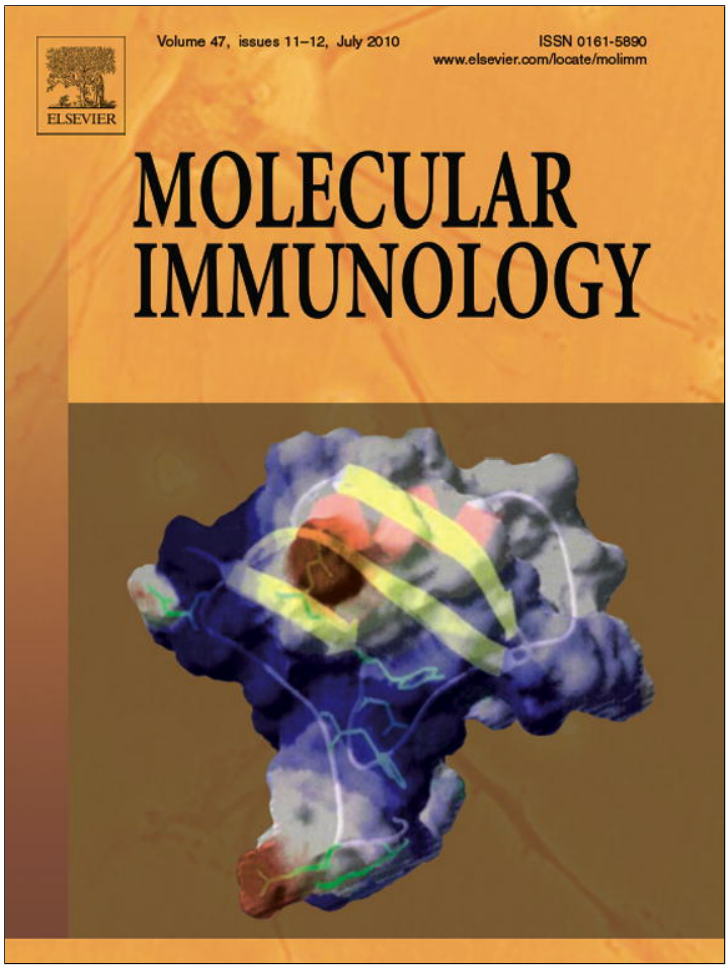

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A recombinant lipoprotein containing an unsaturated fatty acid activates NF-κB through the TLR2 signaling pathway and induces a differential gene profile from a synthetic lipopeptide

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ABSTRACT

The lipid moiety of a novel recombinant lipoprotein, which contains a dengue virus envelope protein domain 3, rlipo-D1E3, has been shown to activate antigen-presenting cells (APCs) as an intrinsic adjuvant. Because the lipid moiety of rlipo-D1E3 contains an unsaturated fatty acid, it is unclear if the receptor usage by bacterially derived lipoproteins is the same as that of the synthetic lipopeptide palmitoyl-3-Cys-Ser-$(Lys)_4$ (Pam3). In the present study, we show that the rlipo-D1E3 lipoprotein can induce the activation of spleen cells and bone marrow-derived dendritic cells (BM-DCs) in wild-type and TLR4-deficient mice, but not in $TLR2^{-/-}$ mice. After analyzing the co-receptor usage of TLR2 using $TLR1^{-/-}$ or $TLR6^{-/-}$ mice, the TLR2 signaling triggered by rlipo-D1E3 and Pam3 could use either TLR1 or TLR6 as a co-receptor. Analysis of the MAPK signaling pathway revealed that rlipo-D1E3 could initiate the phosphorylation of p38, ERK1/2 and JNK1/2 earlier than the synthetic lipopeptide. In addition, the expression levels of IL-23, IL-27 and MIP-1α in BM-DCs stimulated by rlipo-D1E3 were higher than the expression levels in BM-DCs stimulated by Pam3. Taken together, these results demonstrate that different TLR2 ligands can promote various immune responses by inducing different levels of biological cytokines and chemokines.

1. Introduction

Bacterial lipoproteins have been shown to activate antigen-presenting cells (APCs) through either Toll-like receptor (TLR)2 (Alexopoulou et al., 2002; Shimizu et al., 2008; Tawaratsumida et al., 2009; Thakran et al., 2008) or TLR4 (Revets et al., 2005). Importantly, N-terminal lipopeptides that contain di- or triacylated S-(2,3-dihydroxypropyl) cysteines have been proven to be essential moieties for TLR2 activation (BenMohamed et al., 1997; Buwitt-Beckmann et al., 2006). The synthetic analogs of di- or triacylated lipopeptides have been used to study host cell receptor specificity and downstream signaling (Buwitt-Beckmann et al., 2006; Into et al., 2007; Khan et al., 2009; Kiura et al., 2006). Using TLR2-, TLR1- or TLR6-deficient mice, di- or triacylated lipopeptides have been shown to be recognized by TLR2/TLR6 or TLR2/TLR1 heterodimers, respectively (Alexopoulou et al., 2002; Takeuchi et al., 2002). However, some studies have also suggested that di-palmitoylated lipopeptides or lipoproteins can activate NF-κB through a TLR6-independent signaling pathway (Buwitt-Beckmann et al., 2006; Shimizu et al., 2005). These studies indicate that the relationship between the number of fatty acids in the lipopeptide and the usage of a TLR2 co-receptor is still unclear. Kiura et al. found that the diacylated lipopeptide FSL-1 could induce TLR2-mediated Th2 responses (Kiura et al., 2006). In contrast, Infante-Duarte et al. found that an OVA-derived peptide bound to $I\text{-}A^d$ promoted a Th1 phenotype in the presence of *Borrelia burgdorferi* lysates or a synthetic triacylated lipopeptide (Infante-Duarte and Kamradt, 1997). Furthermore, this group also found that priming of TCR-transgenic T cells in the presence of *B. burgdorferi* lysates induced IL-17/TNF-α coproduction (Infante-Duarte et al., 2000). These studies imply that the structural details and configuration of fatty acids within lipopeptides or lipoproteins could have different effects on cell-mediated immunity. Therefore, it is of interest to investigate whether the different lipid structures of recombinant lipopro-

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teins and synthetic lipopeptides could influence various immune responses.

