International Immunology, Vol. 20, No. 2, pp. 199–208
doi:10.1093/intimm/dxm129

Low potency of *Chlamydophila* LPS to activate human mononuclear cells due to its reduced affinities for CD14 and LPS-binding protein

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Keywords: innate immunity, toll-like receptor

Abstract

Chlamydiaceae **are small obligate intracellular parasites and classified as Gram-negative bacteria. Among** ***Chlamydiaceae*****-derived components, LPS is known as an immunomodulator and possesses a unique lipid A structure with longer but fewer acyl chains. In this study, to elucidate the** ***Chlamydiaceae*****-induced immune responses, we evaluated the actions of** ***Chlamydophila psittaci*** **LPS as a** ***Chlamydiaceae*** **LPS on human PBMCs and compared with those of** ***Escherichia coli*** **LPS. Similar to** ***E. coli*** **LPS,** ***C. psittaci*** **LPS bound to monocytes and induced the pro-inflammatory cytokine production in an LPS-binding protein (LBP)-dependent manner. However,** ***C. psittaci*** **LPS was much less potent than** ***E. coli*** **LPS in both the LPS binding and cytokine production. Interestingly, although the binding of** ***C. psittaci*** **LPS was mediated by CD14, Toll-like receptor 4 (TLR4) and CD11b, CD14 and TLR4 but not CD11b were involved in the cytokine production. Of note, ELISA-based binding assays revealed that** ***C. psittaci*** **LPS directly bound to LBP and CD14; however, the affinities were much less than those of** ***E. coli*** **LPS. Together, these observations possibly suggest that** ***Chlamydiaceae*** **LPS has low binding affinities for LPS recognition molecules such as CD14 and LBP and exhibit weak biological activities against host immune cells including monocytes, thereby contributing to the chronic (persistent) inflammatory reactions during infection.**

Introduction

Chlamydiaceae are small obligate, intracellular, Gram-negative bacteria that cause various infectious diseases in humans and animals as common pathogens (1–3). Among *Chlamydiaceae*, *Chlamydia trachomatis*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci* are pathogenic to humans and responsible for infectious diseases involving eyes, urogenital organs and respiratory tract (3, 4). The development of *Chlamydiaceae*-triggered diseases is closely associated with their persistent infection.

During infection, pathogens are eliminated by the combination of innate and acquired immunities. *Chlamydiaceae*, however, have evolved the strategies to escape from host defense by avoiding recognition as pathogens, inhibiting phagolysosome fusion and controlling host cell survival (5–7). Thus, *Chlamydiaceae* can survive in the infected cells and persistently activate host cells at the infection site. Although sustained infection is an important feature of *Chlamydiaceae*, little is known about the mechanisms for the host cell activation. Earlier studies reported that *Chlamydiaceae*-derived components such as LPS, major outer membrane protein (MOMP) and heat shock protein 60 (HSP60) as well as intact bacteria could activate macrophages and endothelial cells (7–10). Among these bacterial components, LPS is known as an immunomodulator, which induces pro-inflammatory cytokine production and mitogenic reaction (11–13). The biological activities of LPS are critically dependent on its lipid A portion, composed of phosphorylated glucosamine disaccharide and several acyl chains. Lipid A moieties of LPS from distinct bacteria species differ in the length, position and number of the acyl chains, and these variations reflect the bioactivities of lipid A (12–14). To date, structures of LPS from three species of *Chlamydiaceae* (*C. pneumoniae*, *C. psittaci* and *C. trachomatis*) have been characterized, and their structures are shown to resemble

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Received 26 December 2006, *accepted* 8 November 2007

Transmitting editor: K. Okumura

Advance Access publication 4 December 2007

the rough form of enterobacterial LPS (such as *Escherichia coli* R mutants and *Salmonella minnesota* R595) (15–21). Of note, *Chlamydiaceae* LPS possesses a unique lipid A structure with longer (C18–C22) but fewer acyl chains (3–5) compared with a typical enterobacterial LPS such as *E. coli* with short hexaacyl chains (C12 and C14). In addition, *Chlamydiaceae* LPS has a weak endotoxic activity compared with *E. coli* LPS. Thus, the low biological activities of *Chlamydiaceae* LPS could permit the relatively weak host immune cell responses during *Chlamydiaceae* infection, which enables the persistent infection in the host cells.

However, it is still unclear why the biological activities of *Chlamydiaceae* LPS is low. Thus, to address this issue, we here investigated the actions of *Chlamydiaceae* LPS on PBMCs using *C. psittaci* LPS as an immunomodulator. The results have demonstrated that the LPS binds to monocytes and induces pro-inflammatory cytokine production from PBMC via the actions on LPS-binding protein (LBP) and the surface receptors CD14 and Toll-like receptor 4 (TLR4); however, *C. psittaci* LPS was much less potent than *E. coli* LPS in both the LPS binding and cytokine production. We further provided the evidence that *C. psittaci* LPS bound to both LBP and CD14 with lower affinities compared with *E. coli* LPS. Our findings suggest that the reduced affinities of *Chlamydiaceae* LPS for LPS-binding molecules may define the weak biological activities against host immune cells, thereby contributing to the chronic (persistent) inflammatory reactions during *Chlamydiaceae* infection.

Methods

LPS and reagents

Chlamydophila LPS was prepared as described by Hussein *et al.* (16) from elementary bodies (EBs) of *C. psittaci* 6BC and provided by Professor Rudolf Toman (Slovak Academy of Science, Slovak Republic). Briefly, *C. psittaci* EBs, which were propagated in yolk sacs of embryonated hen eggs, were suspended in 2 M NaCl (20% v/v), inactivated with 0.2% formaldehyde and centrifuged at 20 000 $\times$ *g*. The pellet was suspended in PBS, digested with trypsin and then extracted with diethyl ether. EBs in the aqueous phase were precipitated by centrifugation, and LPS was extracted from EBs by a phenol–water method and further purified by a preparative PAGE (22). LPS from *E. coli* O111:B4 was obtained from Sigma-Aldrich (St Louis, MO). Alexa Fluor 488 (Alexa488)-labeled *E. coli* O55:B5 was obtained from Invitrogen (Carlsbad, CA, USA). Biotinylation of *C. psittaci* and *E. coli* LPS was performed using biotin-LC-hydrazide (Pierce, Rockford, IL, USA) according to manufacturer's protocol. For quantification, biotinylated LPS was separated by 10% SDS-PAGE, stained with a Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen) and quantitated by comparison with the defined amounts of unlabeled LPS. The molecular weight of *C. psittaci* LPS (rough type) was estimated as 4500 by SDS-PAGE, whereas the smooth-type LPS from *E. coli* O111:B4 is estimated as the average molecular weight of 15 800 (23).

