The Proinflammatory Cytokine Response to *Chlamydia trachomatis* Elementary Bodies in Human Macrophages Is Partly Mediated by a Lipoprotein, the Macrophage Infectivity Potentiator, through TLR2/TLR1/TLR6 and CD14

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The Proinflammatory Cytokine Response to *Chlamydia trachomatis* Elementary Bodies in Human Macrophages Is Partly Mediated by a Lipoprotein, the Macrophage Infectivity Potentiator, through TLR2/TLR1/TLR6 and CD14

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Chlamydiae components and signaling pathway(s) responsible for the production of proinflammatory cytokines by human monocytes/macrophages are not clearly identified. To this aim, *Chlamydia trachomatis*-inactivated elementary bodies (EB) as well as the following seven individual Ags were tested for their ability to induce the production of proinflammatory cytokines by human monocytes/macrophages and THP-1 cells: purified LPS, recombinant heat shock protein (rhsp70, rhsp60, rhsp10, recombinant polypeptide encoded by open reading frame 3 of the plasmid (rpgp3), recombinant macrophage infectivity potentiator (rMip), and recombinant outer membrane protein 2 (rOmp2). Aside from EB, rMip displayed the highest ability to induce release of IL-1β, TNF-α, IL-6, and IL-8. rMip proinflammatory activity could not be attributed to *Escherichia coli* LPS contamination as determined by the Limulus Amoeocyte lysate assay, insensitivity to polymyxin B (50 μg/ml), and different serum requirement. We have recently demonstrated that Mip is a “classical” bacterial lipoprotein, exposed at the surface of EB. The proinflammatory activity of EB was significantly attenuated in the presence of polyclonal Ab to rMip. Native Mip was able to induce TNF-α and IL-8 secretion, whereas a nonlipidated C20A rMip variant was not. Proinflammatory activity of rMip was unaffected by heat or protease K treatments but was greatly reduced by treatment with lipases, supporting a role of lipid modification in this process. Stimulating pathways appeared to involve TLR2/TLR1/TLR6 with the help of CD14 but not TLR4. These data support a role of Mip lipoprotein in pathogenesis of *C. trachomatis*-induced inflammatory responses.

Chlamydiae are important human pathogens due to the wide repertoire of important diseases that they cause (1–5). These microorganisms infect primary epithelial cells with subsequent attraction of monocytes/macrophages (6, 7). They frequently cause chronic inflammatory diseases characterized by the presence of a high number of mononuclear cells (8) that are involved in the pathogenesis by inducing mediators of inflammation. However, few data have been published about chlamydial components involved in inflammatory response, and results are still debated. Chlamydial LPS (9, 10) and heat shock protein (hsp)60 (11, 12) were involved in some reports but not in others (13–15). In signaling pathway, whole bacteria or *Chlamydia* hsp60 have been shown to induce TLR-mediated activation, but the signalizing receptor differed among studies from both TLR2 and TLR4 (16), only TLR4 (17, 18), only TLR2 (14), to largely TLR2 and to a minor extent TLR4 (19). Except LPS and hsp60, chlamydiae components responsible for these effects were unidentified.

To identify chlamydial component(s) able to induce production of proinflammatory cytokines by human monocytes/macrophages, inactivated elementary bodies (EB), one of the two forms presented by chlamydiae in their biphasic developmental cycle, as well as seven individual chlamydial Ags: purified LPS, recombinant hsp70 (rhsp70), rhsp60, rhsp10, recombinant polypeptide encoded by open reading frame 3 of the plasmid (rpgp3), recombinant macrophage infectivity potentiator (rMip), and recombinant outer membrane protein 2 (rOmp2) were carefully purified and tested. Aside from EB, rMip displayed the highest proinflammatory activity, stimulating the synthesis of IL-1β, TNF-α, IL-6, and IL-8. Mip was recently shown to have lipid modification similar to that of other procaryotic lipoproteins and to be exposed at the surface of EB (20). The proinflammatory activity of EB was significantly attenuated in the presence of polyclonal Ab to rMip. Failure of stimulation with a nonlipidated C20A rMip variant as well as after lipase treatment of rMip showed that the proinflammatory activity was dependent upon lipid modification. Use of blocking mAb and human embryonic kidney (HEK)-293 transfected cells

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**Abbreviations used in this paper:** EB, elementary body; hsp, heat shock protein; pgp3, polypeptide encoded by open reading frame 3 of the plasmid; Mip, macrophage infectivity potentiator; Omp2, outer membrane protein 2; WT, wild type; HEK, human embryonic kidney.