The production of lipoproteins for enhancing the antigenicity of recombinant proteins has been a promising approach to develop novel subunit vaccines. A lipoprotein fraction obtained from *Staphylococcus aureus*, a Gram-positive pathogen, was found to be a potent activator of TLR2 (Hashimoto et al., 2006), and the lipid moiety was identified as a diacylated peptide using tandem mass spectrometry (Tawaratsumida et al., 2009). Additionally, a membrane-bound OprI lipoprotein from *Pseudomonas aeruginosa* was used to engineer heterologous fusion proteins in Gram-negative bacteria (Cote-Sierra et al., 2002). OprI was also shown to modulate Th2-driven allergic immune responses *in vivo* for a prolonged period via stimulation of the TLR2 and TLR4 signaling pathways (Revets et al., 2005). OprI appears to induce non-specific immune responses when used as an adjuvant and to facilitate the uptake of the immunogen through an interaction with TLR2/4 on antigen-presenting cells. Recently, we reported a novel technology to produce recombinant rlipo-D1E3 (lipidated dengue virus envelope protein domain 3) at a high level using the special *Escherichia coli* strain C43 (DE3). The mass spectrum analysis of the purified recombinant lipoprotein revealed that it was triacylated at the N-terminus (Chen et al., 2009). We further found that the lipid moiety of rlipo-D1E3 contained one unsaturated fatty acid, and that rlipo-D1E3 could induce the maturation of dendritic cells. However, it was not clear if the double bond-containing recombinant lipoprotein could have different effector mechanisms compared to the synthetic triacylated lipopeptide Pam3.

In this report, we used TLR2-knockout ($TLR2^{-/-}$) and TLR4-deficient mice to study the effector mechanisms and signaling pathways of cells after stimulation with a prototypic dengue vaccine lipo-immunogen (rlipo-D1E3) that has been shown to have intrinsic adjuvant properties (Chen et al., 2009). Our results showed that rlipo-D1E3 could activate antigen-presenting cells through TLR2, but not through TLR4, and the initiation of the signaling pathway mediated by rlipo-D1E3 was similar to that of the tri-palmitoylated lipopeptide Pam3. Both rlipo-D1E3 and Pam3 activated TLR2 signaling independently of either TLR1 or TLR6. Interestingly, we found that rlipo-D1E3-stimulated antigen-presenting cells induced different levels of gene expression when compared to Pam3 stimulation. These results demonstrate that the recombinant lipoprotein has the ability to activate different levels of cytokine and chemokine gene expression downstream of TLR2, implying that the recombinant lipoprotein or the N-terminal lipid moiety of the lipoprotein may be used as a novel vaccine adjuvant.

2. Materials and methods

2.1. Reagents and animals

All chemicals were from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany), and the matrix used for the mass spectrometry analysis was from Promega Co. (Madison, WI, USA). The palmitoyl-3-Cys-Ser-$(Lys)_4$ (Pam3) was purchased from InvivoGen (San Diego, CA, USA). The NF-κB-dependent promoter fused to firefly luciferase was a kind gift from Dr. S.-F. Huang (National Health Research Institutes (NHRI), Taiwan). A reporter plasmid, pRL-TK, that constitutively expresses Renilla luciferase was purchased from Promega Co. (Madison, WI, USA), and a phTLR2flag plasmid was purchased from Addgene (Cambridge, MA, USA). The IL-6, TNF-α and IL-12p40 ELISA kits were from R&D Systems, Inc. (Minneapolis, MN, USA). TLR4-deficient mice (TLR4-def, C3H/HeJ) and TLR2-knockout mice ($TLR2^{-/-}$) were purchased from The Jackson Laboratory. The TLR1 knockout ($TLR1^{-/-}$) and TLR6 knockout ($TLR6^{-/-}$) mice were purchased from Oriental Bioservice, Inc. (Tokyo, Japan) and held in the Animal Center of the NHRI. C57BL/6 and C3H/HeN were purchased from the National Animal Center in Taiwan. All studies were approved by the Institutional Animal Care and Use Committee of the NHRI.

2.2. Production of recombinant lipo-immunogen and non-lipidated immunogen

Based on the consensus domain III of the dengue virus envelope protein (E3), the recombinant lipidated E3 immunogen (rlipo-D1E3) and its recombinant non-lipidated E3 (rE3) counterpart were prepared as described in our previous reports (Chen et al., 2009; Leng et al., 2009). Briefly, the E3 gene, either alone or fused with a lipidation signal DNA sequence, was cloned into the pET-22b vector (+) and expressed in *E. coli* C43 (DE3) or BL21 (DE3). Recombinant lipo-D1E3 and rE3 were purified by immobilized metal affinity chromatography (IMAC), and the amount of residual LPS was negligible (<3 EU/mg) in both preparations.