Neutralizing murine anti-human mAbs used were as follows: anti-CD14 (clone MY4; Beckman-Coulter, Fullerton, CA, USA), anti-TLR4 (clone HTA125; MBL, Nagoya, Japan), anti-CD11b (clone 44; Sigma-Aldrich) and anti-CD11b (clone D12; BD Biosciences, San Jose, CA, USA). Murine FITC-labeled anti-human CD3 (clone UCHT1) and PE-labeled anti-CD19 (clone HIB19) mAbs were purchased from BD Biosciences. Murine anti-human LBP mAb (clone 6G3) was obtained from HyCult Biotechnologies (Uden, Netherlands). Murine control antibodies (IgG) were obtained from eBioscience (San Diego, CA, USA) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Streptavidin–PE conjugate was from eBioscience, and streptavidin–HRP conjugate was from Zymed Laboratories (South San Francisco, CA, USA). Recombinant human LBP, CD14 and TLR4–MD-2 were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of PBMC

Human PBMCs from healthy donors were obtained by density-gradient centrifugation with Lymphoprep (Axis-Shield, Dundee, UK). The purity of PBMC (>95%) was confirmed by FACS staining for CD3, CD19 and CD14, in addition to the microscopic examination of Diff-Quick-stained cells. PBMCs were suspended in AIM-V serum-free medium (Invitrogen) containing 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Sigma-Aldrich). In some experiments, FCS (<0.03 ng ml^{-1} endotoxin; Equitech-Bio, Kerrville, TX, USA) was added to the culture medium.

Binding of LPS to PBMC

PBMCs (1×10^6 cells ml^{-1}) were incubated with biotinylated *C. psittaci* LPS (1000 ng ml^{-1}) or *E. coli* LPS (100 ng ml^{-1}) in the presence or absence of LBP (10 and 100 ng ml^{-1}) or 1% FCS at 37°C for 15 min with gentle shaking. Subsequently, cells were washed with PBS and incubated with streptavidin–PE for 30 min on ice. After washing twice with PBS, cells were analyzed with a FACScan and CellQuest Pro Software (BD Biosciences). For blocking experiments, PBMCs were pre-incubated with indicated concentrations of neutralizing mAbs (4 μg ml^{-1} anti-CD14, 10 μg ml^{-1} anti-TLR4 or 2.5 μg ml^{-1} anti-CD11b) or 10 μg ml^{-1} control IgG at 37°C for 15 min. Alternatively, PBMCs were incubated with excess amounts of unlabeled *C. psittaci* LPS or *E. coli* LPS (100 μg ml^{-1}) in the presence of 10 ng ml^{-1} LBP at 37°C for 15 min. Then, biotinylated *C. psittaci* LPS (1000 ng ml^{-1}) or Alexa488-labeled *E. coli* LPS (100 ng ml^{-1}) was added and incubated for another 15 min. In the case of biotinylated *C. psittaci* LPS, cells were washed and further stained with streptavidin–PE.

Cytokine production from LPS-stimulated PBMC

To measure the production of IL-6, IL-1β and TNF-α from LPS-stimulated PBMC, cells (1×10^6 cells ml^{-1} in 96-well plates) were treated with or without neutralizing mAbs (anti-CD14, anti-TLR4 or anti-CD11b; 2.5–10 μg ml^{-1}) or control IgG (10 μg ml^{-1}) in AIM-V medium containing 1% FCS at 37°C for 1 h and then stimulated with *C. psittaci* LPS (100 and 1000 ng ml^{-1}) or *E. coli* LPS (1, 10 and 100 ng ml^{-1}) for 6 h. Culture supernatants were collected and stored at −80°C for further assay. The amounts of IL-6, IL-1β and

TNF-α in the culture supernatants were determined using Ready-To-Go Cytokine ELISA kit (eBioscience). The detection limits of IL-6, IL-1β and TNF-α were 15.6 pg ml^{-1}.

Interaction of LPS with LBP

The binding of biotinylated LPS to immobilized LBP was measured as described by Scott *et al.* (24). Anti-human LBP mAb (6G3), which recognizes LBP molecules as well as LBP–LPS complexes, was used as a capture mAb. The mAb was diluted to 1 μg ml^{-1} in 0.1 M $NaHCO_3$ (pH 9.0) and absorbed to 96-well ELISA plates (Corning, Acton, MA, USA) overnight at 4°C. The plates were blocked with PBS containing 1% BSA for 2 h at room temperature. After washing with PBS–0.05% Tween 20, LBP in PBS containing 0.1% BSA was added to the plates at indicated concentrations (0.1–200 ng ml^{-1}) for 1.5 h at room temperature. Then, the plates were washed, and biotinylated *C. psittaci* LPS (1000 ng ml^{-1}, 220 nM) or *E. coli* LPS (100 ng ml^{-1}, 6.3 nM) diluted in PBS containing 0.1% BSA was incubated in the plates for 1 h at room temperature. The LPS bound to immobilized LBP was detected using streptavidin–HRP (1:2500 dilution; Zymed Laboratories) and tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). For competition experiments, unlabeled *C. psittaci* LPS (0.22 nM–22 μM) or *E. coli* LPS (0.063 nM–6.3 μM) was pre-incubated in the LBP (100 ng ml^{-1})-captured plates for 30 min, and then biotinylated *C. psittaci* LPS or *E. coli* LPS was added.