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revealed that TLR2/TLR1/TLR6 and CD14 but not TLR4 were involved in mediating these effects.

Materials and Methods

**Bacteria and aged**

EB of *Chlamydia trachomatis* LGV2 strain 434 (inactivated by a photochemical treatment affecting bacterial genomes) were either purchased from Biodisgn International (Milan Analytica) or prepared according to the method of Bollet et al. (21). *Chlamydia* LPS was extracted according to the protocol of Mayer et al. (22). LPS from *Escherichia coli* serotype O55B5 and *Salmonella typhimurium* were purchased from Sigma-Aldrich (Fluka Chemie) and repurified (23) to rule out protein and lipoprotein contamination. Cloning, expression in E. coli, and purification of six different recombinant proteins from *C. trachomatis* (rrip170, rshp60, rshp10, rpp3, rMip, and rOmp2) were previously described (24, 25). To rule out LPS and LPS-associated molecule contamination (26–28), all recombinant proteins were subsequently treated by polymyxin B-agarose (Sigma-Aldrich) and had <10 endotoxin units per milligram, according to the Limulus Amebicolyte Lysace chromogenic assay (BioWhittaker Cambrex), which is an amount that did not stimulate proinflammatory cytokine production by itself. Preparations of native Mip and CD20A rMip variant were previously described (20). Protein concentrations were determined by using a microbicinonic acid protein assay kit (Pierce, Perbio Science). Racemic Pam3CSK4 (Pam3, Cys-Ser-Lys-Lys-OH) and Pam2CSK4, (Pam3-Cys-Ser-Lys-OH) used as synthetic triacylated and diacylated control lipopeptides, respectively, were obtained from EM Microcollections.

**Abs and antisera preparation**

Anti-CD14 (clone MY4, IgG2b) was purchased from Coulter Clone (Beckman Coulter), anti-TLR1 (clone GZD4, F(ab′)2, IgG1) from Hycult Biotechnology (Biocoba), anti-TLR2 and anti-TLR4 (clone TL2.1 and HTA125, respectively, IgG2a) from ImmunoMerkont (AMS Biotechnology). Anti-TLR6 (clone 127, IgG1) was previously described (29). Isotype-matched mouse IgG1 control was purchased from Southern Biotechnology Associates and IgG2a, and IgG2b from Coulter Clone. Preparation of IgG fraction from rMip antisera was previously described (20).

**Human monocytic/macrophage culture**

PBMC from healthy blood donors were isolated by density gradient centrifugation with Ficoll-Hypaque (Amersham Biosciences). Monocytes/macrophages were separated by aggregation, gradient of FBS (Invitrogen Life Technologies), and rosetting (30, 31). Monocyte purity consisted of >=90% CD14+ cells, ≤1% CD3+ cells, and ≤1% CD19+ cells as assessed by flow cytometry. The enriched monocytes were cultured in 24-well plates (105 cells/ml per well) in RPMI 1640 containing 2 mM GlutaMAX I, 5% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml blasticidin S (InvivoGen), and maintained in DMEM (Invitrogen Life Technologies) supplemented with 4.5 g/L glucose, 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 μg/ml blasticidin S (InvivoGen) for 240 h. After 48 h (or indicated time periods) stimulation at 37°C with indicated stimuli, cells were centrifuged at 400 × g for 10 min at 4°C and cell-free supernatants were collected and stored at −70°C until cytokine measurements.

**THP-1 cell culture**

Cells of the human promyelomyelocytic cell line THP-1 (32) were purchased from American Type Culture Collection and were grown in RPMI 1640 medium as described above. For monocytic differentiation, they were seeded in 24-well flat-bottom tissue culture plates at a density of 2.5 × 10^6 cells/l ml per well and allowed to adhere and differentiate 48 h at 37°C in the presence of 10 nM PMA (Sigma-Aldrich). After repeated washing with RPMI 1640, PMA-differentiated THP-1 cells were stimulated at 37°C with indicated stimuli. Cell-free supernatants were harvested after 4 h (or indicated time periods) of incubation and kept at −70°C until cytokine measurements.

