Ureaplasma parvum lipoproteins, including MB antigen, activate NF- κ B through TLR1, TLR2 and TLR6

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Ureaplasma species (*Ureaplasma parvum* and *Ureaplasma urealyticum*) are commonly isolated pathogens from the female reproductive tract and are associated with perinatal diseases in humans. Inappropriate induction of inflammatory responses may be involved in the occurrence of such diseases; however, pathogenic agents that induce the inflammatory response have not been identified in ureaplasmas. In this study, we examined the involvement of Toll-like receptors (TLRs) in the activation of the immune response by *U. parvum* lipoproteins, as well as the *U. parvum* components responsible for nuclear factor κ B (NF- κ B) activation. The Triton X-114 (TX-114) detergent phase of *U. parvum* was found to induce NF- κ B through TLR2. The active components of the TX-114 detergent phase were lipoproteins, such as multiple banded (MB) antigen, UU012 and UU016 of *U. parvum*. The activation of NF- κ B by these lipoproteins was inhibited by dominant negative (DN) constructs of TLR1 and DN TLR6. Thus, the lipoproteins from *U. parvum* were found to activate NF- κ B through TLR1, TLR2 and TLR6. Furthermore, these lipoproteins possessed an ability to induce tumour necrosis factor- α (TNF- α) in mouse peritoneal macrophages.

Received 20 December 2007

Revised 25 January 2008

Accepted 6 February 2008

INTRODUCTION

Ureaplasmas are wall-less bacteria belonging to the *Mycoplasmataceae*. Although *Ureaplasma* species (*Ureaplasma parvum* and *Ureaplasma urealyticum*) are common commensals of the urogenital tract of humans, ureaplasmas have frequently been recognized as important pathogens associated with non-gonococcal urethritis (Shepard, 1954), chorioamnionitis (Cassell *et al.*, 1986; Maher *et al.*, 1994), preterm birth (Jelsema, 2006; Kataoka *et al.*, 2006; Ollikainen *et al.*, 1998), infertility (Taylor-Robinson, 1986; Xu *et al.*, 1997), pneumonia in neonates (Waites *et al.*, 1989) and bronchopulmonary dysplasia in neonates (Schelonka *et al.*, 2005). The mechanisms by which ureaplasmas cause such diseases are unclear, but the inappropriate induction of inflammatory responses may be involved. It has been reported that the levels of interleukin-6 (IL-6), tumour necrosis factor- α (TNF- α), IL-1 β and

IL-8 in intra-amniotic fluid infected with ureaplasmas are increased (Yoon *et al.*, 1998). Moreover, macrophages are capable of producing such cytokines and nitric oxide in response to *U. urealyticum* (Li *et al.*, 2000a, b). However, pathogenic agents such as endotoxin and exotoxin that induce an inflammatory response have not been identified in ureaplasmas.

It has been widely known that Toll-like receptors (TLRs) with the function of pattern-recognition receptors play critical roles in early innate recognition and inflammatory responses by the host against invading microbes (Akira & Takeda, 2004; Kopp & Medzhitov, 1999). Among 10 TLR family members reported, TLR2, TLR4, TLR5 and TLR9 have been implicated in the recognition of different bacterial components. Peptidoglycan (PGN), lipoarabinomannan, zymosan and lipoproteins from various microorganisms are recognized by TLR2 (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999; Lien *et al.*, 1999; Means *et al.*, 1999; Takeuchi *et al.*, 1999, 2000, 2002; Underhill *et al.*, 1999). On the other hand, LPS, bacterial flagellin and bacterial DNA are recognized by TLR4, TLR5 and TLR9, respectively (Hayashi *et al.*, 2001; Hemmi *et al.*, 2000; Hoshino *et al.*, 1999; Poltorak *et al.*, 1998). These TLR family members have been shown to activate nuclear factor κ B (NF- κ B) via IL-1R-associated signal molecules, including myeloid differentiation protein (MyD88), IL-1R-activated kinase (IRAK), TNFR-associated factor 6

Abbreviations: DN, dominant negative; FAM20, synthetic lipopeptide containing N-terminal 20 amino acids of F₀F₁-type ATPase derived from *Mycoplasma pneumoniae*; IL, interleukin; MALP-2, macrophage-activating lipopeptide 2; MB, multiple banded; NF- κ B, nuclear factor κ B; Pam₃CSK₄, (S)-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH, 3HCl; PGN, peptidoglycan; PMF, peptide mass fingerprinting; sN-ALP2, synthesized NF- κ B-activating lipopeptide 2; TLR, Toll-like receptor; TNF- α , tumour necrosis factor- α ; TX-114, Triton X-114.