Interaction of LPS with CD14

The interaction of LPS with CD14 was analyzed by the binding of biotinylated LPS to immobilized CD14 (25). Briefly, ELISA plates were coated with 2.5 μg ml^{-1} CD14 in 0.1 M $NaHCO_3$ (pH 9.0) overnight at 4°C. The plates were blocked with 1× Block Ace (Dainippon Pharmaceutical, Tokyo, Japan) for 2 h at room temperature. After washing with PBS–0.05% Tween 20, biotinylated *C. psittaci* LPS (1000 ng ml^{-1}, 220 nM) or *E. coli* LPS (100 ng ml^{-1}, 6.3 nM) diluted in PBS containing 0.5% BSA was incubated in the plates in the presence or absence of LBP for 1 h at 37°C. Subsequently, the LPS bound to CD14 was detected using streptavidin–HRP and TMB substrate. For competition assay, anti-CD14 mAb (MY4; 5 μg per well), polymyxin B (PMB) sulfate (5 μg per well; Sigma-Aldrich) or unlabeled *C. psittaci* LPS (0.22 nM–22 μM) or *E. coli* LPS (0.063 nM–6.3 μM) was pre-incubated with 100 ng ml^{-1} LBP in the CD14-immobilized plates for 30 min, and then biotinylated *C. psittaci* LPS or *E. coli* LPS was added.

Interaction of LPS with TLR4–MD-2

ELISA plates were coated with recombinant TLR4–MD-2 (2 μg ml^{-1}) in PBS at 4°C overnight. After blocking with PBS containing 2% BSA for 1 h, the plates were washed with PBS–0.05% Tween 20. Biotinylated *C. psittaci* LPS or *E. coli* LPS (10–1000 ng ml^{-1}) diluted in PBS–0.5% BSA was incubated in the plates in the presence or absence of 100 ng ml^{-1} LBP for 1 h at 37°C. Then the binding of LPS to TLR4–MD-2 was determined using streptavidin–HRP and TMB substrate. Inhibition of the binding of biotinylated LPS to TLR4–MD-2 was assessed by the addition of unlabeled LPS (*C. psittaci*: 0.22 nM–22 μM; *E. coli*: 0.063 nM–6.3 μM) as described above.

Statistical analysis

The data were expressed as the mean ± SD and analyzed for significant difference by a one-way analysis of variance and a *post-hoc* test using the StatView program (SAS, Berkeley, CA, USA). Differences were considered statistically significant if a P-value < 0.05. For competition analysis, curve fitting and 50% inhibitory concentration (IC_{50}) determination was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

LBP-dependent binding of C. psittaci LPS to human peripheral blood monocytes

LBP facilitates the binding of LPS to mononuclear phagocytes (monocytes/macrophages) by catalyzing transfer of LPS monomers to CD14 molecules (26, 27). Thus, we first evaluated the binding ability of *C. psittaci* LPS to human peripheral blood monocytes in the presence or absence of LBP using flow cytometry. As shown in Fig. 1(A), in the absence of LBP, biotinylated *C. psittaci* LPS (1000 ng ml^{-1}) somewhat bound to monocytes, and the addition of LBP (10 ng ml^{-1}) increased the binding. Similarly, the binding of biotinylated *E. coli* LPS (100 ng ml^{-1}) to monocytes was enhanced in the presence of LBP (Fig. 1D); however, the binding level of *C. psittaci* LPS was much lower than that of *E. coli* LPS. Almost the same results were observed by the addition of 1% FCS (data not shown). These results indicated that *C. psittaci* LPS could bind to monocytes in an LBP-dependent manner, although the binding of *C. psittaci* LPS was apparently less than that of *E. coli* LPS.

Next, to characterize the LPS receptors on monocytes, we performed a competition assay. The addition of both unlabeled *C. psittaci* LPS (100-fold molar excess) and *E. coli* LPS (28-fold molar excess) blocked the binding of biotinylated *C. psittaci* LPS to monocytes (>48 and >50% inhibition, respectively) (Fig. 1B and C). Similarly, unlabeled *C. psittaci* LPS (355-fold molar excess) partially blocked the binding of Alexa488-labeled *E. coli* LPS to monocytes (62.7% inhibition); however, unlabeled *E. coli* LPS (100-fold molar excess) markedly inhibited the binding of labeled *E. coli* LPS (97.5% inhibition) (Fig. 1E and F). These findings suggest that *C. psittaci* LPS shares the common receptors with *E. coli* LPS on monocytes, but that the binding affinity for *C. psittaci* LPS may be lower than that for *E. coli* LPS.

It has been reported that several cellular receptors such as TLR4 and CD11b as well as CD14 form the clusters on cells and are involved in the LPS signaling (28–31). Thus, we investigated the surface receptors for *C. psittaci* LPS on monocytes using neutralizing mAbs. Anti-CD14 and anti-TLR4 mAbs significantly inhibited the binding of *C. psittaci* LPS to monocytes by 41.8 and 47.4%, respectively (Fig. 2A). Anti-CD11b mAb also inhibited the *C. psittaci* LPS binding by 61%. In contrast, the binding of *E. coli* LPS to monocytes was significantly blocked by anti-CD14 and