2.3. Splenocyte proliferation assay

Splenocytes from C57BL6, TLR4-deficient C3H/HeJ (TLR4-def), or TLR2-knockout ($TLR2^{-/-}$) mice were plated at a concentration of 2×10^5 cells/well in 96-well plates and stimulated with lipopeptides or lipoproteins for 48 h at 37 °C in a 5% CO_2 humidified incubator. During the final 24 h of culture, 1 μCi of $[^3H]$-thymidine was added to each well, and the cells were harvested using a FilterMate automatic cell harvester (Packard, Meriden, CT, USA). The radioactivity incorporated was determined on a TopCount microplate scintillation counter (Packard, Meriden, CT, USA). LPS (0.01 μg/mL) was included in the assay as a positive control. All results are presented as the mean cpm ± standard deviation (SD).

2.4. NF-κB luciferase reporter assay

HEK293 cells were plated onto 24-well plates (2×10^5 cells/well) and co-transfected with 0.1 μg of pFLAG-TLR2, 0.01 μg of pNF-κB-Luc, and 0.01 μg of the pRL-TK internal control plasmid (Promega, Madison, WI, USA) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h, the transfected cells were stimulated with various synthetic lipopeptides or recombinant proteins for 24 h. The cells were lysed so that the luciferase activity could be measured using a dual-luciferase reporter assay system (Promega Co., Madison, WI, USA). Firefly luciferase activities were compared to Renilla luciferase activities for the purposes of normalization. Both firefly and Renilla luciferase activities were monitored using a Berthold Orion II luminometer (Pforzheim, Germany).

2.5. Western blot analysis

The BM-DCs were grown in DMEM or RPMI medium without FBS for 16–18 h and then stimulated with 100 nM of the indicated lipopeptides or lipoproteins for 10, 20, 40, 60, 90, or 120 min. The concentration of the protein in the lysates was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). A total of 50 μg of lysate was electrophoresed per lane in a 10% SDS-PAGE gel. After the resolved lysates in the gel were transferred to a PVDF membrane, the membranes were blocked with 5% milk and 0.05% Tween-20. The membranes were incubated with antibodies against p38, pp38, pERK1/2, or pJNK1/2 in 5% (w/v) BSA, 1× PBS and 0.1% Tween-20. An anti-p38 antibody was used as a control for protein loading in each lane. The membrane was developed using the LumiGLO® Chemiluminescent Substrate system (Millipore, Billerica, MA, USA).

Table 1
The UPL number and primer sequences for mRNA analysis by real-time PCR. Sequences designed for the detection of indicated gene products by real-time PCR are presented.

Gene name	UPL no.	Primers
GADPH	69	Forward: 5′-ggagcggtagcacctcct-3′ Reverse: 5′-ctggttcatcatcgctaatcac-3′
IL-1α	52	Forward: 5′-ttggttaaatgacctgcaaca-3′ Reverse: 5′-gagcgctcacgaacagttg-3′
IL-23	6	Forward: 5′-gagacactgatttgtgggaaaga-3′ Reverse: 5′-aaatgacacatgtcagattgctg-3′
IL-27	38	Forward: 5′-catggcatcacctctctgac-3′ Reverse: 5′-aagggccgaagtgtggta-3′
IL-10	3	Forward: 5′-gctgccgtcattttctgc-3′ Reverse: 5′-tctcactggcccgtcatc-3′
IL-13	17	Forward: 5′-cctctgacccttaaggagcttat-3′ Reverse: 5′-cgttgcacaggggagtct-3′
TGF-β1	72	Forward: 5′-tggagcaacatgtggaactc-3′ Reverse: 5′-cagcagccggttaccaag-3′
MCP-1	62	Forward: 5′-catccacgtgttggctca-3′ Reverse: 5′-gatcatcttgctggtgaatgagt-3′
MIP-1α	40	Forward: 5′-caagtcttctcagcgccata-3′ Reverse: 5′-ggaatcttccggctgtagg-3′
CXCL1	75	Forward: 5′-ttttgtatgtattagggtgaggacat-3′ Reverse: 5′-gcgtgttgaccatacaatatgaa-3′

2.6. Real-time quantitative PCR (qPCR) analysis of gene expression

The BM-DCs were purified to 60–90% using the Dynabeads® Mouse DC Enrichment kit (Invitrogen Dynal AS, Oslo, Norway) according to the manufacturer's instructions. The purified BM-DCs were stimulated using medium alone or 100 nM of Pam3 or rlipo-D1E3 at 37 °C for 2 or 4 h. Total RNA was extracted from the isolated cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. RNA (0.5–1 μg) was reverse-transcribed to cDNA using an oligo-dT primer in a 20 μL volume and SuperScript III RT (Invitrogen, Carlsbad, CA, USA). The mouse Universal Probe Library (UPL) set (Roche, Mannheim, Germany) was used to perform the real-time qPCR assay for gene expression in isolated cell populations (Liu et al., 2007). The specific primers and the UPL number used are listed (Table 1). Target gene expression was normalized to HPRT gene expression. The relative gene expression levels were calculated by comparing the expression levels after various treatment conditions to those obtained using medium alone.

