



<b>Title</b>	A Potential Pathogenic Factor from <i>Mycoplasma hominis</i> is a TLR2-Dependent, Macrophage-Activating, P50-Related Adhesin
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<b>Citation</b>	American Journal of Reproductive Immunology, 72(3), 285-295 <a href="https://doi.org/10.1111/aji.12279">https://doi.org/10.1111/aji.12279</a>
<b>Issue Date</b>	2014-06-17
<b>Doc URL</b>	<a href="http://hdl.handle.net/2115/59366">http://hdl.handle.net/2115/59366</a>
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<b>Type</b>	article (author version)
<b>File Information</b>	2014 M homi LP edited.pdf



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**A potential pathogenic factor from *Mycoplasma hominis* is a TLR2-dependent, macrophage-activating, P50-related adhesin.**

**Running head: Macrophage activation by *M. hominis* P50 adhesin**

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## **ABSTRACT**

**Problem.** *Mycoplasma hominis* has been implicated in many inflammatory conditions of the human urogenital tract in particular amniotic infections that lead to fetal and neonatal disease and preterm labor. The mechanisms responsible are poorly defined.

**Method of Study.** Biochemical and immunological methods were used to extract, purify and characterize an inflammatory component present in *M. hominis*.

**Results.** We isolated and purified to homogeneity a 40 kDa bioactive lipoprotein from *M. hominis* that was a potent TLR2-dependent, CD14-independent activator of the human THP-1 macrophage cell line. Homology searches of the N-terminal sequence revealed that 22 of the first 23 residues were identical to those seen for the phase-variable *M. hominis* p50 adhesin. The truncated P50t lipoprotein importantly retained its adhesive properties for human macrophages.

**Conclusions.** The unique adhesin/macrophage activator may play a key role in *M. hominis* infections by triggering an inflammatory cytokine cascade.

**Key words:** genital mycoplasmosis, adhesin, TLR2, macrophage

## Introduction

Mycoplasmas are wall-less prokaryotes characterized by small genomes, and known as the smallest self-replicating organisms<sup>1</sup>. *Mycoplasma hominis* is a mycoplasma that is isolated from urogenital tract<sup>2,3</sup> and has been considered to be an opportunistic human pathogen. It may also be associated with urogenital infections, postpartum fever, septic arthritis, and pneumonia<sup>1</sup>.

Lipoproteins are integral components of mycoplasmal cell membrane to maintain their structure, and they can be potent initiators of inflammatory reactions in mycoplasmal infections. There have been many reports on biological activities of mycoplasmal lipoproteins/lipopeptides<sup>4-7</sup> that can activate host cells by inducing production of proinflammatory cytokines, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 and IL-6. Therefore, we considered that lipoproteins might be involved in the pathogenicity of *M. hominis*, and we purified and characterized an active lipoprotein from *M. hominis* to determine the association of the organism with its pathogenicity.

Adherence of bacteria to host cells is crucial for infection. For mycoplasmal infection, it is known that many mycoplasmas possess adhesin<sup>8-12</sup>. Some mycoplasmal adhesins are both size and phase variable, and the often the chronic nature of mycoplasmal infection is considered to be a consequence of evasion of the humoral immune response by the variable adherence-associated (Vaa) antigens<sup>13</sup>. The vaa gene encodes a size- and phase-variable *M. hominis* adhesin<sup>11, 14-16</sup>. It has been reported that a single vaa gene is present in each *M. hominis* isolate<sup>12</sup>, and the size of Vaa observed in different isolates ranges from 28 kDa to 72 kDa. This size variation is considered to be a consequence of a

variable number of homologous, exchangeable cassette sequences located in the 3' end of *vaa*<sup>11, 15</sup>. P50 is an *M. hominis* Vaa lipoprotein, with a molecular weight of 50 kDa, which is involved in *M. hominis* cytoadherence<sup>17</sup>. It is known that its antigenicity is also variable<sup>11</sup>. There are many reports on molecular biological studies to elucidate the mechanism of mycoplasmal adhesins' size and antigenic variety. However, there is no report that suggested that mycoplasmal adhesin can activate host cells.

By purification and characterization of an active lipoprotein from *M. hominis*, this study reveals that mycoplasmal adhesin can contribute to mycoplasmal adhesion to, and also activation of, host cells.

## **Materials and methods**

### **Chemicals, enzymes and antibodies**

MALP-2 and lipopolysaccharide (LPS) from *Escherichia coli* R515 were purchased from Alexis Biochemicals (San Diego, CA). Endotoxin-free normal saline (NS) was from Baxter Healthcare (Deerfield, IL). Polymyxin B, n-octyl- $\beta$ -glucopyranoside (OG) and proteinase K were from Sigma (St Louis, MO). Recombinant human interferon (IFN)- $\gamma$  was from R&D systems (Minneapolis, MN). Inhibitors for mitogen-activated protein kinases (MAPKs) were from Calbiochem (La Jolla, CA). Anti-mouse CD14 monoclonal antibody (mAb) and isotype control antibody were purchased from BD Biosciences (San Jose, CA). Anti-MAPKs and phosphorylated MAPKs mAbs were from Cell Signaling Technology (Danvers, MA). Biotinylated anti-human TLR2 mAb (clone TL 2.1) and FITC-conjugated avidin were purchased from eBioscience (San Diego, CA).