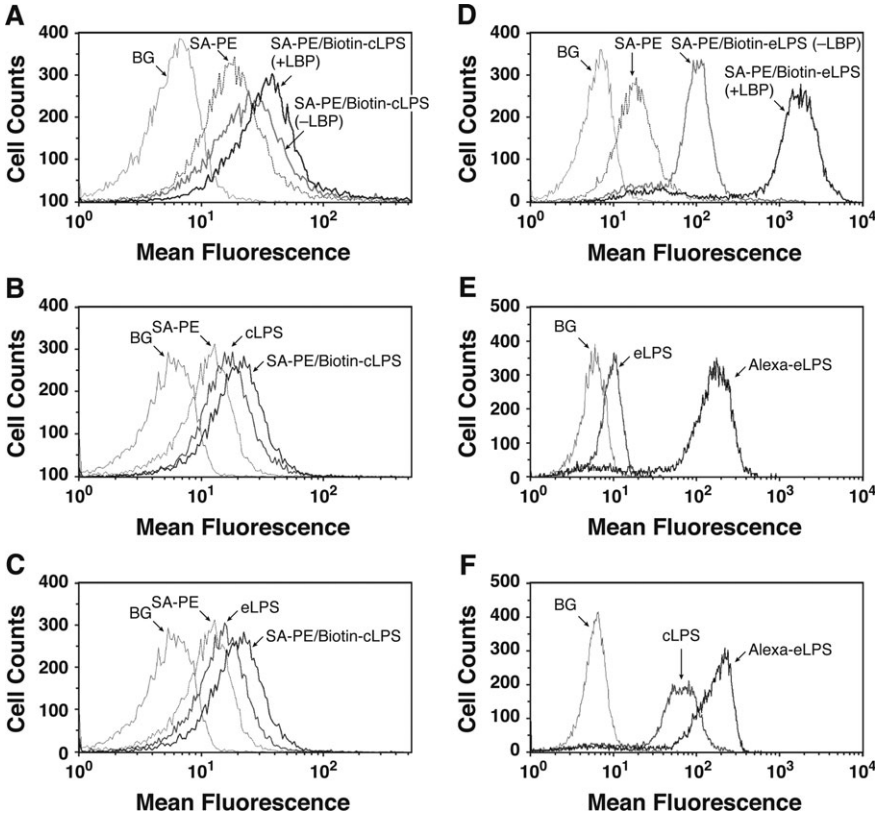

Fig. 1. Flow cytometric analysis of the binding of *Chlamydophila psittaci* LPS to human monocytes. PBMCs (1×10^6 cells ml^{-1}) suspended in AIM-V serum-free medium were incubated with biotin-labeled *C. psittaci* LPS (Biotin-cLPS, 1000 ng ml^{-1}) or *Escherichia coli* LPS (Biotin-eLPS, 100 ng ml^{-1}) in the presence or absence of LBP (10 ng ml^{-1}) (A and D). PBMCs were also incubated with Biotin-cLPS (1000 ng ml^{-1}) in the presence or absence of excess amounts of unlabeled *C. psittaci* LPS (cLPS; 100 μg ml^{-1}, 100-fold molar excess) or *E. coli* LPS (eLPS; 100 μg ml^{-1}; 28.6-fold molar excess) (B and C). Thereafter, the cells were incubated with streptavidin–PE (SA-PE). Alternatively, PBMCs were incubated with Alexa488-labeled *E. coli* LPS (Alexa-eLPS, 100 ng ml^{-1}) in the presence of 10 ng ml^{-1} LBP with or without excess amounts of unlabeled eLPS (10 μg ml^{-1}; 100-fold molar excess) or cLPS (10 μg ml^{-1}; 349-fold molar excess) (E and F). For each samples, 10 000 monocytes were analyzed by flow cytometry. BG, background fluorescence of unlabeled cells; SA-PE, fluorescence of cells stained with SA-PE only.

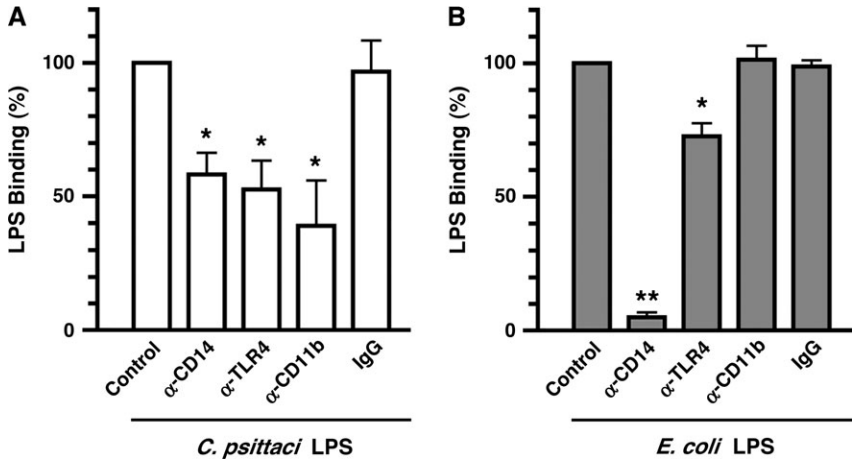

Fig. 2. Effect of blocking mAbs on the binding of *Chlamydophila psittaci* LPS to monocytes. PBMCs (1×10^6 cells ml^{-1}) suspended in AIM-V containing 10 ng ml^{-1} LBP were pre-incubated without (control) or with blocking mAbs (2.5–10 μg ml^{-1}) or control IgG (10 μg ml^{-1}) for 15 min and then added with biotinylated *C. psittaci* LPS (1000 ng ml^{-1}, A) or *Escherichia coli* LPS (100 ng ml^{-1}, B). The mean fluorescent intensity of bound LPS was analyzed by flow cytometry. LPS binding was expressed as a percentage of control. Data are the mean ± SD of three independent experiments. Values are compared between the incubation in the absence (control) and presence of blocking mAbs or control IgG. $^*P < 0.05$, $^{**}P < 0.005$.

anti-TLR4 mAbs (95 and 27.4%, respectively), but not by anti-CD11b mAbs (Fig. 2B).

Pro-inflammatory cytokine production from C. psittaci LPS-stimulated PBMC

It is known that enterobacterial LPS from *E. coli* and *Salmonella* elicits the production of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 from PBMC via the actions on CD14 and TLR4 (13). Thus, we investigated whether *C. psittaci* LPS could also activate PBMC to produce pro-inflammatory cytokines.

Following the stimulation with *C. psittaci* LPS (100 and 1000 ng ml^{-1}), IL-6 was produced from PBMC in a dose-dependent manner, and the production of these cytokines was significantly enhanced by the addition of LBP (Fig. 3A). However, in agreement with the LPS binding (Fig. 1), the cytokine production by *C. psittaci* LPS was much lower than that by *E. coli* LPS (1 and 10 ng ml^{-1}, Fig. 3B). Moreover, experiments using neutralizing antibodies demonstrated that *C. psittaci* LPS-induced production of IL-6 was blocked by not only anti-CD14 mAb but also by anti-TLR4 mAb (Fig. 3C). Interestingly, anti-CD11b mAb, which inhibited the binding of *C. psittaci* LPS, had no effect on the cytokine production. In contrast, anti-CD14 and anti-TLR4 mAbs inhibited *E. coli* LPS-induced cytokine production as well as LPS binding (Fig. 3D). Similar results were observed for IL-1β and TNF-α production (data not shown).

These observations suggest that CD14 and TLR4 but not CD11b are involved in the pro-inflammatory cytokine production from *C. psittaci* LPS-stimulated PBMC.

Interaction of C. psittaci LPS with LBP and CD14

The above observations clearly indicate that the biological activities of *C. psittaci* LPS are lower than those of *E. coli* LPS. To elucidate these differences, we focused on the interaction of *C. psittaci* LPS with the two major LPS-binding molecules LBP and CD14.