3. Results

3.1. Characterization of purified rlipo-D1E3

Previously, the lipid modifications of rlipo-D1E3 were identified and characterized by mass spectrometry (Chen et al., 2009). In order to obtain more information about the lipid modifications, the tryptic fragments of rlipo-D1E3 were further purified using C18 silica resin and analyzed by MS/MS spectrometry. The molecular weight of Pam3 (lipid-Cys-Ser-Lys-Lys-Lys-Lys) was 1509.6, and the y-ions were identified using collision-induced dissociation (CID) MS/MS analysis (Fig. 1A). The mass of the lipid-cysteinyl residue of Pam3 was 892.2 atomic mass units (amus), which matched the predicted value of palmitoyl-3-cysteine ($SNO_6C_{54}H_{102}$). On the other hand, the first major peak of rlipo-D1E3 (lipid-Cys-Ser-Gln-Glu-Ala-Lys) had an *m*/*z* value of 1452.2; the rlipo-D1E3 y-ions were also identified using CID MS/MS analysis, and the mass of the lipid-cysteinyl residues of N-terminal rlipo-D1E3 was found to be 890, which is 2 amu less than that of Pam3 (Fig. 1B). These results suggest that there is a double bond in the lipid moiety of rlipo-D1E3.

3.2. The rlipo-D1E3 stimulates spleen cells to proliferate through TLR2

To investigate if TLR4 or TLR2 signaling could be observed in response to rlipo-D1E3, $TLR2^{-/-}$ and TLR4-deficient mice were used to study the receptor specificity of rlipo-D1E3. Because lipopeptides have been shown to stimulate the proliferation of spleen cells, we isolated spleen cells from wild-type, $TLR2^{-/-}$ and TLR4-deficient mice and stimulated them with lipopolysaccharide (LPS), Pam3, rlipo-D1E3 or non-lipidated E3 (rE3). We found that LPS, Pam3 and rlipo-D1E3 could stimulate the proliferation of wild-type spleen cells, but rE3 failed to induce proliferation (Fig. 2). The spleen cells from $TLR2^{-/-}$ mice did not proliferate after stimulation with Pam3 or rlipo-D1E3. In contrast, spleen cells from TLR4-deficient mice still responded to Pam3 and rlipo-D1E3. These results indicate that TLR2 is the major receptor responsible for the rlipo-D1E3-induced spleen cell proliferation; thus, rlipo-D1E3 is very similar to lipopeptides reported in previous studies (Buwitt-Beckmann et al., 2006; Farhat et al., 2008).

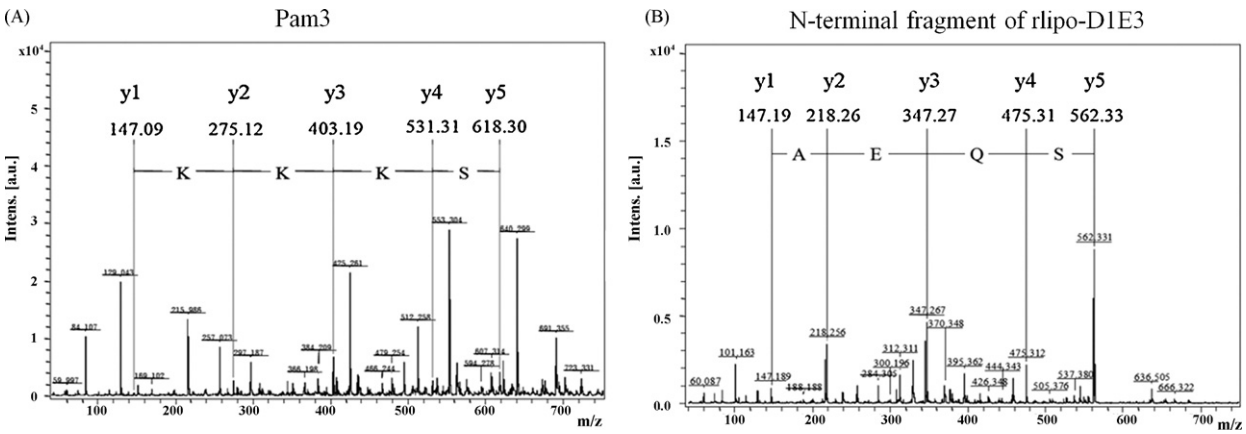

Fig. 1. Identification of the synthetic lipopeptide and purified N-terminal fragments of rlipo-D1E3. (A) The molecular mass of Pam3 is 1509.6 Da, and the sequence is lipid-Cys-Ser-Lys-Lys-Lys-Lys. The y-ions, y1–y5, have been identified, and they confirm that the sequence has no further modifications. The mass of N-acyl-S-diacylglyceryl-cysteinyl from Pam3 is 892.2, which agrees with the expected composition: $SNO_6C_{54}H_{102}$. (B) The y-ions, y1–y5, have been identified in the first major peak of rlipo-D1E3, and they confirm that the sequence has no further modifications. The mass of N-acyl-S-diacylglyceryl-cysteinyl at peak 1452 is 890.0.