(TRAF6) and NF- κ B-inducing kinase (NIK) (Medzhitov *et al.*, 1998).

In this study, we examined the involvement of TLRs in the activation of the immune response by lipoproteins from *U. parvum*, as well as the *U. parvum* components responsible for NF- κ B activation. We observed that the Triton X-114 (TX-114) detergent phase of *U. parvum* could induce NF- κ B through TLR2. The active components of the TX-114 detergent phase responsible for NF- κ B activation were found to be 75 and 55 kDa proteins (P75 and P55, respectively). The NF- κ B-inducing activity of P75 was much higher than that of P55. By analysis with peptide mass fingerprinting (PMF), P75 was matched to a multiple banded (MB) antigen of *U. parvum*, and P55 was found to be a mixture of lipoproteins containing UU012 and UU016. The activation of TLR signalling by P75 and P55 was apparently dependent on TLR1, TLR2 and TLR6. These lipoproteins also induced TNF- α in mouse peritoneal macrophages. Thus, the results indicate that the MB antigen induces inflammatory responses that might be responsible for pathogenesis in *U. parvum* infection. Therefore, the MB antigen might be a candidate molecule for the prevention and therapy of *U. parvum* infection.

METHODS

Cells. Cells of a human kidney cell line, 293T, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 U penicillin G ml⁻¹ and 100 μ g streptomycin ml⁻¹.

TX-114 phase partitioning. *U. parvum* serovar 3 (ATCC 700970) was cultured in PPLO broth, pH 6.0, containing 20 % horse serum, 0.25 % yeast extract and 100 μ g ampicillin ml⁻¹ to the beginning of stationary phase, and then pelleted by centrifugation for 30 min at 12 000 g. TX-114 phase partitioning was performed as described by Feng & Lo (1994, 1999). Briefly, a ureaplasma pellet was suspended in Tris-buffered saline (TBS) containing 1 mM EDTA (TBSE), solubilized by adding TX-114 to a final concentration of 2 %, and incubated at 4 °C for 1 h. The lysate was incubated at 37 °C for 10 min for phase separation. After centrifugation at 10 000 g for 20 min, the upper aqueous phase was removed and replaced by the same volume of TBSE. The procedure of phase separation was repeated twice. The final TX-114 detergent phase was resuspended in TBSE to the original volume, and 2.5 volumes of ethanol were added to precipitate membrane components, followed by incubation at -20 °C overnight. After centrifugation, the pellet was suspended in DMSO. The aqueous phase was washed two times by adding TX-114 to a final concentration of 2 %. The TX-114-insoluble pellet was dissolved in DMSO. The protein concentration of the suspension was measured by Protein Assay CBB Solution (Nacalai Tesque).

Expression vectors. To prepare TLR1, TLR2 and TLR6 expression vectors (pFLAG-TLR1, pFLAG-TLR2 and pFLAG-TLR6), the coding regions of TLR1, TLR2 and TLR6 minus the respective N-terminal signal sequences were amplified by PCR from a cDNA of THP-1 and cloned into the expression vector pFLAG-CMV1 (Sigma), in which a preprotrypsin leader precedes an N-terminal FLAG epitope. Dominant negative (DN) TLR1 and TLR6 expression vectors were constructed by subcloning Toll and IL-1 receptor (TIR) homology domain-deleted TLR1 and TLR6 fragments into pFLAG-CMV1 (pFLAG-dTLR1 and pFLAG-dTLR6, respectively). The NF- κ B

cis-Reporting System containing pNF- κ B-luc was purchased from Stratagene.