As shown in Fig. 1, LBP enhanced the binding of *C. psittaci* LPS to monocytes, suggesting the interaction of *C. psittaci* LPS with LBP. Consistent with this, biotinylated *C. psittaci* LPS (1000 ng ml^{-1}) directly bound to immobilized LBP (⩾50 ng ml^{-1} LBP), and the binding reached a plateau at ⩾100 ng ml^{-1} LBP (Fig. 4A). In contrast, biotinylated *E. coli* LPS (100 ng ml^{-1}) bound to immobilized LBP (⩾10 ng ml^{-1}), and the binding reached a plateau at ⩾50 ng ml^{-1} LBP. Apparently, the binding levels of *E. coli* LPS were higher than those of *C. psittaci* LPS. We subsequently performed the competitive ELISA to determine the binding affinities of *C. psittaci* LPS and *E. coli* LPS to immobilized LBP (100 ng ml^{-1}). Both unlabeled *C. psittaci* LPS and *E. coli* LPS effectively inhibited the binding of biotinylated *C. psittaci* LPS (1000 ng ml^{-1}, i.e. 220 nM) to LBP with IC_{50} values of 8.9 and 0.4 nM, respectively (Fig. 4B). Similarly, unlabeled *C. psittaci* LPS and *E. coli* LPS dose dependently inhibited the binding of biotinylated *E. coli* LPS (100 ng ml^{-1}, i.e. 6.3 nM) to immobilized LBP (IC_{50} values of 230.5 and 43.2 nM, respectively, Fig. 4C). Of note, *C. psittaci* LPS was approximately 5- to 20-fold less potent than *E. coli* LPS in suppressing the binding of biotinylated LPS (*C. psittaci* and *E. coli*) to LBP.

We further analyzed the interaction of *C. psittaci* LPS with CD14. The binding of biotinylated *C. psittaci* LPS (1000 ng

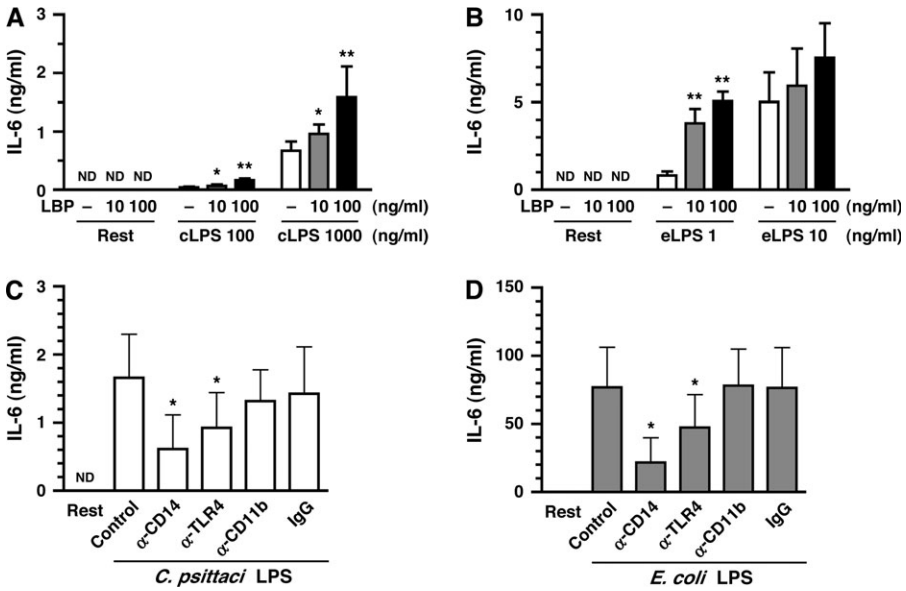

Fig. 3. IL-6 production from *Chlamydophila psittaci* LPS-stimulated PBMC. PBMCs (1×10^6 cells ml^{-1}) suspended in AIM-V were stimulated with *C. psittaci* LPS (cLPS; 100 and 1000 ng ml^{-1}; A) or *Escherichia coli* LPS (eLPS; 1 and 10 ng ml^{-1}; B) in the absence (−) or presence of LBP (10 and 100 ng ml^{-1}) for 6 h. For neutralization experiments, PBMCs suspended in AIM-V containing 1% FCS were incubated without (control) or with appropriate mAbs (2.5–10 μg ml^{-1}) or control IgG (10 μg ml^{-1}) for 1 h prior to LPS stimulation (C and D). Amounts of IL-6 in culture supernatants were measured by ELISA. Alternatively, PBMCs were incubated without cLPS or eLPS in the presence or absence of LBP (Rest). Data are the mean ± SD of five to seven independent experiments. Values are compared between the incubation without (−) and with LBP (A and B) or the absence (control) and presence of blocking mAbs or control IgG (C and D). ND, not detected. *$P < 0.05$, **$P < 0.005$.