### **Organism and culture conditions**

*M. hominis* ATCC 33129 was purchased from American Type Culture Collection (Manassas, VA). It was grown in PPLO broth (BD biosciences) supplemented with 15% heat-inactivated horse serum, 1.5% yeast extract, 0.25% L-arginine HCl, and 500 U/ml of penicillin G, and harvested by centrifugation at  $27,000 \times g$  for 30 minutes. The organisms were then washed three times with NS and frozen in NS at  $-70^{\circ}\text{C}$ .

### **Mice**

Female C57BL/6 wild type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME), and TLR2-deficient C57BL/6 mice (TLR2<sup>-/-</sup>) were obtained from Dr. Thomas Hawn (University of Washington School of Medicine, Seattle, WA), courtesy of

Dr Shizuo Akira (Osaka University, Japan). All the mice were bred in the Animal Resource Center (ARC) at the University of Utah Health Sciences Center. All mice were maintained in specific-pathogen-free conditions at the ARC and were used at 8 to 12 weeks of age. The ARC guarantees strict compliance with regulations established by the Animal Welfare Act.

### **Cell culture and ELISA**

Human monocyte/macrophage cell lines, THP-1, or mouse monocyte/macrophage cell lines, RAW264.7, were adjusted at  $5 \times 10^5$  cells/ml and cultured in 24-well or 96-well flat-bottomed plates in RPMI medium. Murine adherent peritoneal macrophages were cultured as described previously<sup>18</sup>. Cells were stimulated as described<sup>6</sup>, and the amount of TNF- $\alpha$  in cell culture supernatants was determined using human or mouse ELISA kits from eBioscience or BD Biosciences. Results are expressed as the mean  $\pm$  SD of three determinations. Statistical analysis was done using a Student's *t*-test.

### **Preparation of lipoprotein fraction using OG**

The lipoprotein fraction was extracted from *M. hominis* using OG according to the previously-described method<sup>5</sup>. Briefly, a 15-ml volume of cell membrane suspension was treated twice with 15 ml chloroform-methanol (2:1, v/v) at room temperature. Organic solvents were removed from the delipidated interphase in vacuo at 37°C and lyophilized to remove water. The lyophilized material was suspended in 50 mM OG in NS, treated for 6 min in boiling water, and centrifuged at  $20,000 \times g$  for 30 min. The supernatant was collected, filtered through a 0.22  $\mu$ m pore-size filter, and used as OG extracts. The lipoprotein fraction obtained by the extraction was named OG hom.

### **Identification of the active component and lipoprotein purification**

The active component was identified according to the method described previously<sup>19,20</sup>. Briefly, OG hom was passed over a 10% SDS-PAGE gel and the gel was blotted to a 0.45- $\mu\text{m}$  cellulose membrane (Bio-Rad), cut into 2-mm strips, and each dissolved in 1 ml of DMSO. Protein-coated particles were formed by adding sodium carbonate buffer, and then tested for bioactivity.

The active component was purified as described previously. Briefly, OG hom was rerun on SDS-PAGE, the gel was stained with zinc, and the previously-identified active lipoprotein band was cut out from the gel and eluted directly from the gel using Electro-eluter (Bio-Rad, Hercules, CA).

#### **Alkaline hydrolysis**

Alkaline hydrolysis was started by mixing equal volume of 0.2 M sodium hydroxide and 1  $\mu\text{g/ml}$  of purified lipoproteins. Then the mixture was neutralized by adding an equal volume of 0.1 M hydrochloric acid. The effect of alkaline hydrolysis on the activity was determined using THP-1 cells.

#### **Involvement of mitogen-activated protein kinases (MAPKs) in P50t signaling**

Phosphorylation of MAPKs was detected through Western blotting analysis using the antibodies described above, the chemiluminescent substrate from Pierce (Rockford, IL), and Fluor-S MAX (Bio-Rad) or X-ray film. Briefly, THP-1 cells ( $4 \times 10^6$ ) were stimulated with P50t (1  $\mu\text{g/ml}$ ) in a 1.5-ml tube. The cells were harvested after 0, 15, 30 or 60 min incubation and phosphorylated or non-phosphorylated p38, Erk1/2 and SAPK/JNK were detected using appropriate antibodies.

MAPKs inhibitors were purchased from Calbiochem-Millipore (Billerica, MA). THP-1 cells ( $5 \times 10^5$  cells/ml) were preincubated with SB203580 (p38 inhibitor), PD98059 (Erk1/2 inhibitor), SP600125 (SAPK/JNK inhibitor) or NS for 1 h. Then all the cells were stimulated with P50t (500 ng/ml) for 18 h and the amount of TNF- $\alpha$  in the supernatants were determined.

#### **Amino acid sequence and flow cytometric analysis**

Purified protein was dot blotted onto Immobilon PVDF membrane (Millipore, Bedford, MA), and then was excised and Edman sequencing was performed on an ABI Procise sequencer (Applied Biosystems, Foster City, CA). For flow cytometric analysis, THP-1 cells were stimulated and analyzed as previously described<sup>21</sup>.

#### **P50t Adhesion test**

The protein portion of P50t was biotinylated (B-P50t) according to manufacturer's instructions (Pierce). THP-1 cells were prepared in 1.5-ml tubes and preincubated with NS, P50t or anti-human TLR2 mAb at 4°C for 1 h and then incubated with various concentration of B-P50t for 3 h at 4°C with horizontal shaking (150 rpm). The cells were harvested by centrifugation and washed twice with ice-cold NS, treated with SDS-PAGE sample buffer. The SDS-PAGE was performed and proteins were transferred to PVDF membrane. The B-P50t adhered to THP-1 cells was detected using HRP-conjugated avidin, a chemiluminescent substrate (Pierce), and Fluor-S MAX (Bio-Rad).