**PMA-differentiated THP-1 cells were pretreated at 37°C for 1 h with 50 μg/ml human IgG to block Fc receptors and prevent subsequent nonspecific binding of IgG and then stimulated with inactivated *C. trachomatis* EB (5 × 10^7/ml) in presence of rabbit polyclonal anti-rMip IgG or pre-immune IgG (0, 40, or 80 μg/ml). Cell-free supernatants were harvested after 4 h of incubation and kept at −70°C until cytokine measurements.**

**Heat, proteinase K, alkaline, and lipase treatments of rMip**

Heat sensitivity was determined by heating rMip at 100°C for 3 h and proteinase K sensitivity by incubating rMip with 100 μg/ml proteinase K (Promega) in 100 mM Tris-HCl (pH 8.0) at 37°C for 2 h, followed by addition of 200 μM PMSF. Alkaline hydrolysis was performed according to Mahlradt and Frisch (33). Briefly, rMip solution was adjusted to pH 13.0 with sodium hydroxide, incubated at 37°C for 1 h, and neutralized with HEPES buffer after assay. Lipase sensitivity was determined with two glycerol ester hydrolases (E.C. 3.1.1.3) having different side chain specificity (34). Either 1000 U/ml pig pancreas lipase (type VI-S lipase; Sigma-Aldrich) or 10,000 U/ml Bhatipus arhichus lipase (type XI lipase; Sigma-Aldrich) were added to rMip at 37°C for 16 h, in 50 mM HEPES buffer (pH 7.5), 10 mM CaCl2, followed by heating at 100°C before assay (35). As controls, rMip was incubated in buffer with sodium hydroxide or enzymes and PMA-differentiated THP-1 cells were incubated with buffer and enzymes in the absence of rMip. Cell-free supernatants were harvested after 4 h of incubation and kept at −70°C until cytokine measurements.

**CD14, TLR1, TLR2, TLR4, and TLR6 blocking experiments**

Blocking experiments were performed after 1 h pretreatment of PMA-differentiated THP-1 cells with 50 μg/ml human IgG to block Fc receptors and prevent subsequent nonspecific binding of blocking Ab or nonimmune isotype controls. Cells were next incubated at 37°C for 1 h with blocking Ab (5 μg/ml) before stimulation with either 1 μg/ml rMip, inactivated *C. trachomatis* EB (5 × 10^7/ml), 0.01 μg/ml lipopeptides (Pam3CSK4, or Pam3CSK5), or 1 μg/ml E. coli LPS. Cell-free supernatants were harvested after 4 h of incubation and kept at −70°C until cytokine measurements.

**Response of TLR/CD14 cell lines**

HEK-293 cells stably transfected with either the empty plasmid (293-Null) or human TLR1/2, TLR2/6, or TLR2/CD14 genes were purchased from Invitrogen (LabForce) and maintained in DMEM (Invitrogen Life Technologies) supplemented with 4.5 g/L glucose, 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 μg/ml blasticidin S (InvivoGen) for 293-Null, 293-hTLR1/2, and 293-hTLR2/6 and with 50 μg/ml HygroGold (InvivoGen) for 293-hTLR2/CD14. For stimulation experiments, stable transfected cells were seeded into individual wells of a 48-well tissue culture plate at a concentration of 3 × 10^5 cells in 300 μl of complete medium and allowed to adhere overnight. The following day, fresh medium was added, and the cells were stimulated with either 1 μg/ml rMip, inactivated *C. trachomatis* EB (5 × 10^7/ml), 1 or 0.01 μg/ml lipopeptides (Pam3CSK4, or Pam3CSK5), or 1 μg/ml E. coli LPS for 24 h. Culture supernatants were collected, and IL-8 content was analyzed. Results were expressed in terms of fold increase over the IL-8 levels of unstimulated cells.

**Cytokine measurements**

Extracellular release of IL-1β, TNF-α, IL-6, and IL-8 was determined by a sandwich ELISA technique using the DuoSet ELISA Development System (R&D Systems), according to the manufacturer’s instructions. The ELISA detection limits were 2 pg/ml for all tested cytokines. When the distributions in cytokine production were not normal, results were expressed as median and interquartile range.

**Statistical analysis**

Statistical analysis was performed using the SPSS statistical software (for Macintosh, v.10). Kruskal-Wallis and Mann-Whitney U tests were used to compare the levels of inflammatory cytokines produced by human monocytes/macrophages in response to various microbial components. A comparison between two groups was made only when the Kruskal-Wallis test yields statistically significant results. Statistical analysis for PMA-differentiated THP-1 cell and HEP assays were performed using a Student’s t test. Differences were considered significant at p < 0.05.

