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Legionella lipoprotein activates toll-like receptor 2 and induces cytokine production and expression of costimulatory molecules in peritoneal macrophages

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Abbreviations: CpG ODN, synthetic CpG-oligodeoxynucleotides; rPAL, recombinant peptidoglycan-associated lipoprotein; TLRs, toll-like receptors

Abstract

***Legionella* bacterium, an intracellular pathogen of mononuclear phagocytes, causes acute fatal pneumonia, especially in patients with impaired cellular immune responses. Until recently, however, the toll-like receptor (TLR) engagement of bacterial proteins derived from *Legionella* is uncertain. We previously showed that a 19-kDa highly conserved peptidoglycan-associated lipoprotein (PAL) of *Legionella pneumophila* induced the PAL-specific B cell and T cell responses in mice. In this study, we observed that the rPAL antigen of *L. pneumophila,* as an effector molecule, activated murine macrophages via TLR2 and produced proinflammatory cytokines such as IL-6 and TNF-α. In both BALB/c and TLR4-deficient C3H/HeJ mice, pretreatment of macrophages with anti-TLR2 mAb showed severely impaired cytokine production in response to the rPAL. In addition, *in vitro* the rPAL treatment increased the cell surface expression of CD40, CD80, CD86 and MHC I/II molecules. We further showed that the synthetic CpG-oligodeoxynucleotides (CpG ODN) coadministered with the rPAL enhanced IL-12 and IL-6 production and expression of CD40, CD80 and MHC II compared to the rPAL treatment alone. In conclusions, these results indicate that *Legionella* PAL might activate macrophages via a TLR2-dependent mechanism which thus induce cytokine production and expression of costimulatory and MHC molecules.**

Keywords: *Legionella pneumophila*; Legionnaires' disease; lipoproteins; macrophages, peritoneal; Toll-like receptors

Introduction

Legionella pneumophila, a gram-negative intracellular pathogen, is the etiologic agent of Legionnaires' disease and Pontiac fever. The bacterium is also an important cause of epidemic and sporadic pneumonia worldwide. Transmission to humans occurs through inhalation of aerosols containing *L. pneumophila* (Steinert *et al*., 2002). The bacteria reside in the lungs and multiply in the phagosomes of human monocytes (Horwitz *et al*., 1980) and alveolar macrophages (Nash *et al*., 1984) by evading phagosome acidification or phagolysosomal fusion (Horwitz, 1983).

Peptidoglycan-associated lipoprotein (PAL) is an outer membrane protein that is commonly present among *Legionella* species as well as other gram-negative bacteria (Engleberg *et al*., 1991). A 19 kDa Legionella PAL is of particular interest. Our previous study first demonstrated that rPAL of *L. pneumophila*, as a vaccine candidate, was a potent activator of PAL-specific B and T cell responses in BALB/c mice immunized with either the rPAL or plasmid DNA PAL (Yoon *et al*., 2002). Subsequently, the PAL was characterized as a soluble antigen excreted from urine of patients with *Legionella* pneumonia (Kim *et al*., 2003).

Members of the toll-like receptor (TLR) family in cells of the innate immune system recognize specific conserved components of microbes, including lipopolysaccharide, peptidoglycan, lipopeptides, lipoteichoic acid and CpG DNA motifs present in bacterial DNA, and initiate the cascade of the inflammatory response, further activating adaptive immunity through the induction of cytokine production and expression of costimulatory molecules (Akira *et al*., 2001; Barton and Medzhitov, 2002; Dabbagh and Lewis, 2003; Krishnan *et al*., 2007).

Recently there has been broad interest in testing and developing TLR ligands for immune stimulation to enhance antigen-specific responses. Especially, synthetic oligonucleotides containing the CpG motifs (CpG ODN), which are ligands for TLR9, are being developed as a new generation of vaccine adjuvants to enhance immune responses (Krieg and Davis, 2006).

Several studies have reported that TLRs contribute to the host response against *L. pneumophila* during *in vitro* and *in vivo* infection (Girard *et al*., 2003; Kikuchi *et al*., 2004; Akamine *et al*., 2005; Hawn *et al*., 2006, 2007; Fuse *et al*., 2007; Newton *et al*., 2007). However, there have been no studies to elucidate immunostimulatory properties of isolated individual proteins from *L. pneumophila* in terms of engagement of TLRs and linking to elicit the antigen-specific adaptive immune responses.