## **Results**

### **OG extraction of a bioactive 40 kDa lipoprotein(s) from *M. hominis***

The lipoprotein OG extracts from *M. hominis* (OG hom) contained a bioactive component that induced macrophages of the human THP-1 cell line (Fig. 1A) and macrophages of the murine RAW 264.7 cell line (Fig.1B) to produce TNF- $\alpha$  in a dose-dependent manner.

When OG hom was run on an SDS-PAGE gel, the major component was a 40 kDa lipoprotein (Fig. 1C); gel slices when extracted revealed that the peak of macrophage activating activity coincided with the 40 kDa band although significant activity migrated to the dye front. Digestion of OG hom with proteinase K resulted in disappearance of the 40 kDa band and all activity now migrated to the dye front (Fig.1D). These results suggest that the active entity(ies) in OG hom is a lipoprotein(s) and that lipopeptide moieties might be responsible for activity.

### **Purification of the bioactive component in *M. hominis* lipoprotein extracts and derivation of its N-terminal sequence**

Prior to sequence determination, we purified the 40 kDa component to homogeneity by elution, concentration and re-running on SDS-PAGE gels (Fig. 2A). The macrophage-activating potency was retested on THP-1 cells and was still about 50% active down to 16 ng/ml (Fig. 2B). The homogeneous material was subjected to Edman degradation and we obtained the first 22 of 23 amino acid residues in the N-terminal region (Table). Amino acids after the second one were easily identified suggesting that the amino group of the N-terminal amino acid is free. Based on the characteristics of the Edman degradation, the N-terminal amino acid is thought to be cysteine. This is supported by the previous finding that

the N-terminal amino acid of lipoproteins from prokaryotes is cysteine, the Src homology group of which is bound to lipids<sup>22</sup>. This was same as lipopeptides from *M. fermentans* (MALP-2)<sup>4</sup> and *M. salivarium* (FSL-1)<sup>5</sup>, which have a free N-terminal that is diacylated. In addition, it is known that N-terminal of both MALP-2 and FSL-1 are cysteine. Homology searching by BLAST (Table) revealed that the 40 kDa component was a truncated form of the *M. hominis* P50 (Vaa) lipoprotein adhesins, which comprise a series of phase variable truncated molecules that exhibit a highly conserved N-terminal region<sup>12</sup>. The cysteine residue, a known lipid attachment site, at the N-terminus of P50 could not be confirmed by Edman degradation in the 40 kDa truncated molecule, now designated as “P50t”.

#### **Role of a lipid component in bioactivity of P50t**

Alkaline hydrolysis is known to remove ester-linked lipids at the N-terminus of bioactive bacterial lipoproteins<sup>22</sup>. We showed here that alkaline hydrolysis reduced the activity of P50t in a time-dependent manner (Fig. 3A), suggesting that a lipid moiety was important for activity as for other mycoplasmal lipopeptides/lipoproteins<sup>4,5</sup>. In addition, we examined the effect of proteinase K on the bioactivity of the homogeneous preparation, P50t.

Proteinase K completely digested the purified P50t (Fig. 3B) as seen by SDS-PAGE, but had no effect on the bio-activity of P50t to induce TNF- $\alpha$  production to THP-1 cells (Fig. 3C), which was almost identical to that seen for lipoprotein treated with saline. These results suggest that an N-terminal lipopeptide in P50t is the active moiety.

#### **Macrophage receptors for P50t**

TLRs on the surfaces of cells of the innate immune system play an important role in recognizing pathogenic microorganisms by virtue of pathogen-associated molecular

patterns, PAMPs. TLR2 recognizes many microbial lipoproteins, peptidoglycan, lipoteichoic acid<sup>23-25</sup>, and other components whereas LPS is predominantly recognized by TLR4<sup>26</sup>. Other agonists such as MAM superantigen from *M. arthritidis* can be recognized by both TLR2 and TLR4<sup>27</sup>. Since we had previously established that murine macrophages (RAW 264.7 cells) were also activated by P50t (Fig.1B), we tested the ability of peritoneal macrophages from WT mice versus those from TLR2<sup>-/-</sup> mice to produce TNF- $\alpha$  in response to P50t vs LPS (Fig 4A). Cells from TLR2<sup>-/-</sup> mice totally failed to respond to P50t in comparison with cells from WT mice. In contrast, cells from both WT and TLR2<sup>-/-</sup> mice responded similarly to LPS.

Many microbial agonists require a co-receptor for effective recognition. CD14 is a co-receptor for LPS<sup>26</sup> and for some bioactive bacterial lipoproteins<sup>28,29</sup>, whereas MALP-2 is independent of CD14<sup>6</sup>. Murine RAW 264.7 cells were pre-incubated with anti-mouse CD14 mAb for 1 h, and then stimulated with P50t, LPS or NS. Anti-mouse CD14 mAb had no effect on the activity of P50t, or that of MALP-2, whereas it completely inhibited the activity of LPS (Fig. 4 B). Thus P50t is independent of CD14.