**Results**

*Inactivated C. trachomatis* EB and rMip elicited proinflammatory cytokine production by human monocytes/macrophages and PMA-differentiated THP-1 cells

The pathology of *C. trachomatis* infection seems to be related to chronic inflammation, characterized by the dominating presence of macrophages in injured tissues. We have therefore tested the ability of inactivated EB and seven chlamydial components (LPS, rshp70, rshp60, rshp10, rpp3, rMip, and rOmp2) to stimulate the production of various proinflammatory cytokines by healthy blood donor monocytes/macrophages. Inactivated EB as well as rMip induced the release of IL-1β, TNF-α, IL-6, and IL-8 in contrast to
other chlamydial Ags that did not display any consistent stimulatory effects. Both *E. coli* and *S. typhimurium* LPS, used as positive controls, highly induced the release of proinflammatory cytokines, in contrast to chlamydial LPS, as previously reported (10, 15, 36, 37) (Fig. 1). To further define the ability of rMip to stimulate the synthesis of proinflammatory cytokines, dose-response and time course experiments were performed. A dose-dependent stimulation was observed in presence of increasing concentrations of rMip (0.005–5 µg/ml) (Fig. 2). The release of TNF-α was time-dependent with maximal levels being reached after 8 h of culture (Fig. 3). Our data agree with previous findings showing that monocytes/macrophages produced TNF-α shortly after stimulation with lipoproteins (38, 39).

As PMA-differentiated THP-1 cells are resembling to tissue macrophages and are commonly used as models for human monocytes/macrophages (40), they were also tested for their ability to

![FIGURE 1. Cytokine productions by human monocytes/macrophages in response to various bacterial Ags. Human monocytes/macrophages (10^5 cells/1 ml per well) were stimulated by inactivated *C. trachomatis* EB (5 × 10^6/ml), rhp70, rhp60, rhp10, rpgp3, rOmp2, rMip, LPS, *E. coli* LPS or *S. typhimurium* LPS at 1 µg/ml. After 48 h of culture, supernatants were collected and their content in IL-1β, IL-6, IL-8, and TNF-α were analyzed by ELISA. Results were obtained from three different cultures performed in triplicates. Horizontal bar within boxes shows the median, boxes show the interquartile range, and vertical bar shows the 95% confidence interval (values above and below these levels were plotted separately). *, p < 0.05; **, p < 0.005 determined by comparison with unstimulated cells using Mann-Whitney U test.](http://www.jimmunol.org/)

![FIGURE 2. Cytokine productions by human monocytes/macrophages in response to increasing concentrations of rMip. Each value represents the mean ± SD of triplicates from two experiments. *, p < 0.05; **, p < 0.005; ***, p < 0.0001 determined by comparison with unstimulated cells using Mann-Whitney U test.](http://www.jimmunol.org/)
produce cytokines in presence of the bacterial components. As already observed with human monocytes/macrophages, inactivated EB as well as rMip were the most effective stimuli in triggering the synthesis of IL-1β, TNF-α, IL-6, and IL-8. Hsp60 triggered a lower but significant synthesis of IL-1β, TNF-α, IL-6, and IL-8. Hsp70 triggered a very low but significant synthesis of IL-1β, TNF-α, IL-6, and IL-8. Chlamydia LPS stimulated a low IL-8 production, whereas E. coli and S. typhimurium LPS strongly stimulated the synthesis of all the cytokines (Fig. 4). Because rMip was the only chlamydial Ag that consistently stimulated THP-1 cells, dose-response and time course experiments were performed with rMip. At concentrations ranging from 0.001 to 10 μg/ml, rMip elicited a dose-dependent increase in IL-1β, TNF-α, and IL-8 production with maximal response at 10 μg/ml. The production of cytokines was significantly stimulated when cells were incubated with 1 μg/ml rMip (Fig. 5). When comparing Figs. 2 and 5, the proinflammatory activity of rMip was weaker in PMA-differentiated THP-1 cells than in primary human monocytes/macrophages. This difference might be due to different levels of TLRs or CD14 expression at the surface of the cells. In PMA-differentiated THP-1 cells, significant stimulation of TNF-α release was already detected within 2 h and increased rapidly thereafter. At 24 h, TNF-α levels reached peak values and then declined gradually (Fig. 6).