The aim of this study was to determine the role of TLRs in the host response to the purified rPAL antigen of *L. pneumophila* to induce production of proinflammatory cytokines and expression of costimulatory molecules. Furthermore, we examined whether combining treatment of CpG ODN and the rPAL *in vitro* could enhance the rPAL-induced immune responses.

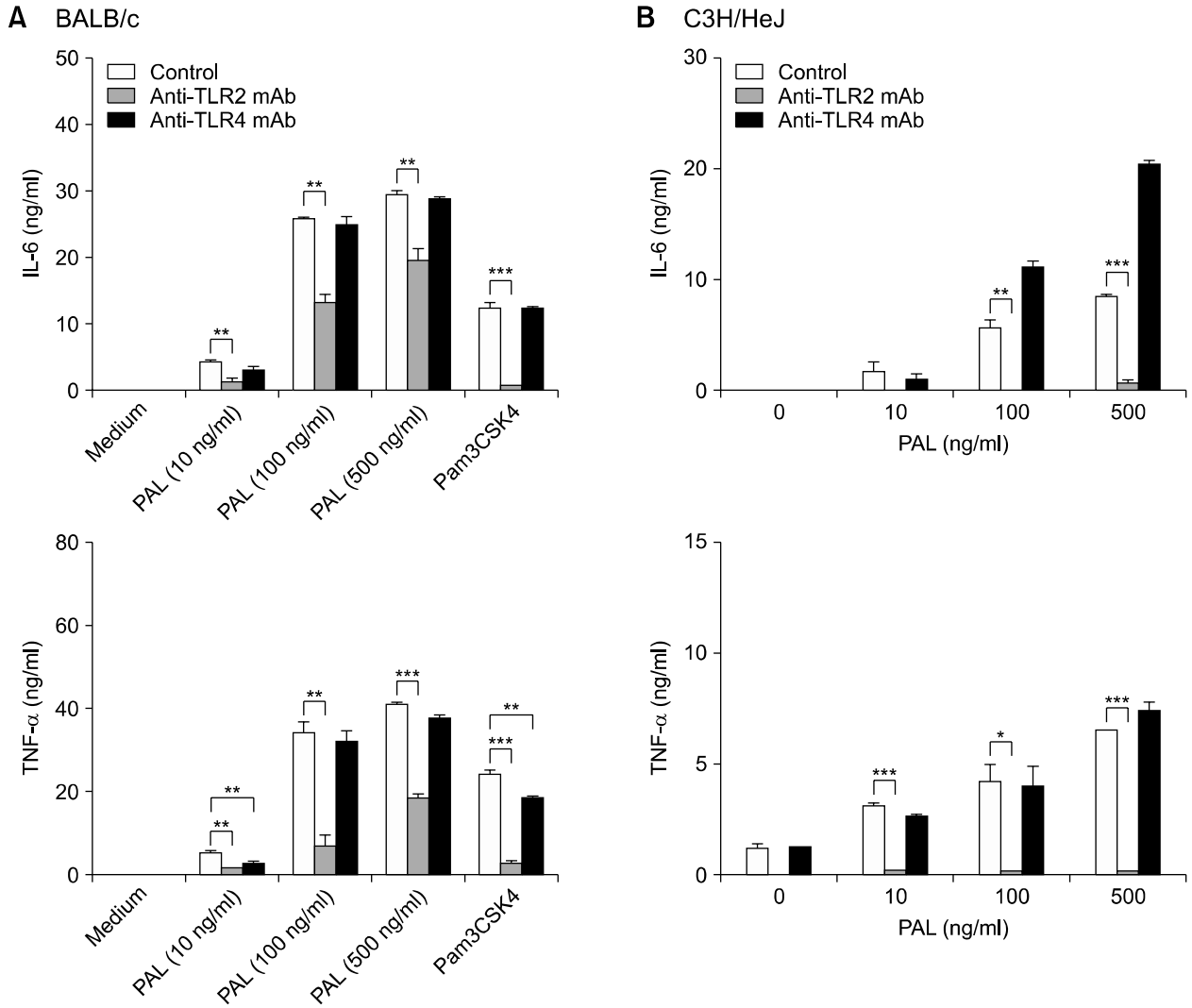

Figure 1. Production of IL-6 and TNF-α in BALB/c mouse macrophages after stimulation with rPAL. Peritoneal macrophages from BALB/c (A) or C3H/HeJ (B) mice were pretreated with or without 10 μg/ml of anti-TLR2 or -TLR4 mAb. After 1h cells were stimulated for 18 h with various concentrations (10, 100 and 500 ng/ml) of rPAL. The amounts of IL-6 and TNF-α in culture supernatants were determined by using ELISA. As a positive control for TLR2-dependant stimulation, TLR2 agonist, Pam3CSK4 (100 ng/ml) was used. Data are expressed as mean ± SD and represent three separate experiments (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

Legionella PAL activates TLR 2-mediated signaling in BALB/c and C3H/HeJ macrophages

To explore the involvement of TLR2 or TLR4 in *Legionella* PAL-induced cell activation, peritoneal macrophages from BALB/c mice were incubated with or without anti-TLR2 and -TLR4 mAb respectively, and then stimulated with various concentrations of the rPAL for 18 h (Figure 1). In the absence of TLR blocking antibodies, the production of IL-6 and TNF-α was increased by the rPAL in a dose-dependent fashion. In experiments with pretreatment of TLR antibodies, the rPAL-induced IL-6 production was significantly inhibited by the anti-TLR2 mAb, but it was unaffected by the anti-TLR4 mAb (Figure 1A). Similarly, the rPAL-induced TNF-α production was significantly inhibited by the anti-TLR2 mAb, but no significant change was observed