#### **Up-regulation of TLR2 on macrophages by P50t**

Some microbial agonists have been shown to upregulate TLR expression on innate immune cells,<sup>30</sup> a process by which host recognition of pathogens is enhanced but which can also contribute to the inflammatory response. This has not yet been shown for mycoplasmal lipoproteins. Macrophage THP-1 cells were incubated with either P50t or with NS and IFN- $\gamma$  as negative or positive controls, respectively. Cells were reacted with FITC-conjugated anti-TLR2 or with a control antibody and were examined using flow cytometry.

Cell surface TLR2 expression on THP-1 cells was up-regulated by P50t as well as with IFN- $\gamma$  as indicated by a marked increase in mean fluorescence intensity (MFI; Fig. 5).

### **Involvement of MAPKs on macrophage activation by P50t**

Stimulation of macrophages by a various microbial agonists is known to cause activation of various MAPKs,<sup>31</sup> which can determine the subsequent signals that lead to MyD88-dependent or MyD88-independent pathways. THP-1 cells were stimulated with P50t for 0, 15, 30 or 60 min, and activation of p38, Erk 1/2, or SAPK/ JNK were examined by Western blotting for the kinetics of phosphorylation. The results showed that p38, Erk 1/2 and SAPK/JNK were all phosphorylated 15-30 min after stimulation with P50t (Fig.6 A). To confirm that these molecules were involved in P50t-induced TNF- $\alpha$  production, THP-1 cells were pretreated for 1 h with inhibitors of p38, Erk1/2 and SAPK/JNK (SB203580, PD98059 and SP600125, respectively) prior to stimulation with P50t. THP-1 cells were preincubated with them for 1 h, and then stimulated with P50t. We showed that all of the MAPK inhibitors significantly decreased the amount of TNF- $\alpha$  induced by P50t in a dose-dependent manner (Fig.6 B).

### ***In vivo* induction of inflammatory cytokines by P50t**

To better assess the potential inflammatory properties of P50t *in vivo*, we intravenously injected NS, LPS, or P50t into WT and TLR2<sup>-/-</sup> mice and their serum cytokine profile was investigated. It was found that although P50t was not as effective as the highly potent agonist LPS, P50t could induce TNF- $\alpha$  (Fig. 7A), IL-6 (Fig. 7B) and IL-12 p40 (Fig. 7C) in the sera of from WT mice but not in the sera from TLR2<sup>-/-</sup> mice (Fig. 7A-C), again

confirming that P50t is dependent on TLR2. As before the TLR4-utilizing LPS induced identical levels of these cytokines in both mouse strains.

### **Adhesive function of P50t**

Finally, we determined whether the P50t truncated molecule retained its adhesive properties. Biotinylated P50t (B-P50t) and THP-1 cells were incubated together at 4°C to avoid the uptake of B-P50t by THP-1 cells<sup>32</sup>. The cells were then washed, treated with SDS-PAGE sample buffer and tested for adhered B-P50t using avidin-HRP. The amount of B-P50t bound on THP-1 cells was found to increase dose dependently with 10 µg/ml being maximal (Fig. 8A). A dose of 2 µg/ml B-P50t was chosen to determine whether unlabeled P50t or anti-hTLR2 antibody could block binding. Preincubation of cells with unlabeled P50t competitively blocked the B-P50t adhesion depending on the dose, with 10 µg/ml unlabeled P50t being the maximum (Fig. 8B). In contrast, preincubation with anti-TLR2 mAb was ineffective (Fig. 8C). These results suggest that P50t retains its function as an adhesin. The failure of anti- hTLR2 antibody to block binding suggests that there are other binding sites for P50t on innate cells other than TLR2.

## Discussion

In this study, it was shown that: i) a truncated form of *M. hominis* adhesin, P50t, can both activate macrophages and adhere to macrophages; ii) the macrophage activation was TLR2 dependent; iii) TLR2 expression on macrophages was upregulated by P50t stimulation; and iv) P50t adhesion was suggested to be TLR2-independent.

Since mycoplasmas lack a cell wall, interest in the association between mycoplasmas and the pathogenicity of their cell surface lipoprotein has increased. There are many reports on the cytokine-inducing activity of lipoproteins/lipopeptides from mycoplasmas, such as MALP-2 from *M. fermentans* lipoprotein<sup>4</sup>, FSL-1 from *M. salivarium* lipoprotein<sup>5</sup>, apolipoprotein A-1 binding lipoproteins/lipopeptides from *M. arthritidis*<sup>19</sup>, adhesive function of Maa1 and Maa2 lipoprotein from *M. arthritidis*<sup>10</sup> and variable P50 lipoprotein from *M. hominis*<sup>17</sup>. In *M. hominis* bioactive lipoprotein, Peltier et al. partially purified a potent 29 kDa lipoprotein, which induced TNF- $\alpha$  production by macrophages<sup>7</sup>, whereas the molecular weight of P50t in this study was 40 kDa. It is speculated that this difference in molecular weight is a result of different methods used to extract the lipoprotein fraction, such as Triton X-114 extraction and OG extraction, or there may be another truncated form of active P50 with a molecular weight of about 29 kDa. Although there are many reports on the pathological roles of mycoplasmal lipoprotein, this is the first report to suggest that mycoplasmal lipoprotein can activate macrophages and help mycoplasmas to adhere to host cells.