Transfection and luciferase assay. Transient transfection was performed by using FuGENE 6 (Roche) according to the manufacturer's instructions. A total of 1×10^5 293T cells were transfected with 0.1 μ g pFLAG-TLR2, 0.01 μ g pNF- κ B-luc, 0.01 μ g of pRL-TK internal control plasmid (Promega) and 0.2 μ g of DN TLRs expressing plasmid in 24-well plates. After 48 h, transfected cells were stimulated with TX-114 detergent phase or purified proteins. After a further 8 h incubation, cells were lysed and assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega). Firefly and *Renilla* luciferase activity was monitored with a Lumat LB 9507 luminometer (Berthold). Normalized reporter activity was expressed as the firefly luciferase value divided by the *Renilla* luciferase value. Relative fold induction was calculated as the normalized reporter activity of the test samples divided by that of the unstimulated samples.

Purification from acrylamide gel. One hundred micrograms of TX-114 detergent phase was separated by 7.5 % glycine-SDS-PAGE gels under reducing conditions. The gels were stained with Quick CBB Plus (Wako). To elute proteins, the stained gel was cut into 5 mm strips and each strip was homogenized with 1 % SDS solution. The homogenized gel was removed by centrifugation at 12 000 g at room temperature. Five volumes of acetone were added to the supernatant, followed by incubation overnight. Proteins were pelleted by centrifugation at 12 000 g and 4 °C, and dissolved in 100 μ l DMSO. The protein concentration of the suspension was measured by Protein Assay CBB Solution (Nacalai Tesque).

PMF. PMF was carried out according to the method of Dr K. Yoshino (Yoshino *et al.*, 2004). Briefly, the Coomassie Brilliant Blue (CBB)- or silver-stained bands obtained from Tricine-SDS-PAGE were excised and sliced into small strips. To remove CBB, the strips were incubated in 50 % methanol/5 % acetic acid for 1 h and washed two times in water. The strips were dehydrated by incubation with 100 % acetonitrile. To alkylate the protein, the strips were incubated at 60 °C for 1 h with 10 mM DTT in 100 mM ammonium hydrogen carbonate, followed by treatment at room temperature for 30 min with 55 mM iodoacetamide (Nacalai Tesque) in 100 mM ammonium hydrogen carbonate. In-gel trypsin digestion was carried out by incubation with 10 μ g trypsin ml⁻¹ (Promega). The digested peptides were eluted by 5 % formic acid (Wako). Peptides were dried *in vacuo* and dissolved in saturated α -cyano-4-hydroxycinnamic acid (Nacalai Tesque) in 50 % acetonitrile and 0.1 % trifluoroacetic acid. The molecular masses of the peptides were measured with an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics). The SWISS-PROT database was searched by MASCOT (Science Matrix).

Macrophage stimulation. To induce peritoneal macrophages, 0.1 mg of OK432 (Chugai Pharmaceutical) was injected into the peritoneal cavity of C57BL mice. Two days later, peritoneal exudate cells as macrophages were harvested and centrifuged. The cell pellets were suspended in serum-free medium (SFM) optimized for macrophage culture (Gibco Invitrogen). Cells were allowed to adhere to 96-well culture plates for 2 h at 37 °C with 5 % CO₂. Non-adherent cells were removed by washing with PBS, and the remaining adherent cells were stimulated with TX-114 detergent phase or purified proteins for 6 h. The production of TNF- α was measured using an ELISA kit (R&D systems).

Statistical analysis. Results expressed as means and SD were compared using one-way ANOVA. The differences between each group were compared by multiple comparisons (Bonferroni *t* test). Differences were considered significant at $P < 0.05$.

Fig. 1. TX-114 phase partitioning. 293T cells were transfected with 0.01 μg pFLAG-TLR2 ml^{-1} , 0.01 μg pNF- κB -luc ml^{-1} and 0.01 μg pRL-TK ml^{-1} . The cells were stimulated with 1.0 μg ml^{-1} culture medium, TX-114-insoluble phase, aqueous phase and TX-114 detergent phase of *U. parvum*. All values represent the means and sds of three assays. ** $P < 0.01$ versus PBS.