Because the response of human monocytes/macrophages varied from one donor to another, the proinflammatory activity of rMip was further studied on PMA-differentiated THP-1 cells to obtain more homogeneous and reproducible data. Based on above results and for practical reasons, the effect of rMip was further examined at 1 μg/ml concentration and at the shortest time point (4 h) required to obtain a significant production of TNF-α.

E. coli LPS contamination is not involved in the production of TNF-α mediated by rMip

Despite the fact that no endotoxin was detected by the Limulus Amoebocyte lysate assay in the highly purified preparation of rMip, the possibility that its proinflammatory activity could be attributed to E. coli LPS contamination was further investigated by testing rMip sensitivity to polymyxin B (50 μg/ml). The ability of rMip to induce TNF-α production by PMA-differentiated THP-1 cells was unaffected by the presence of polymyxin B whereas highly purified E. coli LPS was unable to induce TNF-α production in presence of polymyxin B. In addition, rMip and LPS differed in their serum requirement: in absence of serum, TNF-α production induced by rMip was significantly increased whereas E. coli LPS was devoid of effect as already reported (41–44) (Fig. 7).

Anti-rMip polyclonal IgG partly inhibit the production of TNF-α mediated by C. trachomatis EB

The probable involvement of native Mip in initiation of chlamydial infections has been demonstrated by Lundemose et al. (45) who...
observed a neutralization of the organism in cell culture in presence of anti-Mip Ab. To investigate whether the proinflammatory activity of *C. trachomatis* EB could be attributed to the presence of native Mip exposed at the EB surface (20), experiments were conducted in presence and in absence of rabbit polyclonal IgG anti-rMip. The presence of 80 \( \mu \)g/ml IgG anti-rMip led to significant inhibition of TNF-\( \alpha \) release (33\%, \( p < 0.014 \)) when compared with TNF-\( \alpha \) release in presence of preimmune rabbit IgG (Fig. 8). These results show that native Mip exposed at the EB surface contributes to induce TNF-\( \alpha \)-production when PMA-differentiated THP-1 cells are cultured in presence of *C. trachomatis* EB.

Native Mip but not C20A rMip variant elicited proinflammatory cytokine production

To ascertain that wild-type (WT) rMip activity featured the same stimulatory properties than native Mip, this lipoprotein was purified from *C. trachomatis* EB by immunoprecipitation and tested for its ability to induce the production of TNF-\( \alpha \) by PMA-differentiated THP-1 cells. In addition, to assess the importance of lipid modification, proinflammatory activity of nonlipidated C20A rMip variant was also tested. As shown in Fig. 9, native Mip was able to induce release of TNF-\( \alpha \) and IL-8, in a similar amount than WT rMip. In contrast, no cytokine production was observed when THP-1 cells were cultured in presence of C20A rMip variant suggesting that lipidation plays a major role in proinflammatory activity of native and WT rMip.
Production of proinflammatory cytokines induced by rMip is lipid dependent

Because rMip appeared to have the same proinflammatory activity that native Mip and because it is not feasible to purify adequate amount of native Mip from C. trachomatis for analysis, WT rMip, previously shown to be lipidated as native Mip (20), was used for further investigation. To determine the biochemical nature of rMip proinflammatory activity, experiments were conducted to examine the possible involvement of protein and lipid parts of rMip. To determine the importance of the total protein part, attempts to destroy rMip activity by heat, and proteinase K were tested. Heat treatment was unsuccessful, indicating that protein’s native conformation is not essential for rMip stimulatory activity. Digestion with proteinase K resulted in loss of the 27,6- and 32-kDa bands (20) (data not shown) but did not affect proinflammatory activity. This result suggests that rMip activity may still reside in resulting lipopeptides after proteolytic digestion, as was already reported for a macrophage-activating lipopeptide from Mycoplasma fermentans (46). The involvement of the lipopeptide moiety was confirmed by three different treatments releasing ester-linked fatty acids. Alkaline hydrolysis completely abolished rMip activity and treatment with lipases from two different sources led to significant losses of TNF-α release (76% with pig pancreas and 78% with R. arrhizus lipases, p < 0.05) (Fig. 10). No significant difference was observed between untreated and mock-treated PMA-differentiated THP-1 cells (data not shown). The substitution of rMip with fatty acids in an ester linkage, alkali-labile (47), appears therefore to be crucial for its proinflammatory activity, as reported for other lipoproteins or lipopeptides (33, 48–51). These data confirm that lipid modification of rMip is essential for its ability to stimulate production of proinflammatory cytokines by PMA-differentiated THP-1 cells.

