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Chlamydia pneumoniae Stimulates IFN-γ Synthesis through MyD88-Dependent, TLR2- and TLR4-Independent Induction of IL-18 Release


Recent studies suggest that inflammation plays a central role in the pathogenesis of atherosclerosis, and IFN-γ is a prominent proinflammatory mediator in this context. However, it is unclear what stimuli are responsible for initial stimulation of IFN-γ synthesis in the vessel wall. In the present study, we demonstrate that Chlamydia pneumoniae is an important stimulus for IFN-γ synthesis, and this production depends on release of endogenous IL-18, IL-12, and IL-1, but not of TNF. The production of the proinflammatory cytokines TNF and IL-1β from PBMC by sonicated C. pneumoniae was mediated through TLR2-dependent pathways. In contrast, C. pneumoniae stimulated the production of IL-18 through MyD88-dependent, TLR2-, TLR4-, and CD14-independent pathways, mediated by posttranscriptional mechanisms not involving de novo protein synthesis. In conclusion, C. pneumoniae is a potent stimulus of IFN-γ production, in addition to the proinflammatory cytokines TNF and IL-1β, which may contribute to its proatherogenic effects. Most interestingly, C. pneumoniae is also a potent inducer of IL-18 production through pathways independent of TLR2 and TLR4.


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3 Abbreviations used in this paper: BP, binding protein; ICE, IL-1β converting enzyme; IFU, inclusion-forming units; IL-1ra, IL-1R agonist.
used for the experiments. TLR2-deficient (TLR2(-/-)) and MyD88-deficient (MyD88(-/-)) mice on a C57BL/6J background were kindly provided by Dr. S. Akira (Research Institute of Microbial Diseases, Osaka University, Osaka, Japan), and the control C57BL/6J mice (TLR2 (+/ +) controls) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were fed sterilized laboratory chow (Hope Farms, Woerden, The Netherlands) and water, ad libitum.

C. pneumoniae

C. pneumoniae TW-183 was grown in Hep2 cells, cultured in HEPES-buffered MEM containing 10% FCS, 0.5% (w/v) glucose and 0.5% (w/v) cycloheximide (Flow Laboratories, Irvine, U.K.). After 48, 72, and 96 h, the supernatant containing the cell debris was separated by low speed centrifugation (10 min, 500 g). The infectivity of the Chlamydia-containing supernatant (inclusion-forming units (IFU)) was further tested in Hep2 cells and adjusted to 10⁶ IFU/mL. As a control, supernatants from cultures of uninfected Hep2 cells were collected and prepared in a similar manner as described above. C. pneumoniae was killed by sonication for 10 min, on ice (Bransonic 2200; Branson, Shelton, CT). Sonicated C. pneumoniae was used in all experiments, if not otherwise indicated. The same procedure was followed for the conditioned medium obtained from uninfected Hep2 cells. Heat-killed (30 min, 100°C) C. pneumoniae, Candida albicans strain UC820, and a clinical isolate of Staphylococcus aureus were used in some of the experiments, as indicated.

In a control experiment, the supernatants containing Chlamydia were centrifuged, the cell pellets containing the microorganisms were washed three times in sterile RPMI 1640 and resuspended in culture medium. No differences in cytokine production were detected between the supernatants containing Chlamydia and the repurified preparations (data not shown).

Stimulation of cytokine production in human PBMC

Venous blood was drawn from the cubital vein of healthy volunteers into three 10-mL EDTA tubes (Monoject, s-Hertogenbosch, The Netherlands). The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficol-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMC were washed twice in saline and suspended in cultured medium (RPMI 1640 Dutch modification) supplemented with gentamicin 10 μg/mL, l-glutamine 10 mM, and pyruvate 10 mM. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands), and the number was adjusted to 5 × 10⁵ cells/mL. As a control, supernatants containing Chlamydia were centrifuged, the cell pellets containing the microorganisms were washed three times in sterile RPMI 1640 and resuspended in culture medium. No differences in cytokine production were detected between the supernatants containing Chlamydia and the repurified preparations (data not shown).