RESULTS

TLR2-dependent activation of NF- κB by the TX-114 detergent phase of *U. parvum*

Ureaplasmas are wall-less bacteria, and completely lack LPS and PGN, which are ligands for TLR4 and TLR2, respectively (Weisburg *et al.*, 1989). *U. parvum* has no flagella, the ligand for TLR5 (Glass *et al.*, 2000). Other than these components, therefore, lipoproteins, the ligands for TLR2, are possible candidates for an inflammation-inducing factor in *U. parvum*. In fact, we previously demonstrated that lipoproteins derived from *Mycoplasma pneumoniae* induce NF- κB through TLR2 (Shimizu *et al.*, 2005). To elucidate whether *U. parvum* induces NF- κB through TLR2, we initially performed TX-114 phase partitioning of *U. parvum*. 293T cells were transfected with both a TLR2 expression vector pFLAG-TLR2 and a

reporter vector pNF- κB -luc, in which the luciferase reporter gene was fused to an NF- κB enhancer. When 293T cells transfected with pFLAG-TLR2 were stimulated with the TX-114-insoluble phase and the aqueous phase, the levels of luciferase expression were slightly increased (Fig. 1). In contrast, when the cells were stimulated with the TX-114 detergent phase of *U. parvum*, the expression level was remarkably augmented, and the activity was approximately fivefold higher than those of the TX-114-insoluble phase and the aqueous phase. Upon stimulation with culture medium, the level of luciferase expression was the same as the unstimulated control level. When 293T cells were transfected with an empty vector pFLAG-CMV1, the level of luciferase expression was not augmented (Fig. 1). The results suggest that the TX-114 detergent phase of *U. parvum* induces NF- κB activation through TLR2.

Purification of NF- κB -activating components of the TX-114 detergent phase

To examine the active components of the TX-114 detergent phase, the TX-114 detergent phase was separated by glycine-SDS-PAGE, and the gel was excised into 10 pieces (Fig. 2a). Proteins extracted from each gel piece as described in Methods were incubated with 293T cells transfected with pFLAG-TLR2 and pNF- κB -luc. When the cells were incubated with the proteins extracted from the gel strips, 75 and 55 kDa proteins (P75 and P55, respectively) were found to possess NF- κB -inducing activity (Fig. 2b). The NF- κB -inducing activity of P75 was approximately three times higher than that of P55.

Identification of 75 kDa NF- κB -activating components

First, PMF analysis for P75 was carried out, since the protein was abundant and showed a relatively strong NF- κB -inducing activity. As the result of a database search of SWISS-PROT using MASCOT, P75 was matched to the

Fig. 2. Molecular masses of the active components. (a) TX-114 detergent phase was separated by 7.5 % glycine-SDS-PAGE gels under reducing conditions. Ten major protein bands were elicited from the gels and the proteins were extracted. (b) Eluted solutions were incubated with 293T cells transfected with 0.01 μg pNF- κB -luc ml^{-1} , 0.01 μg pFLAG-TLR2 ml^{-1} and 0.01 μg pRL-TK ml^{-1} at final concentrations of 100 ng ml^{-1} . Luciferase activity was measured as described in Methods. All values represent the means and sds of three assays. * $P < 0.05$ versus PBS; ** $P < 0.01$ versus control.

MB antigen of *U. parvum* (Fig. 3a). The protein score for MB antigen was 102; scores greater than 77 are considered to be significant ($P < 0.05$). MB antigen contains a variable number of repeat sequences and hence shows different molecular masses in different isolates. In *U. parvum* serovar 3, MB antigen contains 42 repeats. The estimated molecular masses are 42 845 and 39 830 Da for the native and processed forms, respectively; however, MB antigen migrates at ~75 kDa by SDS-PAGE (Zheng *et al.*, 1995). MB antigen contains a signal peptide near the N-terminal end followed by a predicted lipid-binding cysteine. These findings indicate that MB antigen should be a lipoprotein (Zheng *et al.*, 1995).