It is important to determine how P50t may be involved in pathogenicity of *M. hominis*. *M. hominis* has been suggested to be associated with bacterial vaginosis, preterm

labor, intra-amniotic infection<sup>33</sup>, and spontaneous preterm birth<sup>34</sup>. Preterm labor is considered to be caused by bacterial infection by modulating cytokine production to favor the production of proinflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ <sup>35-37</sup>. Since P50t induces TNF- $\alpha$  production in macrophages, it is possible for P50t to play a pathological role in preterm labor. Recently, involvement of TLRs in bacterial vaginosis has been suggested by Zariffard et al.<sup>38</sup> They reported that cells in the lumen of the genital tract from women with bacterial vaginosis were found to express abundant TLR4 and TLR2 mRNA. It remains unknown whether or not P50t can directly upregulate TLR2 expression in any cells. However, it is possible to address that as a result of P50t stimulation, TLR2 expression is upregulated in macrophages after 3 days of incubation (Fig. 5). Therefore, *M. hominis* P50t might play important pathological roles in bacterial vaginosis by upregulating TLR2 expression and by inducing TNF- $\alpha$  production, although *M. hominis* is thought to be unable to use this upregulation of TLR2 expression to adhere to host cells (Fig. 8).

It has been reported that P50 is a cytoadhesin of *M. hominis*<sup>17</sup>, and P50 is suggested to be one of the Vaa antigens. Henrich et al. reported that there were some specific truncations of the P50 gene, but the region encoding the N-terminal part of P50 adhesin was present in all of the isolates of *M. hominis* that were tested in the study<sup>16</sup>. Therefore, although it is unknown that whether N-terminal lipopeptides are important for adhesion to host cells, we can at least speculate that all the truncated forms of P50 possess the activity to induce macrophage activation because activity of mycoplasmal lipoproteins are known to reside in their N-terminal lipopeptide portion<sup>4,5</sup>. We consider that the character of P50t is close to that of MALP-2 rather than that of active lipoprotein (MlpD) that was purified

from *M. arthritidis*<sup>29</sup>. First, the MALP-2 N-terminal amino acid is diacylated<sup>4</sup> and that of P50t should be also diacylated, whereas that of the active lipoproteins from *M. arthritidis* is blocked and is thought to be triacylated<sup>29</sup>. Since the N-terminal amino acid sequence after the second P50t amino acid was easily identified, it was suggested that its N-terminal is free and thus diacylated. Second, the activity of both MALP-2 and P50t was CD14-independent (Fig. 4 B), whereas those of purified lipoproteins *M. arthritidis* were CD14 dependent<sup>29</sup>. Third, MALP-2 activates macrophages via activation of p38, Erk1/2, and SAPK/JNK<sup>39</sup>, and this characteristic is identical to P50t. In addition, MALP-2 is known to have some inflammatory effects in vivo<sup>40</sup>, and P50t can also induce TNF- $\alpha$ , IL-6 and IL-12 p40 production in mouse serum by intravenous injection (Fig. 4 C, D, E). Therefore, it was suggested that the characteristics of P50t are similar to those of MALP-2, but not to those of *M. arthritidis*-derived lipoproteins. CD14 and CD36 have been reported to function as co-receptors for the recognition of a MALP-2<sup>41</sup> and a triacylated lipopeptide, Pam<sub>3</sub>CSK<sub>4</sub>, by TLR2<sup>42, 43</sup>. As described above, activity of P50t was independent of CD14, and therefore, CD36 might be important for its activity as well as adhesive activity of P50t because CD36 is crucial for uptake of the diacylated mycoplasmal lipopeptide, FSL-1, by macrophages<sup>32</sup>. Studies are in progress to elucidate the mechanism of how mycoplasmas utilize their surface lipoproteins to adhere to host cells.

## **Acknowledgements**

This work was supported by the Nora Eccles Treadwell Foundation (to BCC) and by Grants-in-Aid for Scientific Research (C23592692) provided by the Japan Society for the Promotion of Science (to AH).

Table. N-terminal amino acid sequence of the 40 kDa active component and *M. hominis* adhesin P50

protein	Amino acid sequence
40 kDa active component	(C*)NDDKLAEKNGKEKADAALKQAN
<i>M. hominis</i> adhesin P50	CNDDKLAEKNGKEKADAALKQAN

Amino acid sequence is expressed by single letter designations

\*could not be determined by Edman degradation

## Figure Legends

**Fig. 1.** The active component is a 40 kDa moiety. (A, B) Dose dependency of OG hom activity to induce TNF- $\alpha$  production by stimulating THP-1 cells (A) and RAW 264.7 cells (B). (C) Molecular mass range of the active components in OG hom was determined using THP-1 cells. (D) Activity migration of proteinase K treated OG hom. The activity was analyzed using the same method used in Fig. 1C. Results are expressed as the mean  $\pm$  SD of three determinations.

**Fig. 2.** Purification and identification of the active component. (A) SDS-PAGE of protein standards (a), OG hom (b), and purified active 40 kDa component (c) was performed with 10% polyacrylamide gel, and stained with silver. (B) Dose dependency of P50t activity to induce TNF- $\alpha$  to THP-1 cells. Results are expressed as the mean  $\pm$  SD of three determinations.

**Fig. 3.** Properties of P50t. (A) Effect of alkaline hydrolysis on the activity of P50t. (B) SDS-PAGE of protein standards (a), P50t treated with normal saline (NS) (b) or proteinase K (c) at 37°C for 2 h. The gel was stained with silver. (C) Effect of treatment with NS or proteinase K on the activity of P50t (500 ng/ml). Results are expressed as the mean  $\pm$  SD of three determinations.