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Cytokine production by murine peritoneal macrophages

Resident peritoneal macrophages from either ScCr and C57Bl/10 mice, or TLR2(-/-) and C57Bl/6J mice, were harvested by injecting 4 mL of sterile PBS containing 0.38% sodium citrate. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM l-glutamine, 100 μg/mL gentamicin, and 2% fresh mouse plasma. Cells were cultured in 96-well microtiter plates (Greiner Bioscience) at 1 × 10⁶ cells/well, in a volume of 100 μL. The cells were stimulated with 100 μL of conditioned medium from uninfected Hep2 cells, or sonicated C. pneumoniae (final concentration, 1 × 10⁵ IFU/mL). After 24-h incubation at 37°C, the supernatants were collected and stored at −70°C until cytokine assays were performed.

Cytokine measurements

Human TNF-α and IL-1β concentrations were determined by specific RIAs, with a detection limit of 20 pg/mL. IFN-γ concentrations were measured by commercial ELISA (Pelikine Compact; Sanguin Laboratories, Amsterdam, The Netherlands), according to the instructions of the manufacturer. Human and murine IL-18 levels were assessed by a commercial ELISA kit (R&D Systems). The detection limit of the assay was 8 pg/mL. Murine IL-1β and TNF-α were determined by specific RIAs (detection limit, 20 pg/mL), as previously described (17).

Statistical analysis

The human experiments were performed in triplicate in a total of seven volunteers, and all data were pooled. The mouse experiments were performed twice in five mice per group, and the data are presented as cumulative results of all experiments performed. The differences between groups were analyzed by Mann-Whitney U test, and, where appropriate, by Kruskal-Wallis ANOVA test. The level of significance between groups was set at p < 0.05. The data are given as mean ± SEM.

Results

C. pneumoniae stimulates IFN-γ production

Stimulation of human PBMC for 24 h with either live or sonicated C. pneumoniae from a culture containing 10⁴ IFU/mL resulted in significant production of TNF and IL-1β (Fig. 1). In addition, IFN-γ production was also induced by C. pneumoniae sonicates when compared with conditioned medium from Hep2 cells (Fig. 1), although C. pneumoniae was less potent in the induction of IFN-γ (240 ± 20 pg/mL) compared with LPS (720 ± 140 pg/mL). Murine IL-1β and TNF-α were determined by specific RIAs (detection limit, 20 pg/mL), as previously described (17).

Previous studies have demonstrated that mature IL-18 is required for the LPS-induced production of IFN-γ, even when IL-12 is present (18). To investigate the role of endogenous IL-18 for the C. pneumoniae-induced IFN-γ synthesis, IL-18BP was added to the PBMC cultures. IL-18BP reduced C. pneumoniae-induced IFN-γ production by 57% (p < 0.03, Fig. 2). Addition of anti-

FIGURE 1. Stimulation of cytokine production by C. pneumoniae. Human PBMC were stimulated for 24 h with either live or sonicated C. pneumoniae from a culture containing 10⁴ IFU/mL.
were measured after 48-h stimulation. One hundred percent IFN-γ or a combination of IL-18BP and anti-IL-12 Abs. IFN-γ concentrations after 24-h incubation time were measured by ELISA. Donors, n = 6; *, p < 0.05 when compared with stimulation by Chlamydia sonicate alone; **, p < 0.01 when compared with stimulation by Chlamydia sonicate alone.

C. pneumoniae is a strong stimulus for IL-18 production

The marked inhibitory effect of IL-18BP on C. pneumoniae-induced IFN-γ implies that Chlamydia is a potent stimulus of IL-18 production. Stimulation of PBMC with sonicated C. pneumoniae resulted in a dose-dependent stimulation of IL-18 production (Fig. 3). C. pneumoniae appeared to be a more potent stimulus of IL-18 production than other stimuli, as shown by the maximal stimulation index (vs conditioned medium) obtained with sonicated (200-fold) or heat-killed (30-fold) Chlamydia, much higher than the stimulation index obtained with LPS (5-fold), C. albi cans (8-fold), and S. aureus (5-fold) (Fig. 3).