between untreated and anti-TLR4 mAb treated macrophages (Figure 1B). We used in parallel Pam3CSK4 as a positive control of TLR2 agonist for the TLR2-dependant stimulation. These findings suggest that *Legionella* PAL might induce cytokine production by interaction with TLR2. We also verified TLR2-dependant stimulation by rPAL in macrophages from LPS-hyporesponsive, TLR4-mutant C3H/HeJ mice, as a control system for TLR4 signaling (Braedel-Ruoff *et al*., 2005). Similar to BALB/c macrophages, C3H/HeJ macrophages significantly increased IL-6 and TNF-α levels in response to rPAL treatment, which was greatly inhibited by TLR2 blocking antibody. There was no inhibition of the cytokine production in cells treated with anti-TLR4 mAb. We further investigated TLR-mediated signaling by the rPAL of *L. pneumophila* at the gene expression level. The mRNA expression of TLR2, TLR4 and MyD88 were analyzed in BALB/c macrophages with or without rPAL treatment by RT-PCR. Compared to the untreated controls, TLR2 and MyD88 mRNA expression was prominent with rPAL treatment (Figure 2). In contrast, the TLR4 mRNA expression was very weak. These findings indicate that *Legionella* PAL-mediated signaling might modulate TLR2 and possibly, MyD88 expression.

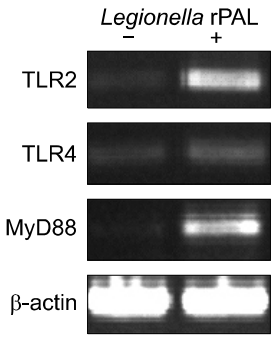

Figure 2. Effect of *Legionella* PAL on TLR2, TLR4 and MyD88 mRNA expression. Peritoneal macrophages from BALB/c mice were treated with or without 1 μg/ml of rPAL for 18 h. Total RNA was then extracted from the macrophages and subjected to RT-PCR, as described in Methods section. The β-actin gene expression was examined as a positive control.

Coadministration of CpG ODN with Legionella PAL synergistically enhances cytokine production in murine macrophages

We next examined whether cytokine production by *Legionella* PAL antigen might be further enhanced