**Fig. 4.** Receptor(s) for P50t. (A) TNF- $\alpha$  production of peritoneal macrophages of WT mice or TLR2<sup>-/-</sup> mice stimulated with NS, P50t (0.2  $\mu$ g/ml) or LPS (0.1  $\mu$ g/ml) (A). (B) RAW 264.7 cells were preincubated with NS, isotype control Ab or anti mouse CD14 mAb (10  $\mu$ g/ml) for 1 h. The cells were then stimulated with LPS (20 ng/ml), MALP-2 (20 ng/ml) or P50t (50 ng/ml). †,  $p < 0.05$ .

**Fig. 5.** Upregulation of TLR2 by P50t. Flow cytometric analysis of cell surface expression of TLR2 on THP-1 cells. THP-1 cells ( $1 \times 10^6$  cells/ml) were stimulated with NS (A), IFN- $\gamma$  (10 U/ml) (B), or P50t (1  $\mu$ g/ml) (C) for 3 days. TLR2 upregulation was expressed by a shift in the histogram peak.

**Fig. 6.** MAPKs involvement in P50t activity. (A) Activation of MAPKs on macrophages by P50t. THP-1 cells were stimulated with P50t, and phosphorylated (P-) or non-phosphorylated p38, Erk1/2 and SAPK/JNK were detected. (B) Effects of various MAPK inhibitors on the activity of P50t. Results are expressed as the mean  $\pm$  SD of three determinations. †,  $p < 0.05$ .

**Fig. 7.** In vivo induction of cytokines. Induction of TNF- $\alpha$  (A), IL-6 (B) and IL-12 p40 (C) in C57BL/6 and C57BL/6 TLR2 (-/-) mouse sera injected with NS, LPS and P50t. The amount of LPS and P50t were 1  $\mu$ g/mouse and 2  $\mu$ g/mouse, respectively. Results are expressed as the mean  $\pm$  SD of three determinations. †,  $p < 0.05$ .

**Fig 8.** Adherence of P50t to THP-1 cells. THP-1 cells were incubated with various B-P50t concentrations for 3 h (A), or preincubated with various P50t concentrations and then incubated with B-P50t (2  $\mu$ g/ml) (B) or preincubated with various anti human TLR2 mAb concentrations and then incubated with B-P50t (2  $\mu$ g/ml) (C) in 1.5-ml at 4°C. After 3 hours incubation, adhered B-P50t was detected using avidin-HRP.