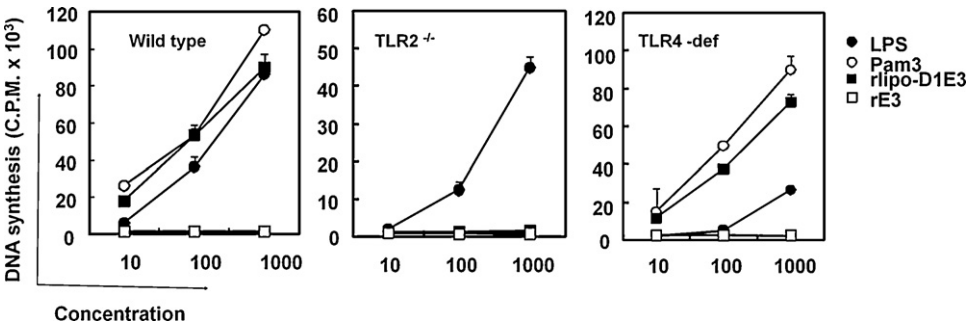

Fig. 2. DNA synthesis in splenocytes from TLR2$^{-/-}$, TLR4-deficient and wild-type mice after stimulation with rlipo-D1E3. Spleen cells were isolated from wild-type, TLR2$^{-/-}$ and TLR4-deficient mice and plated at a density of 2×10^5 cells/well in 96-well plates. The cells were incubated with various concentrations of LPS (0–1000 ng/mL), Pam3 (0–1000 nM), rlipo-D1E3 (0–1000 nM) or rE3 (0–1000 nM) for 48 h. During the final 24 h, 1 μCi of [^{3}H]-thymidine was added to each well to measure DNA synthesis. The data represent the mean ± SD of triplicate samples.

3.3. The rlipo-D1E3-induced cytokine production from BM-DCs is mediated by TLR2

BM-DCs derived from wild-type, TLR2$^{-/-}$ and TLR4-deficient mice were used as a model to study the activation of antigen-presenting cells. As shown in Fig. 3A, rlipo-D1E3 was capable of stimulating the production of TNF-α, IL-6 and IL-12p40 from the BM-DCs of wild-type and TLR4-deficient mice but not from the BM-DCs of TLR2$^{-/-}$ mice. The stimulation profile of rlipo-D1E3 was found to be similar to that obtained with the TLR2 agonist, Pam3. In contrast, the stimulating effect of LPS was lost in TLR4-deficient cells but remained in TLR2$^{-/-}$ and wild-type cells. CpG could stimulate BM-DCs from wild-type, TLR2$^{-/-}$ and TLR4-deficient mice to secrete cytokines. These results clearly confirm that the activation of BM-DCs by rlipo-D1E3 is due to the TLR2-activating function and not to the activation of TLR4. Furthermore, wild-type, TLR2$^{-/-}$ or TLR4-deficient mice were immunized with rlipo-D1E3 to evaluate whether the antibody titer would be decreased in TLR2$^{-/-}$ mice. The mice were immunized twice at 2-week intervals, and serum was collected 2 weeks after the final immunization. The anti-rE3 antibodies were analyzed at a 1:500 or 1:2500 dilution by ELISA. We found that rlipo-D1E3-immunized wild-type and TLR4-deficient mice produced equivalent titers of anti-rE3 antibodies, but anti-rE3 antibody titers were decreased when TLR2$^{-/-}$ mice were immunized (Fig. 3B). These results again demonstrate that TLR2 is the responsive receptor for rlipo-D1E3 to enhance immune responses. The heterodimerization of TLR2 with TLR1 or TLR6 has been documented to induce downstream signaling. It has been reported that triacylated lipopeptides (i.e., Pam3) induce cell activation through TLR1/TLR2 heterodimers (Thakran et al., 2008), whereas diacylated lipopeptides (i.e., the macrophage-activating lipopeptide from *Mycoplasma fermentans* (MALP2)) activate cells

