are thought to play important roles not only in the regulation of gene expression and cell growth but also in the initiation of a proapoptotic signaling pathway in response to various cellular stressors.

MAPKs include extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 MAPKs. p38 MAPKs and JNKs play pivotal roles as stress-activated protein kinases in triggering apoptosis. Two well-studied inhibitors were used for the determination of the involvement of MAPKs in LPfer-induced cell death. The selective inhibitor of p38 MAPK SB203580 clearly attenuated the cytocidal activity of LPfer, whereas the selective inhibitor of MEK1/2 PD98059, which blocked the activation of ERKs, had no effect, suggesting that p38 MAPK is an important mediator of cell death induced by mycoplasmal LP. However, it remains unknown how the p38 MAPK-mediated apoptotic pathway is directly regulated downstream of TLR2/6 and MyD88.

In light of these findings, the stimulation of TLR2/6 with mycoplasmal LP possibly induces sequential MyD88-dependent bifurcate responses, including NF-κB activation as an early event mediated by FADD and IRAK-4, as well as apoptosis as a later event mediated by both FADD-caspase-8 and p38 MAPK (Fig. 5).

**Fig. 5** Schematic model of mycoplasmal LP-induced bifurcate cell responses via TLR2/6

**Regulation of Mycoplasmal LP-induced Cellular Reactions by Extracellular ATP**

LPS are well-studied bacterial components and are known to initiate potent proinflammatory responses towards various host cells after recognition by several cellular factors, including TLR4, MD-2, LPS-binding protein and CD14. However, several studies have suggested that the activation of macrophages by LPS is regulated by extracellular ATP (ATP) and some purinergic receptors. Extracellular purine nucleotides bind to cell surface receptors, which are designated as purinergic P2 receptors. P2 receptors are divided into two groups: P2X receptors,
ligand-gated cation channels, and P2Y receptors coupling with G proteins. It has been reported that P2X and P2Y receptor subtypes, including P2X\textsubscript{1}, P2X\textsubscript{7}, P2Y\textsubscript{2} and P2Y\textsubscript{11}, which are sensitive to ATP\textsuperscript{41-44}, regulate the differentiation, activation or proliferation of various immunocytes. It is now considered that P2X receptors, particularly P2X\textsubscript{7}, are candidates for the regulation of the biological activity of LPS\textsuperscript{39-38}.

Therefore, we investigated whether ATP\textsubscript{o} and/or P2 receptors modulate(s) the TLR2/6-mediated biological activities of mycoplasmal LP/lipopeptides, because most of the biological activities of LPS are shared by microbial LP/lipopeptides. First, the effects of ATP\textsubscript{o} on the mycoplasmal LP/lipopeptides-induced activation of THP-1 cells were investigated. It was found that the FSL-1/MALP-2-induced TNF-\alpha production was augmented by the presence of ATP in a dose-dependent manner (Fig. 6)\textsuperscript{45}. In addition, the transcriptions of IL-1, IL-6 and IL-8 as well as TNF, but not IL-10, induced by these lipopeptides were also upregulated by ATP\textsubscript{o}\textsuperscript{45}. In contrast, the transcriptional levels of TLR2, TLR6, P2X\textsubscript{1}, P2X\textsubscript{7}, P2Y\textsubscript{2} and P2Y\textsubscript{11} were not changed after stimulation with mycoplasmal lipopeptides and/or ATP\textsubscript{o}. These findings indicate that ATP\textsubscript{o} has the activity to upregulate mycoplasmal LP/lipopeptides-induced proinflammatory cell activation via TLR2/6. Furthermore, the effects of ATP\textsubscript{o} on mycoplasmal LP-induced cell death were investigated. As a result, ATP\textsubscript{o} was found to augment the magnitude of LP/\textsubscript{per}-induced cell death features, including LDH release and caspase-3 activation, in lymphocytic and monocytic cell lines\textsuperscript{46}. Therefore, it is likely that ATP\textsubscript{o} is an important regulator for the recognition of mycoplasmal LP/lipopeptides by TLRs, which leads to the initiation of cell activation and death.

In the immune system, ATP\textsubscript{o} triggers many cellular reactions, such as the release of IL-1, exocytosis of granula containing superoxide and reactive oxygen species, phagocytosis, giant cell formation, chemotaxis, cell adhesion to endothelium, cytolysis, and cell apoptosis via P2 receptors\textsuperscript{47}. To investigate which ATP receptors regulate the activities of mycoplasmal LP/lipopeptides, the effects of pyridoxal phosphate 6-azophenyl 2',4'-disulfonic acid (PPADS) and periodate-oxidized ATP (oATP) on the activity of ATP\textsubscript{o} were examined, because PPADS is an antagonist for P2X receptors (P2X\textsubscript{1}—P2X\textsubscript{7}) and P2Y receptors except for P2Y\textsubscript{2} and P2Y\textsubscript{11}\textsuperscript{48,49}, and oATP is an antagonist for P2X receptors, especially P2X\textsubscript{7}\textsuperscript{50,51}. It
was found that both antagonists for P2 receptors attenuated the activity of ATPo\textsuperscript{35,46}, indicating that mycoplasmal LP/lipoproteins-induced cell reactions are regulated through the interaction of P2X receptors, especially P2X\textsubscript{7} receptor, with ATPo.

Thus, ATPo and P2X receptors are important regulators of the biological activities of mycoplasmal LP/lipoproteins. Furthermore, it is possible that ATPo can regulate the biological activities of pathogen-associated lipid derivatives such as LPS and mycoplasmal LP/lipoproteins through its interaction with P2X receptors.

**Conclusion**

Our studies have showed that mycoplasmal LP can induce proinflammatory as well as proapoptotic effects in host cells via recognition by TLR2/6 and regulation by extracellular ATP and P2X receptors. Although the exact roles of mycoplasmal LP in mycoplasmal infections are still unclear, these observations may provide novel aspects of the pathological roles of mycoplasmal LP. Additionally, in future study, elucidation of the exact mechanism of the recognition of mycoplasma LP/lipoproteins by TLR2/6 may give an understanding of the various biological activities of mycoplasmas leading to the disruption of host defense.

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