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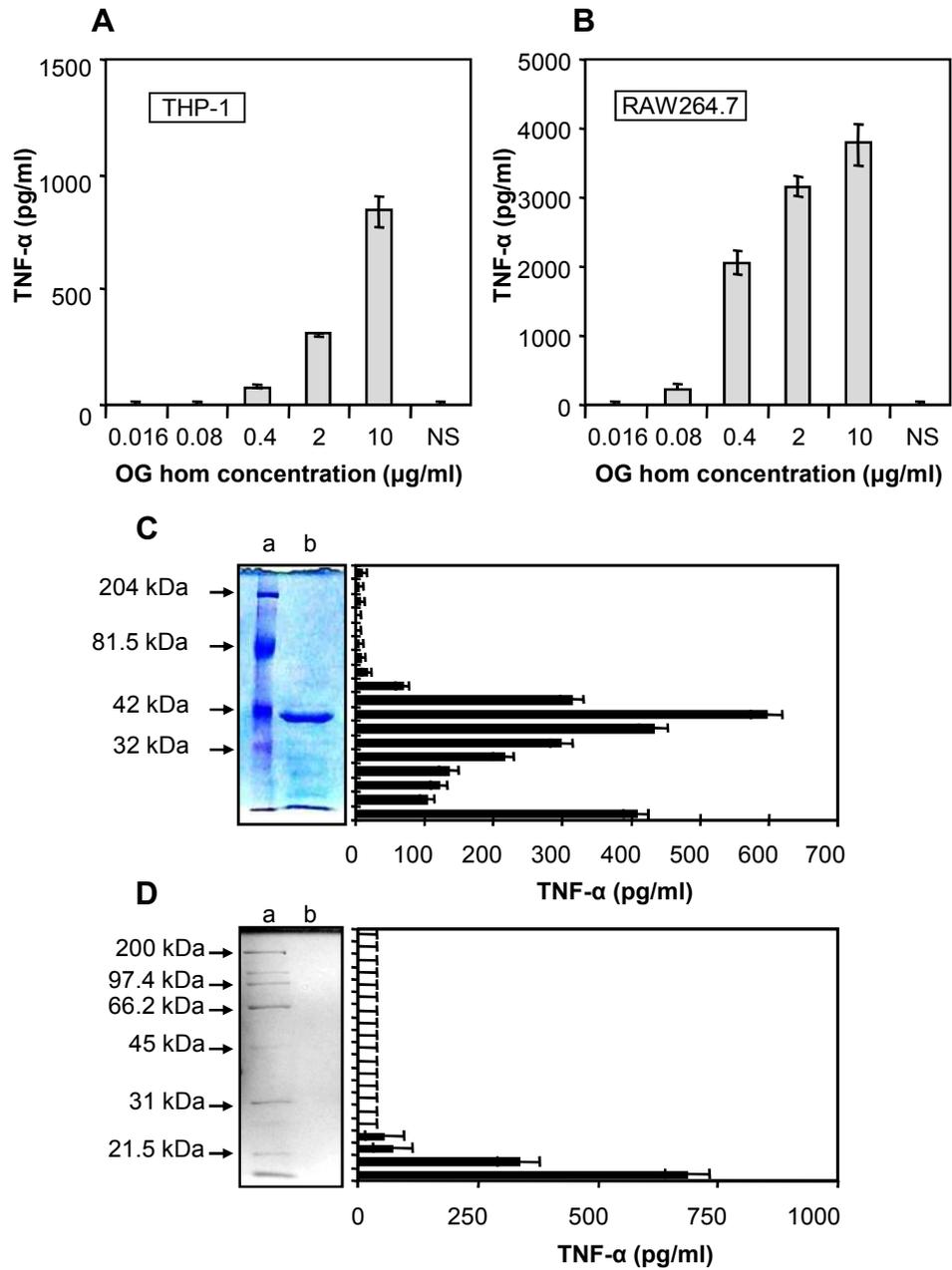
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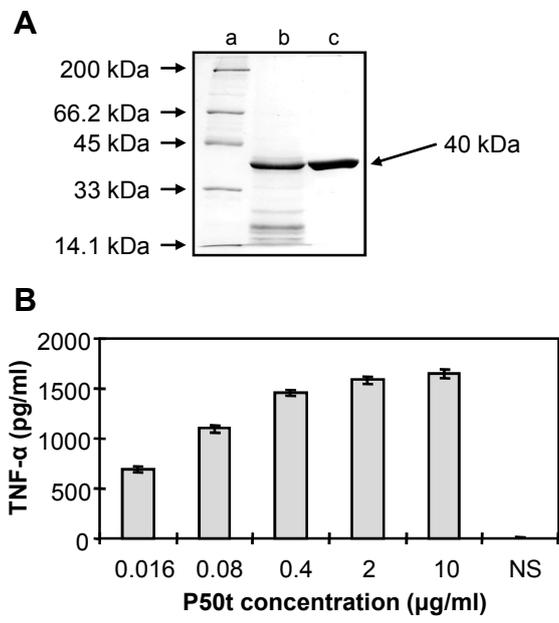
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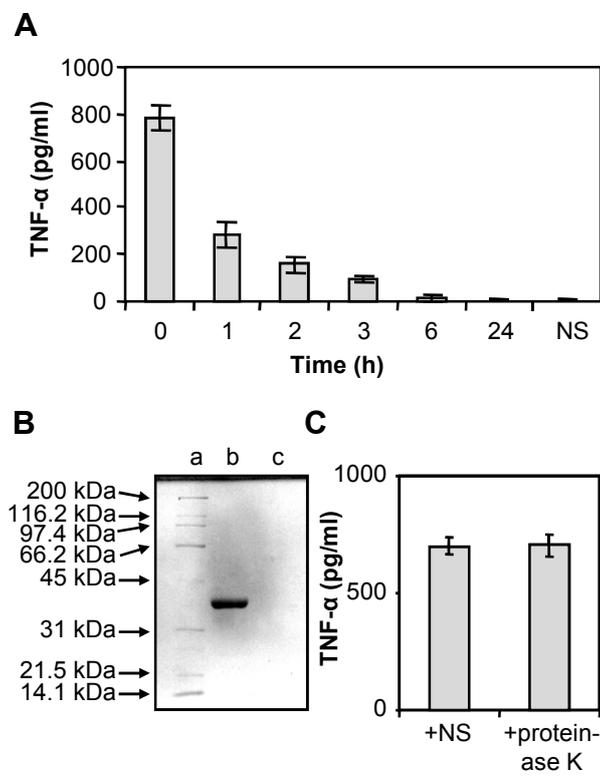
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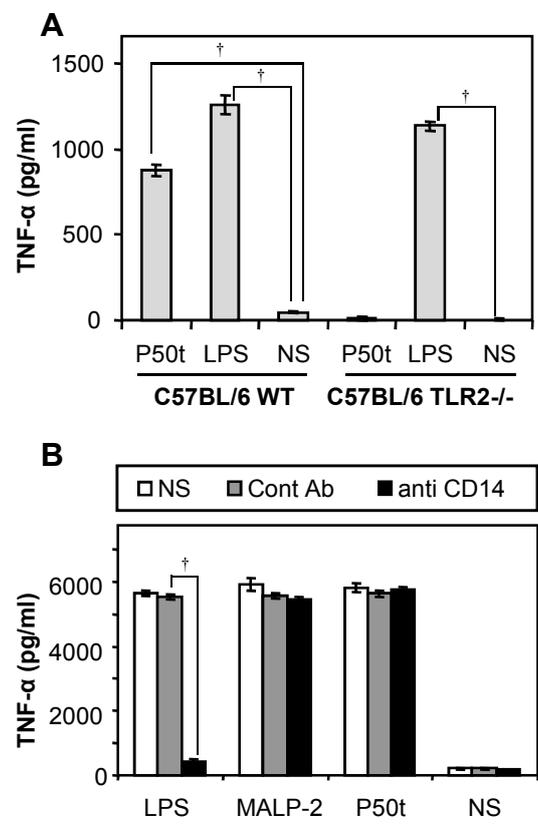
**Fig. 1**