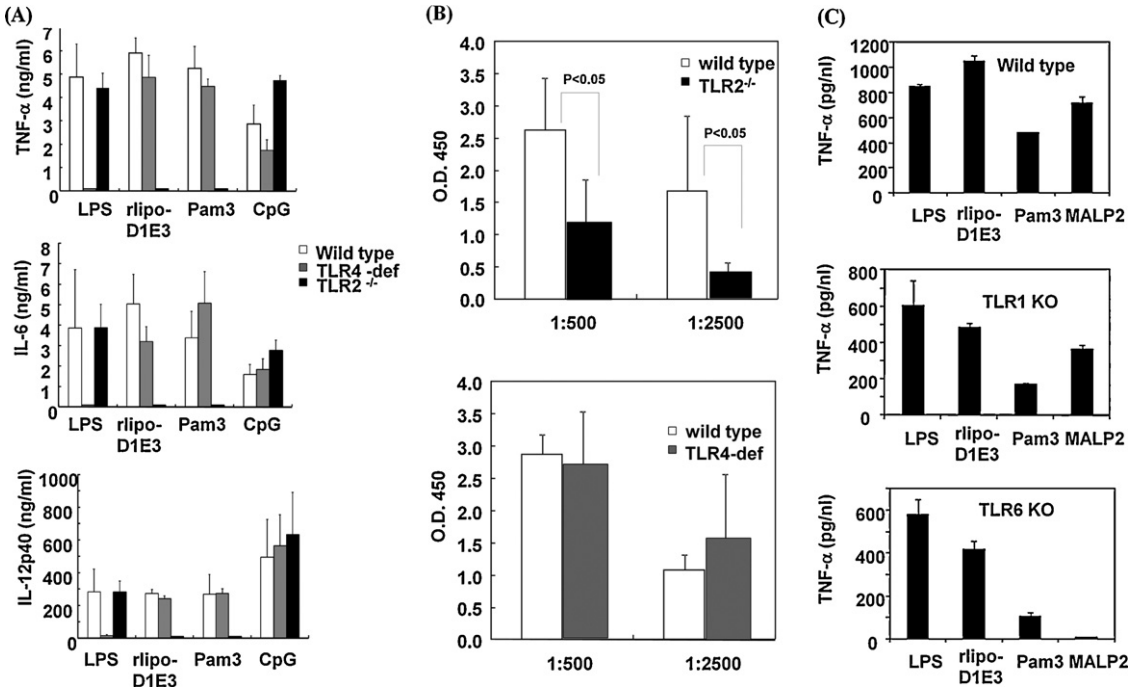

Fig. 3. The effector responses of BM-DCs after stimulation with rlipo-D1E3. (A) BM-DCs from wild-type, TLR4-deficient and TLR2$^{-/-}$ mice were cultured in medium alone or in medium supplemented with LPS (0.01 μg/mL), rlipo-D1E3 (100 nM), Pam3 (100 nM) or CpG (10 μg/mL). After incubation for 24 h, the supernatants were harvested and analyzed for TNF-α, IL-6 and IL-12p40 by ELISA. The data are expressed as the mean ± SD from three independent experiments. □, wild-type mice; ■, TLR4-deficient mice; ■, TLR2$^{-/-}$ mice. (B) The wild-type, TLR4-def, and TLR2$^{-/-}$ mice were immunized twice with 20 μg of rlipo-D1E3 at a 2-week interval. Serum samples were collected at week 4, and anti-rE3 antibody titers were determined using a sandwich ELISA. Sera were from 5 individual mice from each group. Significance was determined using a Student's *t*-test. (C) BM-DCs from wild-type, TLR1$^{-/-}$ and TLR6$^{-/-}$ mice were cultured either in medium alone or in medium supplemented with LPS (0.01 μg/mL), rlipo-D1E3 (100 nM), Pam3 (100 nM) or MALP2 (100 nM). After incubation for 24 h, the supernatants were harvested and analyzed for TNF-α by ELISA. The data represent the mean ± SD of triplicate samples.

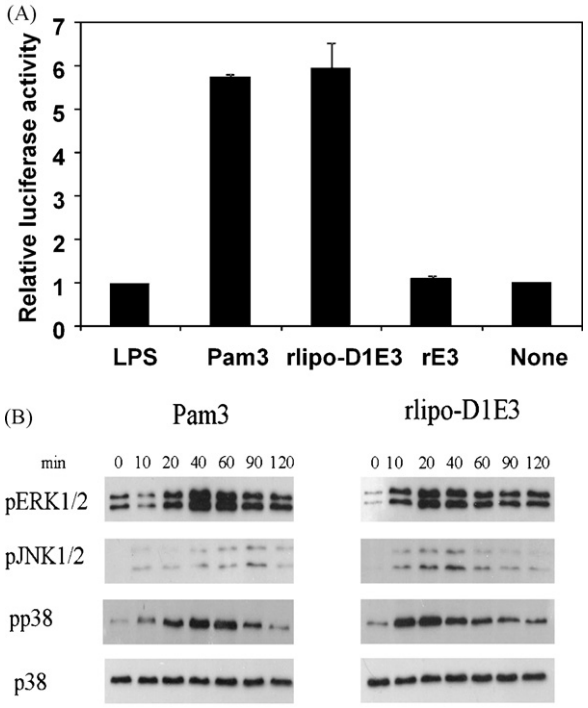

Fig. 4. Intracellular signaling after stimulation with rlipo-D1E3 or Pam3. (A) The 293T cells were transfected with 0.1 μg/mL of hTLR2flag in the presence of 0.01 μg/mL of pNF-κB-Luc and 0.01 μg/mL of pRL-TK. After 24 h, cells were stimulated with rlipo-D1E3 or Pam3 as indicated and cultured for another 5 h. The activation of NF-κB to express firefly luciferase was normalized relative to TK-driven Renilla luciferase expression. The relative luciferase activity was normalized to 1 in the absence of stimulation. The data represent the mean ± SD from triplicate samples. (B) BM-DCs were stimulated with 100 nM of Pam3 or rlipo-D1E3 for 10, 20, 40, 60, 90 or 120 min at 37 °C. Cell lysates were extracted and separated by 10% SDS-PAGE. After blotting for phosphorylated p38, ERK1/2, and JNK1/2, the membranes were developed with a chemiluminescent HRP substrate. The total p38 level was used for normalization.

through TLR2/TLR6 heterodimers (Buwitt-Beckmann et al., 2006; Thakran et al., 2008). In order to study the co-receptor usage for rlipo-D1E3, the BM-DCs from TLR1 or TLR6 knockout mice (TLR1$^{-/-}$ and TLR6$^{-/-}$) were stimulated with rlipo-D1E3, Pam3 or MALP2. Fig. 3C shows that rlipo-D1E3 and Pam3 could stimulate BM-DCs from TLR1$^{-/-}$ or TLR6$^{-/-}$ mice to secrete TNF-α, but MALP2 could not stimulate BM-DCs from TLR6$^{-/-}$ mice to secrete TNF-α. These data further show that rlipo-D1E3 does not act through TLR1- or TLR6-dependent TLR2 activation.

3.4. The kinetics of signaling downstream of rlipo-D1E3 stimulation differ from those induced by the synthetic lipopeptide, Pam3