Fig. 3. PMF. Amino acid sequences of MB antigen (a), UU012 (b) and UU016 (c). Box, signal peptide; *lipid binding cysteine; underlined type, mature protein; bold type, peptides detected by MS.

Cooperation of TLR2 and TLR6 for NF- κ B activation

Diacylated lipopeptides, including macrophage-activating lipopeptide 2 (MALP-2), have been reported to be recognized by mouse TLR2 cooperatively with TLR6 (Takeuchi *et al.*, 2001). To investigate whether MB antigen is also recognized by both TLR2 and TLR6 for NF- κ B activation, we transfected a plasmid encoding DN TLR6 (pFLAG-dTLR6) into 293T cells with both pFLAG-TLR2 and pNF- κ B-luc. Initially, the levels of expression of TLR1 and TLR6 in 293T cells were analysed by RT-PCR. Both TLR1 and TLR6 were found to be transcribed in 293T cells (data not shown). The effect of DN TLR6 on the expression of TLR2 was also analysed by flow cytometry. The level of TLR2 expression was almost constant, irrespective of the expression of DN TLR6 or DN TLR1 (data not shown). When the cells transfected with control vector were stimulated with P75, the levels of NF- κ B activation were increased (Fig. 4). When the cells were transfected with DN TLR6, the levels of NF- κ B activation were strikingly decreased. These results suggest that NF- κ B activation by P75 is dependent on TLR6. As a control, synthetic lipopeptides derived from *M. pneumoniae* (Shimizu *et al.*, 2008) were used in this assay. When DN TLR6-transfected cells were stimulated with a synthetic diacylated lipopeptide, FAM20 (synthetic lipopeptide containing N-terminal 20 amino acids of F₀F₁-type ATPase derived from *Mycoplasma pneumoniae*), the level of NF- κ B activation was decreased by DN TLR6. Meanwhile, when the cells were stimulated with a synthetic triacylated lipopeptide sN-ALP2 (synthesized NF- κ B-activating lipopeptide 2), NF- κ B activation was unaffected by DN TLR6 (Fig. 4).

Fig. 4. Cooperation of TLR1, TLR6 and TLR2 for NF- κ B induction by MB antigen. 293T cells were transfected with 0.2 μ g ml⁻¹ pFLAG-dTLR6 or pFLAG-dTLR1, 0.01 μ g pFLAG-TLR2 ml⁻¹, 0.01 μ g pNF- κ B-luc ml⁻¹ and 0.01 μ g pRL-TK ml⁻¹. The cells were stimulated with 10 ng P75 ml⁻¹, 100 ng P55 ml⁻¹, 10 ng FAM20 ml⁻¹ or 100 ng sN-ALP1 ml⁻¹. All values represent the means and sds of three assays. ** $P < 0.01$ versus vector.

Cooperation of TLR1 and TLR2 for NF- κ B activation

Triacylated bacterial lipopeptides such as Pam₃CSK₄ have been reported to be recognized by murine TLR1 in association with TLR2 (Takeuchi *et al.*, 2002). We next determined whether P75 is recognized by both TLR1 and TLR2 for NF- κ B activation. We transfected a plasmid encoding DN TLR1 (pFLAG-dTLR1) into 293T cells with both pFLAG-TLR2 and pNF- κ B-luc. When the cells were transfected with control vector, the stimulation with P75 augmented the levels of NF- κ B induction compared to background levels. In contrast, upon transfection with DN TLR1, the NF- κ B activation by P75 was significantly decreased (Fig. 4). As for the control, the NF- κ B activation by the diacylated lipopeptide (FAM20) and the triacylated lipopeptide (sN-ALP2) was decreased by DN TLR1 (Fig. 4). These results indicate that the cooperation of TLR1 is required for the NF- κ B activation by P75.