CD14, TLR1, TLR2, and TLR6 but not TLR4 are involved in rMip activation of THP-1 cells

If rMip is able to induce proinflammatory cytokines in human monocytes/macrophages, the specific host receptors that mediate its activation is unknown. As TLRs and CD14 are usually involved in bacterial lipoprotein recognition (52), experiments were conducted in the presence and in the absence of function-blocking mAbs against CD14, TLR1, TLR2, TLR4, and TLR6. These receptors are expressed on cell surface of both human monocytes and monocytic cell line THP-1 (29, 53, 54). The pretreatment of THP-1 cells with MY4, an Ab blocking the mCD14-part of the LPS receptor (55), led to significant inhibition (p < 0.05) of rMip-mediated TNF-α production, indicating that rMip binds to mCD14 at the LPS-binding site as already observed for sponchoetal lipoproteins (53, 56, 57). The pretreatment of THP-1 cells with TL2.1, a specific blocker of extracellular human TLR2 (58) or GD2.4, a mAb blocking TLR1 (59), led to significant inhibitions (p < 0.05) of rMip-mediated TNF-α production, whereas no significant effect was observed neither in presence of HTA125, an anti-TLR4 blocking mAb (60, 61) nor in presence of the clone 127, an anti-TLR6 blocking mAb (29). The combination of anti-TLR2 with either anti-TLR6 or -CD14 led to clear inhibitions (p < 0.005), and the combination of anti-TLR2 with anti-TLR1 to an almost complete inhibition (p < 0.0001) of TNF-α secretion, as shown in Fig. 11A. When inactivated C. trachomatis EB were used as stimulant, the combination of anti-TLR2 with anti-CD14, anti-TLR1, or anti-TLR6 induced significant inhibitions (p < 0.05 to 0.005), whereas the combination of anti-TLR4 with anti-CD14 provoked no significant reduction of EB-mediated TNF-α synthesis, as shown in Fig. 11B. Because TLR1 and TLR2 have been shown to be required for recognition of triacylated lipopetides, such as Pam3CSK4 (62–64) whereas TLR6 and TLR2 were required for recognition of diacylated lipopeptides such as Pam2CSK4 but not for recognition of triacylated lipopeptides (63, 65), these two prototypic synthetic lipopeptides were used as controls and tested in the same conditions. When Pam3CSK4 was used as stimulant, the pretreatment of THP-1 cells with the combination...
of anti-TLR2 with anti-CD14, anti-TLR1, or anti-TLR6 led to significant inhibitions \((p < 0.05 \text{ to } 0.0001)\) of TNF-\(\alpha\) secretion, the association of anti-TLR2 with anti-TLR1 being the most inhibitory, as shown in Fig. 12A. When Pam\(_3\)CSK\(_4\) was used as stimulant, the pretreatment of THP-1 cells with the combination of anti-TLR2 with anti-TLR1 or anti-TLR6 led to significant inhibitions \((p < 0.05 \text{ to } 0.0001)\) of TNF-\(\alpha\) secretion, the association of anti-TLR2 with anti-TLR6 being the most inhibitory, as shown in Fig. 12B. These data show no clear-cut segregation of Pam\(_3\)CSK\(_4\) and Pam\(_2\)CSK\(_4\) interactions with TLR2/TLR1 or TLR2/TLR6 (62–65) but rather a tendency of Pam\(_3\)CSK\(_4\) to stimulate THP-1 cells more efficiently via TLR2/TLR1 and Pam\(_2\)CSK\(_4\) via TLR2/TLR6. Because \(E.\ coli\) LPS is commonly used as TLR4 and CD14-ligand (66), it was also used as control and tested in the same conditions. The pretreatment of THP-1 cells with the combination of anti-TLR4 with anti-CD14 led to significant inhibitions of TNF-\(\alpha\) secretion \((p < 0.005)\), whereas no inhibition was obtained with the combination of anti-TLR2 with anti-CD14, as shown in Fig. 12C. In all these experiments, no significant change of cytokine production was induced by nonimmune mouse IgG used as controls instead of each mAb.
CD14, TLR1, TLR2, and TLR6 are involved in rMip activation of HEK-293 cell lines expressing human TLR1/2, TLR2/6, or TLR2/CD14