It is well known that TLR-mediated signal transduction occurs through the NF-κB activation pathway. We already showed that rlipo-D1E3 and Pam3 could activate BM-DCs through TLR2. To investigate whether the different fatty acid modifications between Pam3 and rlipo-D1E3 have any effect on cellular signaling, the activation of the NF-κB or MAPK pathway was investigated. We transfected 293T cells with hTLR2flag, pNF-κB-Luc and pRL-TK for 24 h and then stimulated the cells with LPS, Pam3, rlipo-D1E3, and rE3 for 5 h. The relative firefly luciferase activity induced by the NF-κB promoter was determined using Renilla luciferase expression. Fig. 4A shows that both Pam3 and rlipo-D1E3 could induce 5–6-fold increases in relative luciferase activity compared to the stimulation with LPS or rE3. This result indicated that both Pam3 and rlipo-D1E3 could activate NF-κB through TLR2. Furthermore, the phosphorylation of p38, ERK1/2 and JNK1/2 in BM-DCs was measured at different time intervals (0, 10, 20, 40, 60, 90 and 120 min) using phospho-specific antibodies. The rlipo-D1E3 was found to induce the phosphorylation of p38, ERK1/2, and JNK1/2 earlier than Pam3. The highest rlipo-D1E3-induced levels of p38, ERK1/2 and JNK1/2 phosphorylation were observed at 20 min in BM-DCs (Fig. 4B). In contrast, the highest phosphorylation levels of p38, ERK1/2 and JNK1/2 induced by Pam3 were at 40, 40 and 90 min, respectively. The stimulation of antigen-presenting cells by rlipo-D1E3 induced the same MAPK phosphorylation as Pam3, but the kinetic profiles induced by both agonists were different. Based on this data, we were interested in the gene expression profiles of antigen-presenting cells after stimulation with both TLR2 agonists.

3.5. The rlipo-D1E3 lipoprotein induces different cytokine and chemokine gene expression levels in BM-DCs

To determine whether rlipo-D1E3 elicits different downstream gene expression than Pam3, we examined the gene expression profile of cytokines and chemokines in BM-DCs after stimulation with Pam3 or rlipo-D1E3. The Th1/Th17-associated cytokine genes (IL-1α, IL-23 and IL-27) (Apte and Voronov, 2008; Hunter, 2005), Th2-associated cytokine genes (IL-10, IL-13 and TGF-β) (Bechetoille et al., 2006; Young et al., 2000) and chemokines secreted by activated dendritic cells (MCP-1, MIP-1α and CXCL1) (Allavena et al., 2000; Rossi and Zlotnik, 2000) were selected for analysis. The gene expression of the control group (medium alone) was set to 1 to normalize the gene expression levels after stimulation. Induction levels of gene expression higher than 2-fold were considered to be significant. The rlipo-D1E3 lipoprotein stimulation was found to induce higher gene expression levels than Pam3 of IL-1α, IL-23, IL-27, IL-13, MCP-1 and MIP-1α. In contrast, the IL-10, TGF-β and CXCL1 expression levels were similar after Pam3 or rlipo-D1E3 stimulation (Fig. 5A). The protein production of IL-23, IL-27 and MIP-1α was measured after stimulation to confirm the gene expression analysis. We found that rlipo-D1E3 induced higher levels of IL-23, IL-27 and MIP-1α secretion than Pam3 (Fig. 5B). The upregulation of Th1/Th17-associated genes (IL-1α, IL-23 and IL-27) indicates that the adjuvant properties of rlipo-D1E3 preferentially stimulate Th1/Th17 immune responses. These results suggest that rlipo-D1E3 and Pam3 activate BM-DCs through the same receptor (TLR2) and a similar MAPK signaling pathway. However, they may induce different gene expression levels to regulate immune responses.

4. Discussion

Most native bacterial lipoproteins activate innate immune responses through TLR2 that is present on antigen-presenting cells (Giambartolomei et al., 2004; Massari et al., 2006; Shimizu et al., 2005, 2008; Thakran et al., 2008). However, the recombinant lipoprotein OspA from *B. burgdorferi* was found to induce similar levels of antibody titers in immunized TLR2-deficient mice when compared to immunized wild-type mice (Yoder et al., 2003). These results indicated that the recombinant OspA had adjuvant properties independent of TLR2. Furthermore, the recombinant lipoprotein OprI from *P. aeruginosa* was shown to stimulate dendritic cells through TLR2 or TLR4 ligation (Revets et al., 2005) when the lipoprotein was expressed in *E. coli* (Cote-Sierra et al., 1998). These controversial results led us to analyze the lipid structures and effector mechanisms of a recombinant lipidated dengue virus vaccine candidate, rlipo-D1E3, which has been shown to have intrinsic adjuvant properties (Chen et al., 2009), specifically because the information on the structure of lipid moieties in bacterially derived lipoproteins is limited. Recently, the lipid moiety of a 30–35 kDa