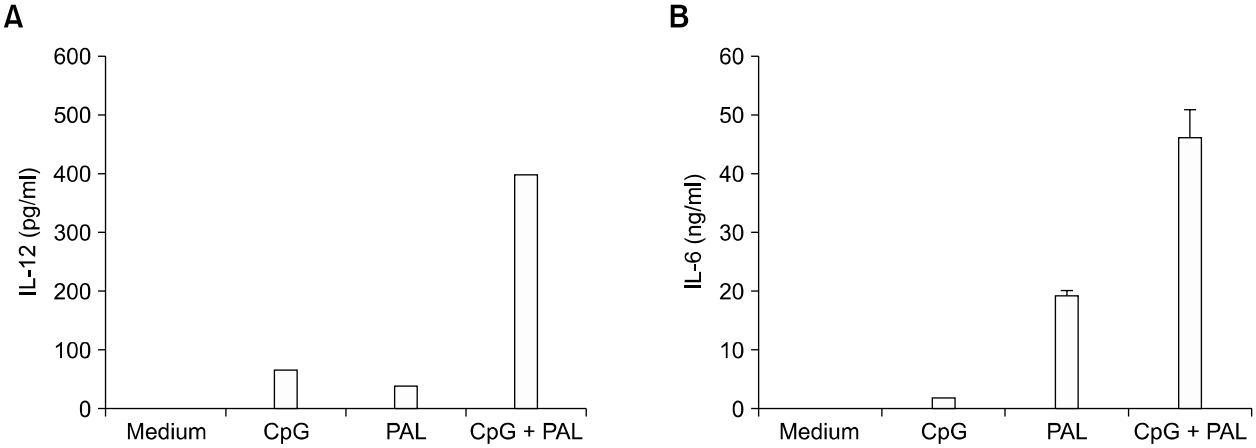

Figure 3. Co-stimulation of *Legionella* PAL with CpG ODN induces significantly increased production of IL-12 and IL-6. BALB/c mice macrophages were incubated for 18 h with culture medium, CpG ODN (3 μg/ml), rPAL (100 ng/ml), and the combination of CpG ODN and rPAL. The cell supernatants were collected and analyzed by ELISA for IL-12p40 (A) and IL-6 (B) levels. Results are expressed as means of triplicate wells ± SD.

by coadministration with CpG ODN. IL-12 production was observed in macrophages when treated with CpG ODN, the rPAL or both (Figure 3A). Interestingly, combined treatment of CpG ODN and the rPAL, increased IL-12 production by up to sevenfold, compared to the rPAL alone. As expected, CpG ODN alone induced minimal release of IL-6 (Figure 3B). However, the levels of IL-6 production were significantly increased by coadministration of CpG ODN and the rPAL. These results indicate that the combination of CpG ODN and *Legionella* PAL might synergistically activate macrophages to produce cytokines.

Legionella PAL increases expression of cell surface molecules in murine macrophages

To elucidate whether the rPAL or the rPAL plus CpG ODN can induce expression of cell surface molecules in macrophages, in terms of activation of the adaptive immune responses, peritoneal macrophages were analyzed by flow cytometry after 24 h incubation with the rPAL, CpG ODN, or CPG ODN plus the rPAL (Figure 4). The rPAL-treated macrophages demonstrated increased expression of the B7 family (CD80 and CD86), MHC II and in particular, CD40 molecules compared to the untreated control. However, coadministration of CpG ODN and the rPAL was associated with