To further ensure proper identification of receptors involved in the recognition of rMip and to assess their respective contribution, a cell model using HEK-293 cells transfected with human TLR1/2, TLR2/6, or TLR2/CD14 genes was used. Null cells lacking TLRs and CD14 responded to rMip by producing a 2.8-fold increase of IL-8 release in absence of stimulation, indicating that rMip was able to slightly stimulate these cells in absence of TLRs and CD14 but no response was observed with other stimulants. In contrast, HEK-293 cells expressing either hTLR1/2, or hTLR2/6, or hTLR2/CD14 responded to all stimulants, except Pam3CSK4 and Pam2CSK4. The highest stimulatory effect of Pam3CSK4 was obtained under the condition of hTLR1/2 coexpression that led to a 11-fold increase of IL-8 release (p < 0.005) but was no more able to stimulate cells expressing hTLR2/6. However, Pam3CSK4 was still able to stimulate HEK-293 cells coexpressing hTLR1/2 and coexpressing hTLR2/6 that led to a 9- and 5-fold increase of IL-8 release, respectively (p < 0.005), as shown in Fig. 13. These results agree with those obtained in blocking experiments except that blocking of both TLR2 and TLR6 slightly inhibited Pam3CSK4 activation and that blocking of both TLR2 and CD14 did not inhibit Pam3CSK4 activation. These discrepancies could be explained by different expression ratios of TLR6 and CD14 in HEK compared with THP-1 cells. Taken together, these data indicate that rMip, inactivated C. trachomatis EB, Pam3CSK4, and Pam3CSK4 are only partially TLR1-, TLR6-, and CD14-dependent. These results are therefore partially contradictory to previous reports identifying TLR1 as the sole coreceptor for triacylated lipopeptides (62), and TLR6 as the sole coreceptor for diacylated lipopeptides (67) while supporting results of other groups (68–70).

Discussion

The present study demonstrates that among seven individual chlamydial components, rMip was the most effective to induce a proinflammatory cytokine response in human monocytes/macrophages (peripheral blood monocytes and THP-1 cell line). Even if rMip responses were lower than those induced by E. coli and S. typhimurium LPS, they were significant in contrast to Chlamydia LPS, as already reported (10, 15, 36, 71). IL-8 was the dominant cytokine induced by rMip in vitro and this observation must be related to the cytokine patterns present in vivo during infection by chlamydiae where high levels of IL-8 were reported in tears from children with trachoma, as well as in endocervical secretions obtained from women infected with Chlamydia (72).

The proinflammatory activity induced by WT rMip was not due to E. coli LPS contamination, as determined by the Limulus Amoeboocyte lysate assay, insensitivity to polymyxin B, different serum requirement, and absence of inhibition by anti-TLR4 Abs. The stimulatory activities of WT rMip were similar to those of native Mip and appeared to be dependent upon lipid modification because nonlipidated C20A rMip variant was devoid of effect on cytokine release. In addition, WT rMip activity was greatly reduced by alkaline hydrolysis or treatment with lipases but was unaffected by heat or proteinase K treatments. The cell receptors involved in rMip and C. trachomatis EB cellular activation were determined using two independent assays that clearly demonstrated the involvement of TLR1/2 and CD14 but not TLR4. The main receptors involved in rMip activation were TLR1/2 but the co-presence of TLR1, TLR6, and CD14 was not absolutely required