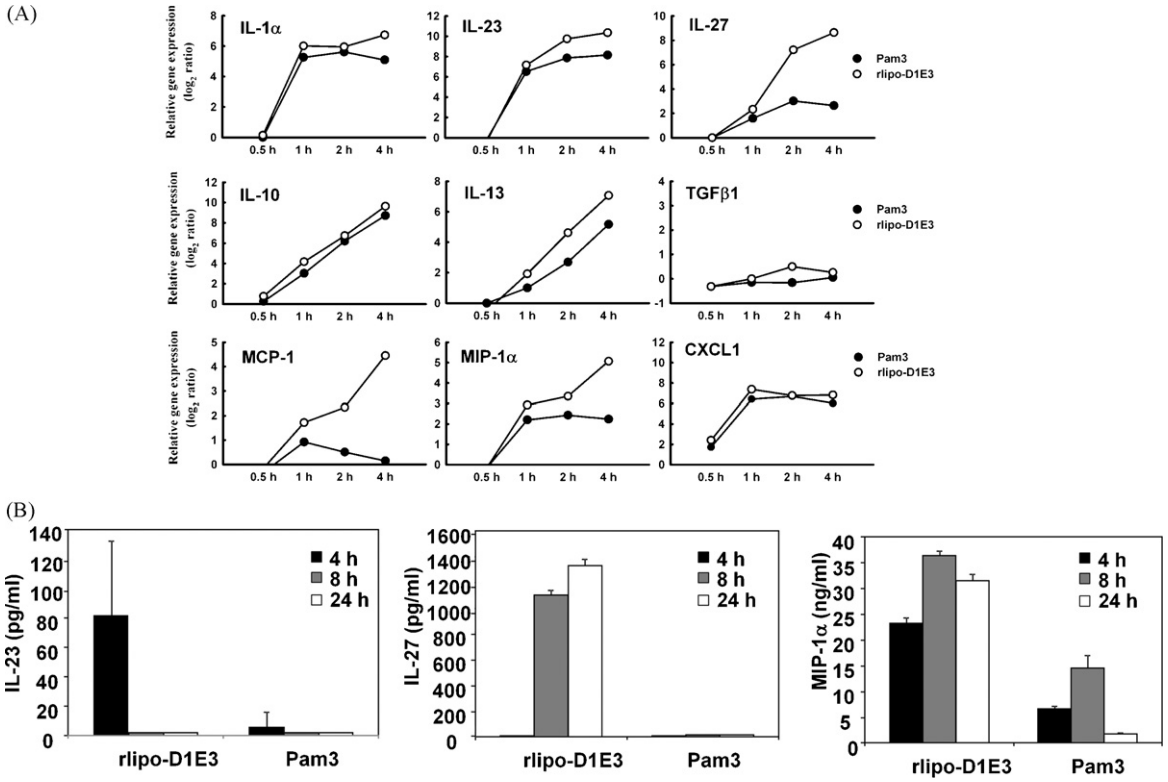

Fig. 5. The gene expression profile of cytokines and chemokines in murine BM-DCs. (A) The purified BM-DCs were stimulated with 100 nM of Pam3 or rlipo-D1E3 for 0.5, 1, 2 or 4 h at 37 °C. Cells were harvested, and the RNA was extracted for real-time qPCR analysis. The gene expression was normalized to HPRT mRNA. To compare the gene expression among the stimulated groups, the gene expression level was set to 1 in the control (medium alone) group. The data shown are representative of 2 independent experiments. (B) The purified BM-DCs were stimulated with 100 nM of Pam3 or rlipo-D1E3 for 4, 8 or 24 h at 37 °C. Supernatants were harvested, and the concentration of IL-23, IL-27 and MIP-1α was determined using ELISA kits. The data shown are representative of 3 independent experiments.

lipoprotein derived from *S. aureus* that stimulates cells through TLR2 was identified as a diacylated protein by MS/MS spectrometry (Tawaratsumida et al., 2009). Post-translational lipid modifications in lipoproteins vary among bacteria (Giambartolomei et al., 2004; Revets et al., 2005; Shimizu et al., 2005, 2008; Tawaratsumida et al., 2009).

The effector mechanisms of a lipoprotein that contains an unsaturated fatty acid have not been reported in previous studies. To understand the effector mechanisms, we studied the binding specificity of rlipo-D1E3 to cellular receptors and the subsequent intracellular signaling pathways accessed. Our results demonstrated that the major receptor used by rlipo-D1E3 was TLR2, not TLR4. Therefore, the recombinant lipoprotein acting through TLR2 may have different effects than other TLR2 agonists on APCs. Although Buwitt-Beckmann et al. (2006) found that the heterodimerization of TLR2 with TLR1 or TLR6 did not lead to differential intracellular signaling, the rlipo-D1E3-induced intracellular signaling was unknown. To address this, the same amount of Pam3 and rlipo-D1E3 was used to stimulate BM-DCs, and we found rlipo-D1E3 to induce higher levels of inflammatory cytokine and chemokine RNA transcripts (IL-1α, IL-23, IL-27, IL-13, MCP-1 and MIP-1α) (Fig. 5). IL-1α has been shown to stimulate NK cells to secrete IFN-γ for maximal IL-12-driven Th1 responses (Weaver et al., 1988). The IL-12 family members, IL-23 and IL-27, can prolong IFN-γ production by effector T cells (Smits et al., 2004) and induce the early production of IFN-γ in naïve Th cells (Pflanz et al., 2002), respectively. Recently, IL-23 and IL-27 were found to influence Th17 cell activities by promoting the differentiation of Th17 cells or by directly antagonizing the development of Th17 cells, respectively (Goriely et al., 2008). The upregulation of cytokines and chemokines after stimulation with rlipo-D1E3 indicated that the recombinant lipoprotein might have different effector functions as compared to Pam3. In contrast, Farhat et al. used FSL-1 (TLR1-dependent), Pam2C-SK4 (TLR1- and TLR6-dependent), or PamOct2C0 (VPGVG)-4VPGKG (TLR6-dependent) to stimulate murine dendritic cells and analyzed the phosphorylation of MAPK and gene expression profiles. They concluded that the lipoproteins present in different pathogens might activate different TLR dimers but use the same signaling pathways for gene activation (Farhat et al., 2008). We are currently interested in investigating whether the unique double bond of the lipid moiety or the D1E3 domain of rlip-D1E3 is contributing to the induction of higher levels of cytokine and chemokine gene expression. We are purifying the N-terminal lipid moiety of rlipo-D1E3 for further studies. Taken together, the elevated expression levels of cytokines and chemokines imply that the effector mechanisms of rlipo-D1E3 differ from those triggered by Pam3. The N-terminal lipid moiety of rlipo-D1E3 could potentially be used as a novel adjuvant to stimulate immune responses in vaccine development.

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