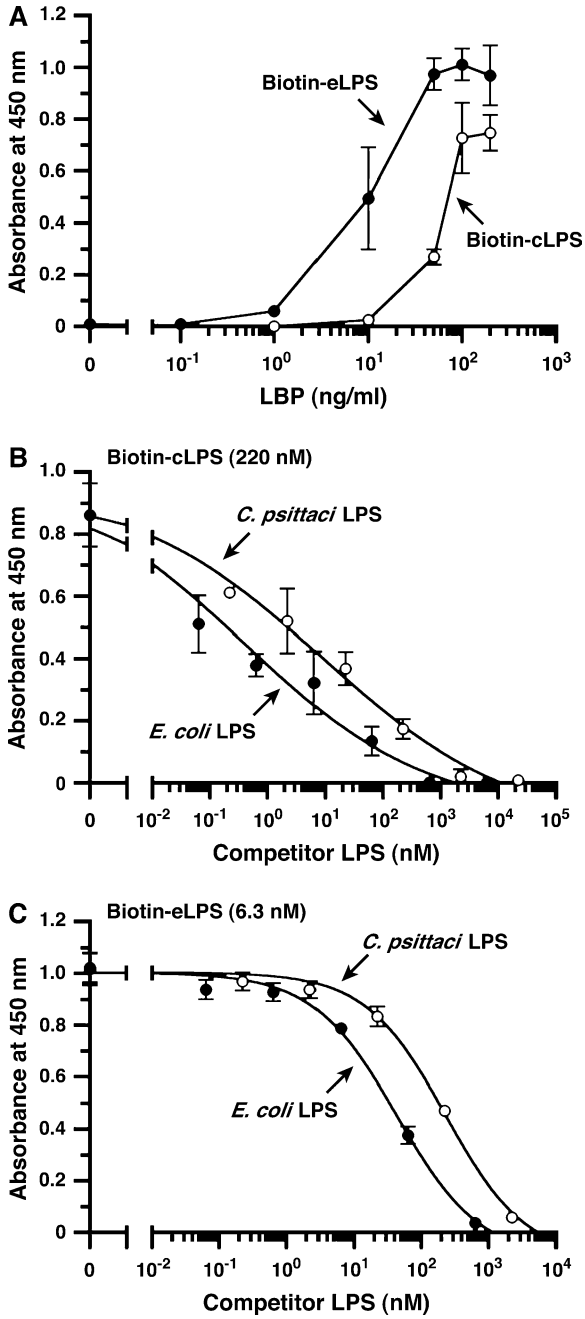

Fig. 4. Binding profiles of *Chlamydophila psittaci* LPS to immobilized LBP. (A) Increasing amounts of LBP (0.1–200 ng ml^{-1}) were immobilized to anti-LBP mAb-coated microplates, and biotinylated *C. psittaci* LPS (Biotin-cLPS; 1000 ng ml^{-1}, 220 nM) or *Escherichia coli* LPS (Biotin-eLPS; 100 ng ml^{-1}, 6.3 nM) was added to the plates. (B) Biotin-cLPS (1000 ng ml^{-1}, 220 nM) was incubated in the LBP (100 ng ml^{-1}, 6.3 nM)-immobilized plates in the absence or presence of unlabeled *C. psittaci* LPS (0.22 nM–22 μM) or *E. coli* LPS (0.063 nM–6.3 μM). (C) Biotin-eLPS (100 ng ml^{-1}, 6.3 nM) was incubated in the LBP (100 ng ml^{-1}, 6.3 nM)-immobilized plates in the absence or presence of unlabeled *C. psittaci* LPS (0.22 nM–2.2 μM) or *E. coli* LPS (0.063–630 nM). Finally, bound LPS was detected by the sequential addition of streptavidin–HRP, TMB and 1 M H_2SO_4. Data are the mean ± SD of three independent experiments.

ml^{-1}) to immobilized CD14 was enhanced in the presence of increasing concentrations of LBP (Fig. 5A). Of importance, neutralizing anti-CD14 mAb almost completely blocked the binding of *C. psittaci* LPS to immobilized CD14 in the presence of LBP (Fig. 5B). Furthermore, PMB, a cationic cyclic peptide, which binds to lipid A moiety and prevents LPS–LBP interaction, also abolished the *C. psittaci* LPS binding to CD14. These findings clearly indicate that *C. psittaci* LPS can bind to CD14 in an LBP-dependent manner. Similarly, the binding of biotinylated *E. coli* LPS (100 ng ml^{-1}) to immobilized CD14 was enhanced by increasing concentrations of LBP, and the binding was significantly inhibited by anti-CD14 mAb and PMB (Fig. 5A and B). Next, we assessed the binding affinity of *C. psittaci* LPS to CD14 in the presence of LBP. As shown in Fig. 5(C), unlabeled *C. psittaci LPS* and *E. coli* LPS inhibited the binding of biotinylated *C. psittaci* LPS (220 nM) to CD14 in a dose-dependent fashion (IC_{50} values of 335.0 and 12.8 nM, respectively). Likewise, unlabeled *C. psittaci* LPS and *E. coli* LPS blocked the binding of biotinylated *E. coli* LPS (6.3 nM) to CD14 with IC_{50} values of 81.3 and 12.3 nM, respectively (Fig. 5D).

Taken together, these observations indicate that the binding affinities of *C. psittaci* LPS for both LBP and CD14 are much lower than those of *E. coli* LPS.

Interaction of C. psittaci LPS with TLR4–MD-2 complex

It is generally accepted that LPS captured by CD14 is transferred to TLR4–MD-2 signaling complex (28, 30). Thus, we finally investigated the binding of *C. psittaci* LPS to immobilized TLR4–MD-2 complex using ELISA and compared with that of *E. coli* LPS.

As shown in Fig. 6(A), both *C. psittaci* LPS and *E. coli* LPS bound to immobilized TLR4–MD-2 in an LBP-dependent manner. However, the binding of *C. psittaci* LPS was much lower than that of *E. coli* LPS, which can be explained by the lower affinity of *C. psittaci* LPS for LBP as compared with *E. coli* LPS (Fig. 4A).

Further, the competition assay indicated that unlabeled LPS dose dependently inhibited the binding of biotinylated *C. psittaci* or *E. coli* LPS to TLR4–MD-2 complex (Fig. 6B and C). However, in contrast with the cases of LBP and CD14 (Figs 4B and C and 5B and C), unlabeled *C. psittaci* LPS and *E. coli* LPS blocked the binding of biotinylated *C. psittaci* LPS to TLR4–MD-2 (Fig. 6B, IC_{50} values of 1.40 and 1.81 nM, respectively) and the binding of biotinylated *E. coli* LPS to TLR4–MD-2 (Fig. 6C, IC_{50} values of 7.20 and 4.14 nM, respectively) with similar affinities.

These observations suggest that the weak biological activity of *C. psittaci* LPS can be defined by the low capacity of *C. psittaci* LPS to bind with LBP and CD14 rather than TLR4–MD-2 complex.

Discussion

Chlamydiaceae can survive in the infected cells, and persistently activate host cells at the infection site, thereby inducing sustained immunological response (3, 7, 13, 32–34). To date, *Chlamydiaceae*-derived factors such as LPS, MOMP and HSP60 are reported to be potential activators of host cells (7–10).