FIGURE 13. The production of IL-8 by HEK-293 cell lines expressing human TLR1/2, TLR2/6, or TLR2/CD14 upon rMip or inactivated C. trachomatis EB activation. Null, hTLR1/2, hTLR2/6, and hTLR2/CD14 cells were stimulated with 1 μg/ml rMip, inactivated C. trachomatis EB (5 × 10³/ml), 1 or 0.01 μg/ml lipopeptides (Pam3CSK4 or Pam2CSK4), or 1 μg/ml E. coli LPS for 24 h. Culture supernatants were collected, and IL-8 content was analyzed. The results are expressed in terms of fold increase over the IL-8 levels of unstimulated cells. Each value represents mean ± SD of triplicates from three independent experiments. *, p < 0.05; **, p < 0.005; ***, p < 0.0001 determined by one sample t-test; mean is significantly different from 1.
because activation was possible by TLR1/2, TLR2/6, or TLR2/CD14. In these assays, the receptors involved in Pam3CSK4 and Pam3CSK4 activation did not completely agree with the original concept, according to which triacylated lipopeptides recognize TLR2/TLR1 heteromers, whereas diacylated lipopeptides recognize TLR2/TLR6 heteromers (63, 65, 73) but agree with other reports (68–70). Indeed, the ability of Pam3CSK4 to stimulate HEK-293 cells coexpressing hTLR1/2 and, to a lesser extent, hTLR2/6 has been reported (68) and Pam3CSK4 has been shown to exhibit some activity toward TLR2/6 when used at high concentrations (69). Pam3CSK4 has been shown to exhibit comparable activities toward both the human TLR2/1 and TLR2/6 pairs (69) and macrophages of TLR6-deficient mice to be fully responsive to Pam3CSK4 (70). In fact the number of acyl-residues, the peptide sequence and the whole molecular structure of the lipopeptide/lipoprotein have been shown to be responsible for TLR2/1- or TLR2/6-dependent signaling (70, 74). The involvement of CD14 in rMip activation agrees with other studies showing that bacterial lipopolysaccharides (53, 56, 75, 76), Pam3CSK4 (53, 77), and Pam3CSK4 (68) interact with CD14 to cause cytokine induction. These results agree with reports showing a predominant role of TLR2 in C. trachomatis (78) as well as C. pneumoniae recognition (14, 19, 79) and with results of Netea et al. (14) attributing proinflammatory cytokine production to non-LPS components. The fact that rMip and EB act through the same receptors and that anti-rMip Ab is able to partly inhibit EB-mediated TNF-α release suggest a role of native Mip, present at the EB surface (20), or other surface lipopolysaccharides in EB recognition and macrophage activation in natural infection. If IL-1β, TNF-α, and IL-6 release in response to native Mip or other lipopolysaccharides may aid in eradicating Chlamydia infection (80–83), particularly if their levels increase early (83), these cytokines may also promote long-term tissue damage. As an example, high levels of TNF-α have been associated with detrimental effects in ocular chlamydial infection (84). Moreover, Mip-induced IL-8 can be deleterious (83), particularly in case of chronic secretion. Indeed, IL-8 promotes the infiltration of neutrophils that are not only inefficient in resolving chlamydial infections but can release proteases that damage cells. In addition and as reported for other bacterium-associated lipopolysaccharides (85), Mip or other lipopolysaccharides could be released from EB surface (either from living bacteria or from bacteria lysed as a result of effective host defense or the activities of certain antibiotics) and retained inside tissues where they might activate resident cells and perpetuate inflammation even after the eradication of live bacteria with antibiotic therapy. Lipopolysaccharides are considered as crucial virulence factors in inflammatory processes and in pathogenesis of several important bacterial infections, such as those triggered by Mycobacterium tuberculosis (76), Neisseria gonorrhoeae (86), Listeria monocytogenes (58, 87), Brucella abortus (88), and members of Enterobacteriaceae family (89, 90). In organisms such as Borrelia burgdorferi and Treponema pallidum, which lack LPS, bacterial lipopolysaccharides are known to play an important role in pathogenesis (49, 75, 88, 91–93). In chlamydiae, LPS is a major structural component of all chlamydiae species (22) but compared with the LPS of enterobacteria, it has much lower endotoxin activity (10, 71, 94, 95) because its lipid A is highly hydrophobic and has unique structural features with the presence of unusual, long-chain fatty acids (96). Chlamydia LPS is unable to elicit inflammation in experimental animals (13) and is a weak inducer of the inflammatory cytokine response (10, 36, 37), as shown in the present study. All these data support the hypothesis that in chlamydiae, Mip or other lipopolysaccharides might play a key role in pathogenesis. The fact that Mip is present in the membrane of EBs as well as reticulate bodies (97) and was recently shown to be surface exposed by two different approaches (20) reinforces its potential role in pathogenesis of Chlamydia infection. If the exact chemical nature of Chlamydia-derived monocyte/macrophage stimulators is not known, the fact that receptors involved in Mip recognition are similar to those involved in C. trachomatis EB recognition is supportive of the involvement of Mip or other Chlamydia-associated lipopolysaccharides as inflammatory active element of EB.

In conclusion, this study is the first report about a chlamydial lipoprotein displaying proinflammatory properties. As Mip appears to be present in different species of the Chlamydiaceae family (20), it could have an important role in the inflammatory aspects of trachoma, reactive arthritis, or attherosclerosis.

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Disclosures

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References