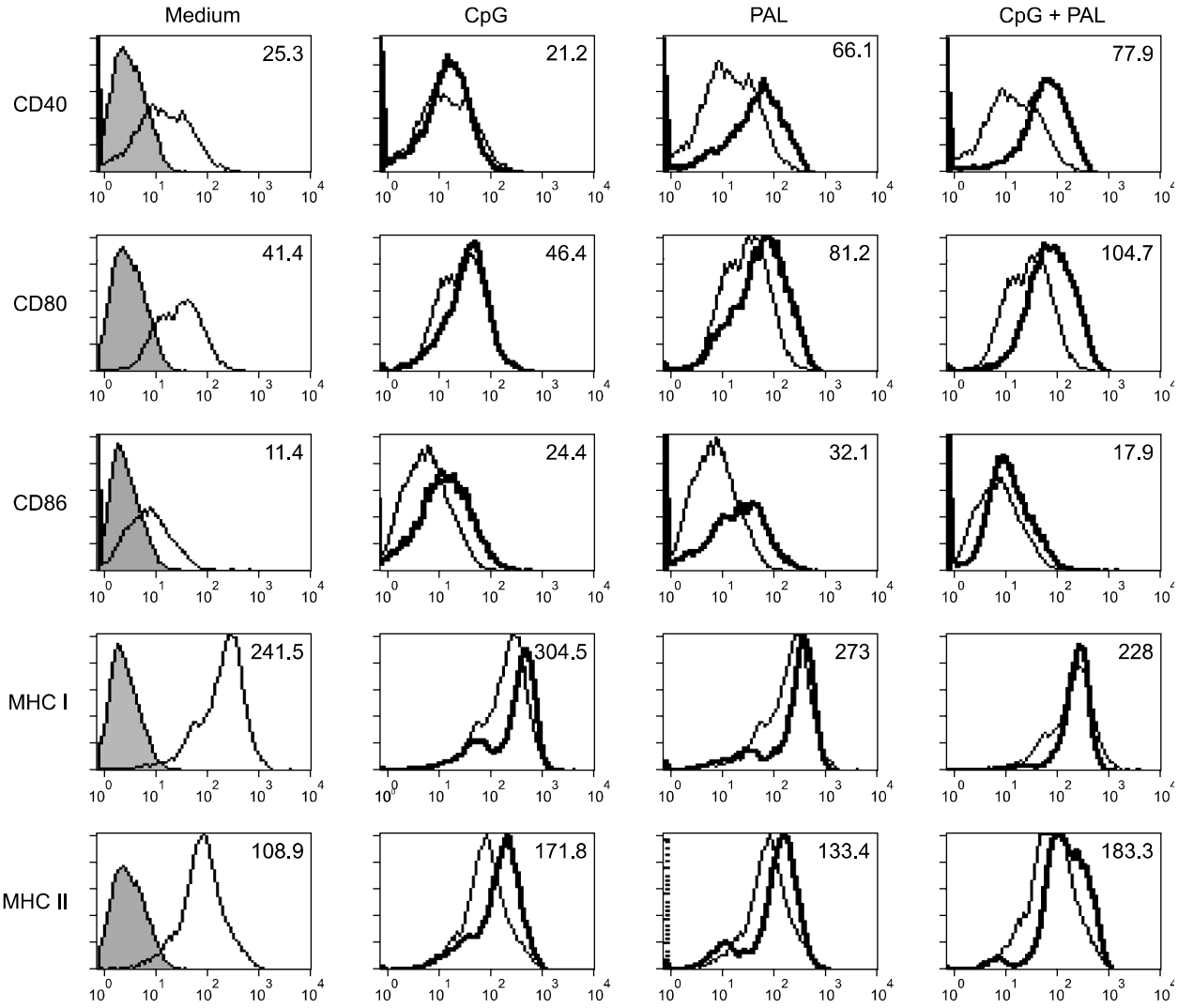

Figure 4. Effect of PAL or CpG ODN on the surface expression of costimulatory and MHC molecules. Peritoneal macrophages (1×10^6 cells/ml) were cultured with media alone, CpG ODN (10 μg/ml), rPAL (10 μg/ml) or CpG ODN plus rPAL, and analyzed for expression of CD40, CD80, CD86, MHC I and MHC II by flow cytometry, as detailed in the Methods section. Gray filled histograms: isotype matched controls, Thin-line histograms: untreated cells, Thick-line histograms: CpG, rPAL or CpG+rPAL treated cells. Mean Fluorescence Intensity (MFI) was given on the top right upper corner of each panel.

little increase in expression of MHC II, CD40 and CD80 and decrease in expression of CD86 and MHC I, compared to the rPAL or CpG ODN alone. Collectively, these results indicate that macrophage activated with *Legionella* PAL upregulates expression of costimulatory molecules, and especially, upregulation of CD40 molecule may participate in T or B cell activation as a predominant costimulatory signal. In addition, CpG ODN could modulate the PAL-specific immune responses.

Discussion

Several studies have identified the role of TLR2 in innate immune responses against *L. pneumophila* infection, although other TLRS might also contribute to innate immunity against this organism (Akamine *et al*., 2005; Archer and Roy, 2006; Hawn *et al*., 2006; Fuse *et al*., 2007). TLR2 plays a pivotal role in the bacterial recognition and host resistance against the intracellular growth of *L. pneumophila* (Akamine *et al*., 2005; Fuse *et al*., 2007). TLR2 deficient mice attenuated IL-12p40 production (Akamine *et al*., 2005), and showed higher mortality to *L. pneumophila* infection compared to wild-type and TLR4-deficient mice (Hawn *et al*., 2006; Fuse *et al*., 2007). The purified LPS of *L. pneumophila* has been shown to be involved in TLR2-mediated macrophage activation (Girard *et al*., 2003; Braedel-Ruoff *et al*., 2005; Fuse *et al*., 2007), however, other bacterial components of *L. pneumophila* have not yet been studied.