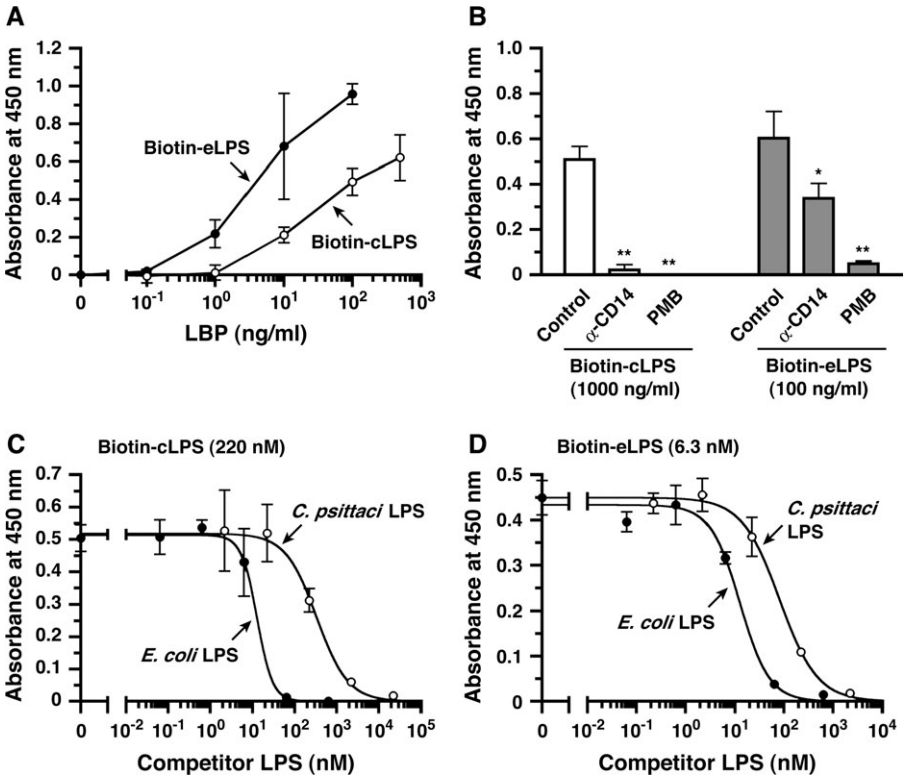

Fig. 5. Binding profiles of *Chlamydophila psittaci* LPS to immobilized CD14. (A) CD14 (2.5 μg ml^{-1}) was absorbed to the microplates. After blocking, biotinylated *C. psittaci* LPS (Biotin-cLPS, 1000 ng ml^{-1}) or *Escherichia coli* LPS (Biotin-eLPS, 100 ng ml^{-1}) was incubated in the presence of the indicated concentration of LBP. (B) The CD14-immobilized plates were incubated without (control) or with neutralizing anti-CD14 (5 μg per well) or PMB (5 μg ml^{-1}) in the presence of 100 ng ml^{-1} LBP and Biotin-cLPS (1000 ng ml^{-1}) or *E. coli* LPS (100 ng ml^{-1}). (C) Biotin-cLPS (1000 ng ml^{-1}, 220 nM) was incubated with 100 ng ml^{-1} LBP in the CD14-immobilized plates in the absence or presence of unlabeled *C. psittaci* LPS (0.22 nM–22 μM) or *E. coli* LPS (0.063 nM–6.3 μM). (D) Biotin-eLPS (100 ng ml^{-1}, 6.3 nM) was incubated with 100 ng ml^{-1} LBP in the CD14-immobilized plates in the absence or presence of unlabeled *C. psittaci* LPS (0.22 nM–2.2 μM) or *E. coli* LPS (0.063–630 nM). Bound LPS was finally detected using streptavidin–HRP. Data are the mean ± SD of three independent experiments. In panel B, values are compared between the absence (control) and presence of anti-CD14 mAb or PMB. *$P < 0.05$, **$P < 0.005$.

In this report, we first demonstrated that *C. psittaci* LPS, a *Chlamydiaceae* LPS, can directly bind to human monocytes in an LBP-dependent manner (Fig. 1), although its structure is distinct from enterobacterial LPS (16, 17, 19). Furthermore, competition assays suggested that *C. psittaci* LPS shares the common surface receptors with *E. coli* LPS on monocytes; excess *E. coli* LPS almost completely inhibited the binding of *C. psittaci* LPS to monocytes and vice versa. Moreover, neutralizing mAbs against LPS receptors (CD14 and TLR4) significantly blocked the binding of *C. psittaci* LPS as well as *E. coli* LPS (Fig. 2). These results clearly indicate that *C. psittaci* LPS can interact with CD14 and TLR4, despite its unique structure. Consistent with our observation, Heine *et al.* also demonstrated that LPS from *C. trachomatis* E could activate HEK293 cells expressing CD14 and TLR4–MD-2, suggesting that *Chlamydiaceae*-derived LPS can recognize CD14 and TLR4 as with enterobacterial LPS. We further provided the evidence that *C. psittaci* LPS is capable of not only binding to PBMC but also activating them to produce cytokines, although *C. psittaci* LPS is less potent than *E. coli*. Moreover, consistent with the LPS binding, the production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) from PBMC was augmented by LBP (Fig. 3A and C and data not shown) and significantly inhibited by anti-CD14 and anti-TLR4 (Fig. 3B and D).

Previous studies showed that certain receptors facilitate the LPS binding to CD14 and TLR4 (14,28–30). In this context, CD11b, an α subunit of β2 integrin complement receptor type 3 (also called Mac-1) is associated with CD14 and TLR4 (35–38). Interestingly, neutralizing anti-CD11b mAb substantially inhibited the binding of *C. psittaci* LPS to monocytes, suggesting that CD11b is involved in the LPS binding (Fig. 2A). However, anti-CD11b, which blocked the binding of *C. psittaci* LPS to monocytes, did not interfere with the cytokine production. In agreement with our findings, it has been revealed that extracellular domain of Mac-1 is involved in the LPS binding but cytoplasmic domain is not required for LPS-mediated intracellular signaling (36). Thus, CD11b may only facilitate the binding of *C. psittaci* LPS to the cell surface.