**Fig. 2**



**Fig. 3**



**Fig. 4**

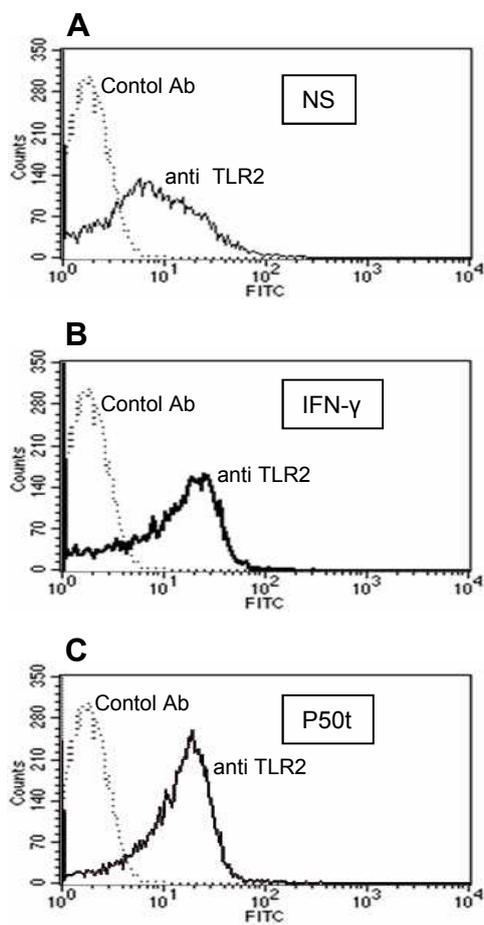
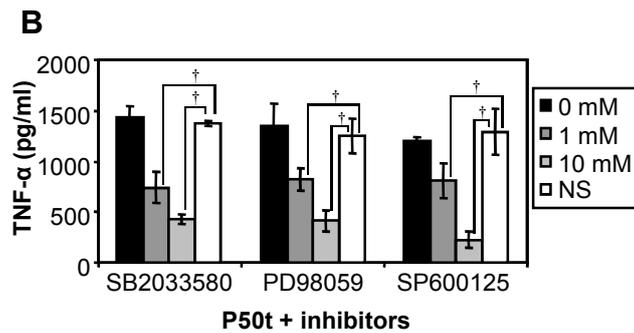
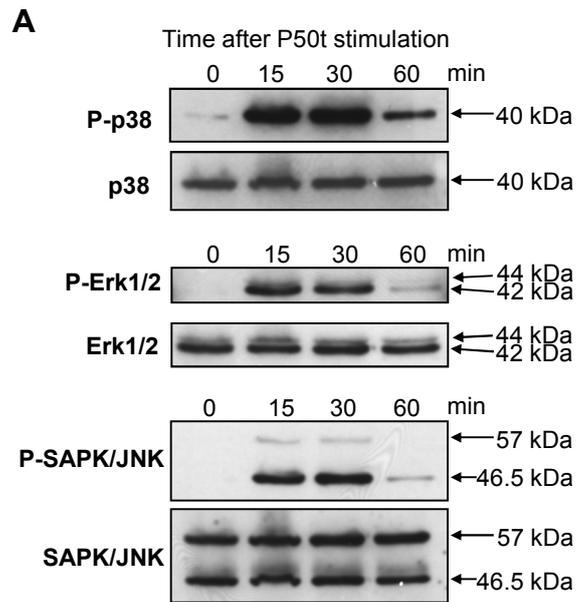


Fig. 5



**Fig. 6**

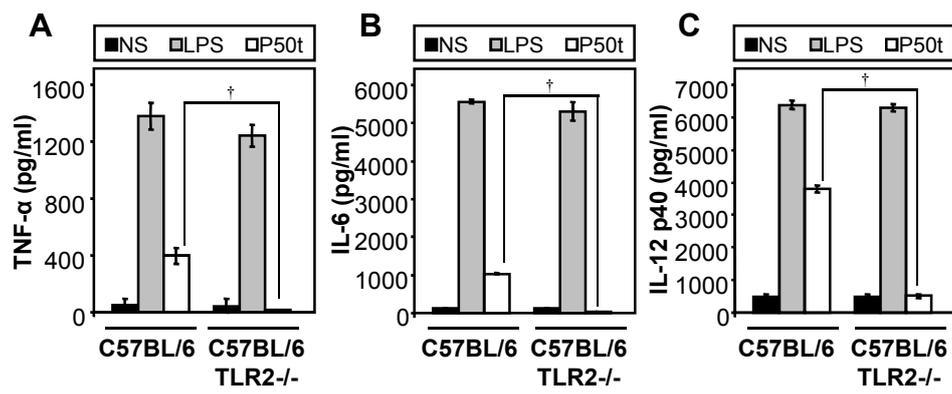
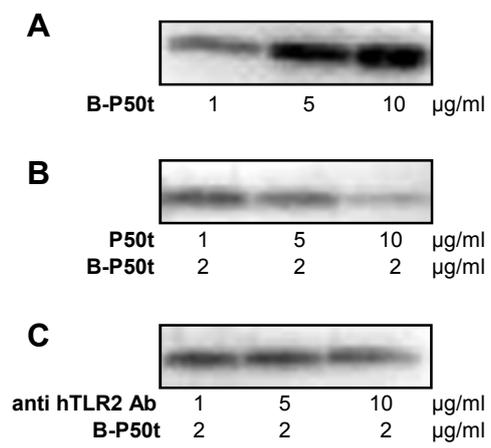


Fig. 7



**Fig. 8**