In the present study, we have demonstrated that the 19 kDa, purified rPAL of *L. pneumophila*, one of cell-wall associated lipoproteins, could significantly induce production of proinflammatory cytokines such as IL-6 and TNF-α in macrophages from BALB/c or TLR4 deficient C3H/HeJ mice. The responses were significantly inhibited by TLR2 blocking antibody, but not affected by TLR4 blocking antibody, indicating that *Legionella* PAL appears to be a potent TLR2 agonist. This finding is consistent with the general notion that TLR2 recognizes various bacterial lipoproteins (Brightbill *et al*., 1999; Hirschfeld *et al*., 1999; Lien *et al*., 1999; Takeuchi *et al*., 1999).

Recently, Fuse *et al*. (2007) reported TLR2 as a recognition molecule of *L. pneumophila* LPS for induction of macrophage inflammatory protein-2 (MIP-2). They demonstrated that MIP-2 production was reduced in lungs from TLR2-deficient mice infection model and bone-marrow-derived macrophages in response to *L. pneumophila* LPS. One interesting observation of their study is that higher concentrations of LPS are able to overcome the lack of TLR2, suggesting the involvement of other factors. Therefore, it might be interesting to examine whether *Legionella* PAL as a TLR2 ligand involve in pulmonary inflammatory responses or MIP-2 production in murine infection models.

Legionella bacterium replicates in alveolar macrophages, but the disease results from a complex series of interactions with host (Cianciotto, 2001). Although *Legionella* species-common PAL antigen has been characterized as the most abundant protein of the soluble fractions (Kim *et al*., 2003), its role in pathogenesis is unknown. In the present study, *in vitro* rPAL-stimulated macrophages induced effectively IL-6 and TNF-α production in dose-dependent fashion, mediated by TLR2 signaling. This suggests that the PAL-induced cytokine mobilization can be either protective or detrimental, depending on the extent of the cytokine released during the innate immune responses. Newton *et al.* (2000) observed that survival of BALB/c mice from sublethal challenge of *L. pneumophila* was associated with elevated acute phase cytokines (TNF-α, IL-1β and IL-6), and susceptible IL-4 deficient mice showed higher levels of the cytokines. In addition, the PAL of gram-negative bacteria, as a naturally occurring TLR2 agonist, is shed by bacteria into circulation of sepsis animal and induces cytokine production by macrophages (Hellman *et al*., 2002; Liang *et al*., 2005). All these findings support that TLR2-mediated macrophage activation by *Legionella* PAL might play in part an important role in pathogenesis during the acute stage of *Legionella* infection.

In the present study, we have also shown that TLR2 as well as MyD88 mRNA expression was prominent in the rPAL-treated macrophages compared to the untreated controls. This indicates that TLR2-mediated signaling by the rPAL might be involved in initiating the MyD88 dependent response, although further studies using TLR2-deficient or MyD88-deficient mice are needed to confirm this. It is not clear whether additional MyD88-dependent, TLR2-independent pathways is crucial for full protection against *L. pneumophila* (Archer and Roy, 2006; Hawn *et al*., 2006). Moreover, the potential ability of the rPAL to activate other TLRS remains to be determined in future experiments.

As our previous study has shown that *Legionella* PAL induces strong PAL-specific antibody and cellular responses in BALB/c mice immunized with pcDNA3-PAL or the rPAL (Yoon *et al*., 2002), we are especially interested in developing strategies to enhance *Legionella* PAL-induced adaptive immunity by stimulating TLR-mediated innate immune responses. In our present study, coadministration of CpG ODN, as a TLR9 agonist, with the rPAL induced synergistic production of inflammatory cytokines (IL-12p40 and IL-6) compared to the rPAL

alone. CpG ODN is known to directly stimulate B cells and DCs, thereby enhancing the production of Th1-biased cytokines (IFN-γ and IL-12) as well as proinflammatory cytokines (IL-1, IL-6, IL-18 and TNF) (Krieg and Davis, 2006). Coadministration of CpG ODN with the rPAL antigen might favorably shift the rPAL-induced Th2 biased response to Th1 responses (Chu *et al*., 1997; Yoon *et al*., 2002). Especially, marked increase of IL-12 production would be beneficial in controlling early dissemination of *L. pneumophila* (Tateda *et al*., 2001a, 2001b). Together with these data, cooperated signaling via TLR2 and TLR9 stimulation might be used as one of new strategies to promote Th1 responses for vaccine development against various infection diseases.