Identification of 55 kDa NF- κ B-activating components

We next tried to identify P55. The results of PMF showed that P55 was a mixture of lipoproteins UU012 and UU016 of *U. parvum* (Fig. 3b, c). The protein scores for UU012 and UU016 were 89 and 113, respectively; scores greater than 77 are considered to be significant ($P < 0.05$). It was technically difficult to separate UU012 and UU016 because of their similar molecular masses and isoelectric points. The estimated molecular mass of UU012 is 56 682 and 53 735 Da for the native and processed form, respectively. Similarly, that of UU016 is 57 241 and 54 198 Da for the native and processed form, respectively. Both UU012 and UU016 contain a signal peptide near the N-terminal end followed by a predicted lipid-binding cysteine. These findings indicate that UU012 and UU016 are also lipoproteins (Glass *et al.*, 2000).

Cooperation of TLR1, TLR2 and TLR6 in NF- κ B activation by P55

We next determined whether P55 is dependent on TLR1 or TLR6 to induce NF- κ B activation. We transfected a plasmid encoding DN TLR1 and DN TLR6 into 293T cells with both pFLAG-TLR2 and pNF- κ B-luc. When the cells were transfected with the control vector, stimulation with P55 augmented the levels of NF- κ B activation. In contrast, upon transfection with DN TLR1 or DN TLR6, the levels of NF- κ B activation were significantly decreased compared with the control (Fig. 4). These results indicate that NF- κ B activation by P55 is also dependent on TLR1 and TLR6.

Induction of TNF- α by TX-114 detergent phase, and by P75 and P55

To investigate whether these components derived from *U. parvum* actually induce inflammatory cytokines in

macrophages, the levels of TNF- α production by mouse peritoneal macrophages stimulated with TX-114 detergent phase, P75 or P55 were measured by ELISA. Peritoneal macrophages treated with PBS failed to produce TNF- α . On the other hand, the levels of TNF- α production were augmented by TX-114 detergent phase, P75 and P55 (Fig. 5). These results suggest that TX-114 detergent phase, P75 and P55 possess the ability to induce inflammatory cytokines, including TNF- α .

DISCUSSION

In this study, we demonstrated that lipoproteins from *U. parvum* activate NF- κ B through TLR1-, TLR2- and TLR6-dependent pathways in 293T cells, and induce TNF- α in mouse peritoneal macrophages. The lipoproteins were found to be MB antigen, UU012 and UU016 of *U. parvum*. In this study, it was difficult to separate UU012 and UU016, since they are very close in molecular mass (53 735 and 54 198 Da, respectively) and isoelectric point (8.6 and 8.4, respectively). In the current study, we did not determine which protein of UU012 and UU016 has the NF- κ B-inducing activity. However, both proteins are assumed to be lipoproteins with the ability to induce NF- κ B activation based on their amino acid sequences. Interestingly, MB antigen was abundant in the TX-114 detergent phase, and its ability to induce NF- κ B activation was higher than that of the mixture of UU012 and UU016. These results indicate that MB antigen might be a key factor to induce inflammatory responses.

It is known that triacylated lipoproteins are recognized by TLR1 and TLR2, whereas diacylated lipoproteins are recognized by TLR2 and TLR6. In TLR1-deficient mice,

Fig. 5. TNF- α induction by TX-114 detergent phase, P75 and P55. A total of 3×10^5 peritoneal macrophages per well obtained from C57BL mice were stimulated with $1.0 \mu\text{g ml}^{-1}$ of TX-114 detergent phase, $10 \text{ ng P75 ml}^{-1}$ or $10 \text{ ng P55 ml}^{-1}$ in a 96-well plate for 6 h. Levels of TNF- α in the culture supernatants were measured using ELISA kits. All values represent the means and SDs of three independent experiments. ** $P < 0.01$ versus PBS.