Recent studies have suggested that *Chlamydiaceae*-induced production of IFN-γ is regarded as one of the potential factors modulating the pathogen growth and regulated by IL-18, an IFN-γ-inducing factor (33, 34, 39–41). In this regards, we have evaluated the production of these cytokines from *C. psittaci* LPS-stimulated PBMC. However,

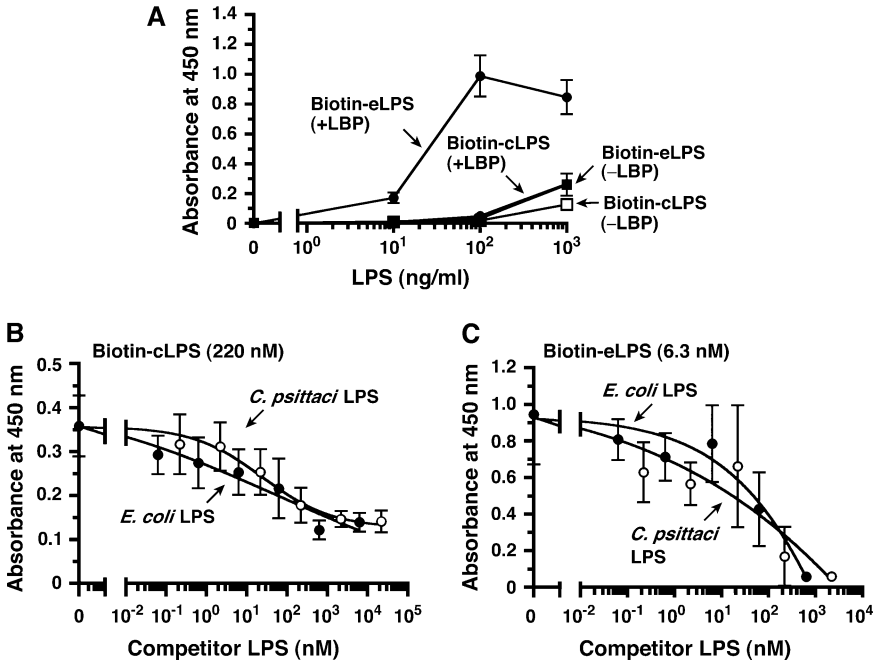

Fig. 6. Binding of *Chlamydophila psittaci* LPS to immobilized TLR4–MD-2 complex. (A) TLR4–MD-2 complex (2 μg ml^{-1}) was absorbed to the microplates. After blocking, biotinylated *C. psittaci* LPS (Biotin-cLPS) or *Escherichia coli* LPS (Biotin-eLPS) was incubated in the absence (–LBP) or presence (+LBP) of 100 ng ml^{-1} LBP. (B) Biotin-cLPS (1000 ng ml^{-1}, 220 nM) was incubated with 100 ng ml^{-1} LBP in the TLR4–MD-2-immobilized plates in the absence or presence of unlabeled *C. psittaci* LPS (0.22 nM–22 μM) or *E. coli* LPS (0.063 nM–6.3 μM). (C) Biotin-eLPS (100 ng ml^{-1}, 6.3 nM) was incubated with 100 ng ml^{-1} LBP in the TLR4–MD-2-immobilized plates in the absence or presence of unlabeled *C. psittaci* LPS (0.22 nM–2.2 μM) or *E. coli* LPS (0.063–630 nM). Bound LPS was finally detected using streptavidin–HRP. Data are the mean ± SD of five independent experiments.

negligible amounts of IL-18 and IFN-γ could be produced from *C. psittaci* LPS-stimulated PBMC (data not shown). Thus, the IFN-γ and IL-18 production is likely to be mainly regulated by other *Chlamydophila*-derived constituents than LPS. Identification of those constituents should be important for understanding of pathogen growth during *Chlamydiaceae* infection.

It is noteworthy that the binding capacities and biological activities of *C. psittaci* LPS were lower than those of *E. coli* LPS (Figs 1–3). Thus, we postulated that the lower activities of *C. psittaci* LPS may result from its weak interaction with LBP and/or receptor molecules. By employing the ELISA-based assays, we confirmed not only the direct binding of *C. psittaci* LPS to LBP but also the LBP-dependent interaction of LPS with CD14, which is in good agreement with the LPS binding to monocytes analyzed by flow cytometry (Figs 1, 4 and 5). Supporting our hypothesis, the binding of *C. psittaci* LPS to LBP and CD14 was much less than that of *E. coli* LPS. Indeed, higher concentrations of *C. psittaci* LPS were required for inhibiting the binding of *E. coli* to LBP and CD14, while *E. coli* LPS effectively competed with the binding of *C. psittaci* at the low concentrations (Figs 4 and 5). Based on IC_{50} values, the binding affinity of *C. psittaci* LPS for LBP was at least 5-fold lower than that of *E. coli* LPS, and the binding affinity for CD14 is at least 6-fold lower than *E. coli* LPS. Thus, it is reasonable to speculate that the structural differences between *C. psittaci* and *E. coli* LPS may affect the binding profiles of these LPS. Furthermore, the low binding affinities of *C. psittaci* LPS with LBP and CD14 are assumed to reduce the efficiency of LPS-induced signaling.

Moreover, the ELISA-based binding experiments revealed that *C. psittaci* LPS and *E. coli* LPS can directly bind to TLR4–MD-2 complex in an LBP-dependent manner, and the binding of *C. psittaci* LPS was much lower than that of *E. coli* LPS. Interestingly, however, the competition experiments showed that both unlabeled *C. psittaci* LPS and *E. coli* LPS inhibited the binding of biotinylated *C. psittaci* LPS and *E. coli* LPS to TLR4–MD-2 complex with similar affinities. Taken together, the low binding affinities of *C. psittaci* LPS with LBP and cell-surface CD14 but not TLR4–MD-2 are most likely to define its weak bioactivities.

It has been proposed that persistent infection with *Chlamydiaceae* in host cells is closely associated with chronic inflammation (3, 7, 33, 34). In the present study, we have suggested that *C. psittaci* LPS play a role in inflammatory responses via the weak activation of host immune cells such as PBMC. Recent study indicated that *Chlamydiaceae*-derived unknown components can be recognized by nucleotide-binding oligomerization domain 1 (NOD1) to activate endothelial cells and induce IL-18 production (42, 43). Thus, *Chlamydiaceae* LPS and other components are likely to cooperatively stimulate host cells and contribute to the persistent infection. It still remains unknown whether *Chlamydiaceae* LPS can activate other target cells such as endothelial cells, which differently express LPS receptors from monocytes/macrophages. Detailed analysis of the actions of *Chlamydiaceae* LPS on

these cells leads to a better understanding of *Chlamydiaceae*-induced immune responses in the host.

Funding

Institute for Environmental and Gender-specific Medicine, Juntendo University School of Medicine; High Technology Research Center, Juntendo University; Scientific Research from Japan Society for the Promotion of Science; 21st Century COE Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Abbreviations

Alexa488	Alexa Fluor 488
EB	elementary body
HSP60	heat shock protein 60
IC_{50}	50% inhibitory concentration
LBP	LPS-binding protein
MOMP	major outer membrane protein
PMB	polymyxin B
TLR4	Toll-like receptor 4
TMB	tetramethylbenzidine

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