Rogers and colleagues (2007) reported that exposure of bone marrow derived DCs to *L. pneumophila* increased expression of TLR2 and TLR4 and enhanced expression of costimulatory (CD40 and CD86) and MHC molecules. In the present study, we have shown that macrophages treated with the rPAL induced increased expression of CD40, CD80, CD86 and MHC I/II, and in particular, the combined treatment of CpG ODN and the rPAL had prominently increased the expression of CD40, which might lead to a greater chance of interaction between CD40 and CD40L and macrophage activation for IL-12 production (Grewal *et al*., 1997; Mathur *et al*., 2006). Therefore, these data support that *Legionella* PAL antigen is fully competent for stimulation of immune responses by expression of costimulatory and MHC molecules as well as cytokine production, and the addition of CpG ODN might expect to induce the synergistic effect via activation of TLR2- and TLR9-mediated signaling pathways.

In summary, our results clearly demonstrated the involvement of TLR2 in the recognition of *Legionella* PAL by murine peritoneal macrophages. Furthermore, we suggest that *Legionella* PAL as a vaccine antigen may activate the innate immunity at least through the TLR2-mediated signaling pathway, and then link to the development of the PAL-specific adaptive immunity. Furthermore, the selective targeting of bacterial cell components to TLR stimulation would appear to be a great assurance for vaccine development against infectious diseases.

Methods

Production and purification of a recombinant lipoprotein

The full protein-coding region of the pal gene of 19 kDa lipoprotein (Engleberg *et al*., 1991) was PCR-amplified from a DNA template derived from *L. pneumophila* serogroup 1 strain with a pair of primers: the *Xba*I containing sense primer 5'-TCTAGATTGTGGAATGAAAGCCGGATCGT-3' and *Sal*I containing anti-sense primer 5'-GTCCACCCATGAGGCGAAAGGAAGCATC-3'. The amplified DNA was digested with *Xba*I and *Sal*I, and ligated into an *Xba*I/*Sal*I-cut pMAL-c2X vector (New England Biolabs Inc., Ipswich, MA). The plasmid construct was used to transform *Escherichia coli* BL21(DE3)*pLysS* (Novagen, Madison, WI) for the production of the recombinant PAL protein fused to a 43 kDa maltose-binding protein (MBP). The *pal* gene with in frame cloning in the plasmid construct was identified by sequence analysis. The MBP-PAL fusion protein was purified using the pMAL protein fusion and purification system (New England Biolabs Inc.) according to the manufacturer's instructions. The 63 kDa purified fusion protein was cleaved with factor Xa at 23°C for 24 h and then applied to DEAE-sepharose resin. In this step, a free rPAL (19 kDa) was separately collected from the flow-through fraction. The protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The protein was identified by SDS-PAGE and immunoblotting with rabbit anti-rPAL IgG (Kim *et al*., 2003) (data not shown). To eliminate contaminating LPS from the rPAL preparation, all reagents were prepared with endotoxin-free solutions. The rPAL used in the experiments was tested for the LPS concentration by the Limulus amebocyte lysate test (BioWhitttaker, Walkersville, MD) and contained less than 20 pg of endotoxin per ml.

Preparation of peritoneal macrophage cells

Six-week-old female BALB/c and C3H/HeJ mice were purchased from Dae Han Bio Link Inc. (Seoul, Korea). Mouse peritoneal macrophages were isolated by peritoneal lavage with ice-cold sterile physiologic saline 5 days after intraperitoneal injection of 3 ml of 3% (w/v) sterile brewer thioglycollate (Becton Dickinson, Franklin Lakes, NJ). The cells were washed, resuspended in RPMI 1640 containing 10% FBS (Gibco, Grand island, NY), 1% penicillin-streptomycin (Gibco) and plated in 96-well flat bottomed plates (Nunclon Surface, Roskilde, Denmark) at a concentration of 1×10^5 cells per well. Following incubation in a humidified 5% CO_2 incubator for 24 h at 37°C, the cells were washed three times with culture medium before the specific treatment.