Pam₃CSK₄ containing three acyl chains fails to induce cytokines, including TNF- α , while MALP-2 containing two acyl chains can induce cytokines (Takeuchi *et al.*, 2002). In TLR6-deficient mice, MALP-2 fails to induce cytokines, but Pam₃CSK₄ can induce them (Takeuchi *et al.*, 2001). In this study, MB antigen and the mixture of UU012 and UU016 induced NF- κ B activation through TLR1-, TLR2- and TLR6-dependent pathways. Curiously, it appears to be unclear whether mycoplasmas and ureaplasmas have triacylated lipoproteins. In triacylated lipoproteins, three fatty acids are bound to the N-terminal cysteine residue: two in a diacylglyceride that is linked through a thioether bond to the thiol group and one to the amine group (N-acylation). Chemically identified lipoproteins from *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma salivarium* and *Mycoplasma gallisepticum* are not N-acylated (Jan *et al.*, 1996a, 2001; Muhlradt *et al.*, 1997, 1998; Shibata *et al.*, 2000). An N-acyltransferase gene responsible for N-acylation has not been detected in the *M. pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma penetrans* and *U. parvum* genomes (Fraser *et al.*, 1995; Glass *et al.*, 2000; Himmelreich *et al.*, 1996; Sasaki *et al.*, 2002) either. However, a study of the ratio of N-amide and O-ester bonds in *M. gallisepticum* and *Mycoplasma mycoides* suggests that such mycoplasmas possess diacylated and triacylated lipoproteins (Jan *et al.*, 1996b). Furthermore, the resistance to Edman degradation of proteins from *M. mycoides* also indicates the presence of N-acylation (Chambaud *et al.*, 1999). We previously tested the TLR dependency of synthetic diacylated or triacylated lipopeptides derived from *M. pneumoniae*. The synthetic triacylated lipopeptides, such as sN-ALP2, induce NF- κ B activation through TLR1 and TLR2, whereas diacylated lipopeptides, such as FAM20, induce through TLR1, TLR2 and TLR6 (Shimizu *et al.*, 2007, 2008) (Fig. 4). These results indicate that MB antigen, UU012 and UU016 may be diacylated lipoproteins.

Ureaplasmas are known to be associated with non-gonococcal urethritis (Shepard, 1954), chorioamnionitis (Cassell *et al.*, 1986; Maher *et al.*, 1994), preterm birth (Jelsema, 2006; Kataoka *et al.*, 2006; Ollikainen *et al.*, 1998), infertility (Taylor-Robinson, 1986; Xu *et al.*, 1997), pneumonia in neonates (Waites *et al.*, 1989) and bronchopulmonary dysplasia in neonates (Schelonka *et al.*, 2005). The urease and phospholipase produced by ureaplasmas have been implicated as virulence factors (De Silva & Quinn, 1986; Ligon & Kenny, 1991). In addition, inappropriate induction of inflammatory responses may be involved in the development of these diseases (Yoon *et al.*, 1998). In this study, we identified lipoproteins, such as MB antigen, UU012 and UU016, that can induce inflammatory responses. The functions of MB antigen are unknown, but it has been reported to be a major component of *U. parvum* and has been detected in various serovars (Watson *et al.*, 1990; Zheng *et al.*, 1992). Since there is an association between the amino acid sequences of lipoproteins and TLR dependency and

stimulatory activity (Buwitt-Beckmann *et al.*, 2005), the size variation of MB antigen in different serovars may affect the TLR dependency and stimulatory activity. To our knowledge, this study is the first report of a virulence factor of *U. parvum* that induces an inflammatory response. However, further study is needed to clarify the *in vivo* activity of the lipoproteins.

Considering the interaction between TLR and lipoproteins such as MB antigen, the inhibition of the interaction may result in the reduction of the inflammatory response induced by *U. parvum*. Accordingly, our data suggest that such lipoproteins are potential therapeutic targets. The lipoproteins and their derivatives might be suitable candidates as antigens for vaccination, causing the induction of a specific antibody capable of inhibiting TLR signalling. Alternatively, the derivatives may work as antagonists that inhibit TLR signalling. Furthermore, several reports indicate that mycoplasma-derived lipoproteins possess immune modulation and adjuvant activities (Link *et al.*, 2004; Rharbaoui *et al.*, 2002, 2004; Romero *et al.*, 2004). Thus, lipoproteins capable of interacting with TLR might become potential molecules in the development of new therapeutics against *U. parvum* infection.

ACKNOWLEDGEMENTS

We thank Dr T. Ishikawa (Fukuoka Industrial Technology Center) for MS analysis. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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Edited by: J. Renaudin