Cytokine ELISA

For blockade of the TLRs, macrophages (1×10^5 cells) were preincubated with 10 μg/ml of anti-TLR2 mAb (clone T2.5, eBioscience, CA) or anti-TLR4 mAb (clone MTS 510, eBioscience) for 1 h. Macrophages were pulsed with rPAL (10-500 ng/ml), Pam3CSK4 (100 ng/ml), CpG ODN (3 μg/ml) or CpG ODN (3 μg/ml) plus rPAL (100 ng/ml) for 18 h at 37°C in a humidified 5% CO_2 environment. The cell supernatants were collected and stored at -20°C. IL-12p40 (BioSource, Camarillo, CA), IL-6 (BioSource) and TNF-α (Serotec, Oxford, UK) levels were determined by ELISA kits according to the manufacturers' instructions. The optical density was measured at 450 nm by a VERSAmax

reader (Molecular Devices Co., Sunnyvale, CA). Pam3CSK4, a synthetic lipopeptide was purchased from Invivogen (San Diego, CA). CpG ODN was purchased from Bioneer Co. (Daejeon, Korea). The sequences of the CpG ODN used were 5'-TCCATGACGTTCCTGACGTT-3' (CpG DNA, ODN1826).

RT-PCR

The peritoneal macrophages (1×10^6 cells) were stimulated with 1 μg/ml of the rPAL for 18 h at 37°C. Total RNA was isolated from macrophage cultures using Trizol reagent (Invitrogen, Camarillo, CA) according to the manufacturer's protocol. To synthesize the first strand cDNA, reverse transcription of total RNA was performed with avian myeloblastosis virus RTase (Promega, Madison, WI). For the PCR amplification, the sample was heated at 94°C for 5 min, and then subjected to 30 amplification cycles of 1 min at 94°C, 30 s at 60°C, 1 min at 72°C, and 5 min at 72°C. PCR products were visualized with ethidium bromide in 1.5% agarose gels. The specific primer sets used were as follows: 5'-TTCCTACAGTGAGCAGGATTCCCAT-3' and anti-sense: 5'-CTTTTCGATGGAATCGATGATGTGT-3' for TLR2, sense: 5'-TAGCCATTGCTGCCAACATCATCCA-3' and anti-sense: 5'-TGCCAGAGCGGCTGCTCAGAAACT-3' for TLR4, sense: 5'-GGTCCATTGCCAGCGAGCTAATTG-3' and anti-sense: 5'-AATCAGTCGCTTCTGTTGGACAC-CT-3' for MyD88, and sense: 5'-AAGACCTCTATGCCA-ACACAGTGCT-3' and anti-sense: 5'-CCACACAGAGTAC-TTGCGCTCAGG-3' for β-actin.

Flow cytometry

The peritoneal macrophages (1×10^6 cells/ml) were incubated with CpG ODN (10 μg/ml), rPAL (10 μg/ml) or the combination of CpG ODN and rPAL for 24 h. The cells were harvested and washed with ice-cold PBS containing 2% FBS and stained with FITC- or PE-conjugated mAbs against CD40 (clone 3/23) (Santa cruze, Biotec. Inc.), CD80 (B7-1, clone 16-10A1) (eBioscience), CD86 (B7-2, clone GL-1) (eBioscience), MHC I (clone 34-1-2S) (eBioscience) and MHC II (clone M5/114.15.2) (eBioscience). In all experiment the cells were first treated with aggregated 1 μg/ml 2.4G2 (anti-FcrRII and -III: BD Phar-Mingen, San Diego, CA) to block nonspecific IgG binding. Cells for MHC I and II molecules were simultaneously stained with the indicated FITC- or PE-conjugated mAb for 30 min at 4°C. After immunolabeling, cells were washed in PBS with FBS, fixed in 1% paraformaldehyde. Ten thousand events were acquired on FACScan flow cytometer (Becton Dickinson, San Jose, CA). FACS data were analyzed using CellQuest software (Becton Dickinson).

Statistical analysis

All data were expressed as means ± SD. Data analysis was performed using the Sigma stat software (Systat Software Inc., San Jose, CA). Differences between group means were tested for statistical significance by one-way ANOVA and Duncan method post-hoc comparison. A P value of < 0.05 was considered statistically significant.

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