Stimulation of cytokines by C. pneumoniae does not involve TLR4 and CD14

Preincubation of human PBMC for 1 h with anti-TLR4 Abs did not influence the stimulation of TNF, IL-1β, and IL-18 by C. pneumoniae (Fig. 4A). As expected, the anti-TLR4 Abs significantly down-regulated the production of TNF after stimulation by E. coli LPS (85% inhibition, p < 0.01, Fig. 4, inset). To investigate the role of CD14, we preincubated PBMC with a neutralizing anti-CD14 Ab. The anti-CD14 Ab did not influence the production of cytokines induced by chlamydial Ags (Fig. 4A), whereas it almost completely blocked the cytokine induction by E. coli LPS (94% inhibition, p < 0.01, Fig. 4, inset). Blockade of the heparan sulfate-like glycosaminoglycan chlamydial receptor with heparin was not able to down-modulate the synthesis of either TNF, IL-1β, or IL-18 (95–117% of control production, p > 0.05). Heparan sulfate alone did not stimulate cytokine production (data not shown).

Stimulation of TNF and IL-1β, but not of IL-18, depends on TLR2

In control experiments, the anti-TLR2 Ab significantly reduced the TNF production induced by peptidoglycan by 55–70% (p < 0.05). In contrast, the anti-TLR2 Ab did not influence LPS-induced production of TNF (Fig. 4). We confirm earlier data from our group that blockade of TLR2 by the neutralizing Ab resulted in the reduction of proinflammatory cytokine production induced by sonicated C. pneumoniae: 76% inhibition of TNF production, and 94% inhibition of IL-1β production (p < 0.01, Fig. 4B). In contrast, no effect of the anti-TLR2 Ab on the induction of IL-18 could be demonstrated (7% inhibition, p > 0.05, Fig. 4B).

Blocking TLR2, but not TLR4, with specific antagonistic Abs significantly decreased Chlamydia-induced release of IFN-γ by 40–65% (from 459 ± 154 to 243 ± 99 pg/ml, p < 0.05). This effect is most likely mediated through initial inhibition of endogenous IL-1 release, as >90% of IL-1β release was inhibited by the anti-TLR2 Ab, and IL-1 has proved essential for effective Chlamydia-induced IFN-γ production (see above).

Stimulation of IL-18 production by C. pneumoniae

Human PBMC were stimulated with culture medium, LPS (10 ng/ml), heat-killed or sonicated Chlamydia at three different concentrations: 10³, 10⁴, or 10⁵ IFU/ml. IL-18 concentrations after 24-h incubation time were measured by ELISA. Donors, n = 6; *, p < 0.01 when compared with stimulation by conditioned medium.
Induction of proinflammatory cytokines by C. pneumoniae in peritoneal macrophages of MyD88<sup>-/-</sup>, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice

Peritoneal macrophages from all the mouse strains tested did not release detectable proinflammatory cytokines after incubation with culture medium alone. In addition, peritoneal macrophages isolated from TLR2<sup>-/-</sup> mice produced 60–75% less TNF and IL-1β compared with cells isolated from control TLR2<sup>+/+</sup> (C57BL/6J) mice (p < 0.05, Fig. 5A), supporting the hypothesis that proinflammatory cytokine stimulation by C. pneumoniae is mainly mediated by TLR2. In contrast, macrophages isolated from the TLR4-deficient ScCr mice did not produce detectable cytokines after stimulation with LPS, but displayed a cytokine production after stimulation with C. pneumoniae similar to that of control C57BL/10 mice: 82 ± 10% of control TNF production, and 89 ± 15% of IL-1β production in controls (p > 0.05, Fig. 5B). C. pneumoniae stimulated significantly less IL-18 production in the murine macrophages of MyD88<sup>-/-</sup>, but not TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice (Fig. 5).

The role of de novo protein synthesis for the cytokine induction by C. pneumoniae

As a recent study has demonstrated that IL-18 release induced by Chlamydia trachomatis in epithelial cells is independent of de novo protein synthesis (16), we assessed whether similar mechanisms apply for induction of cytokines by C. pneumoniae. Blockade of de novo protein synthesis with cycloheximide has almost completely blocked the production of TNF and IL-1β, whereas the release of IL-18 induced by C. pneumoniae was not influenced (Fig. 6). This demonstrates that IL-18 release by C. pneumoniae is independent of de novo protein synthesis. In contrast, addition of the ICE inhibitor to the cells stimulated with Chlamydia decreased the IL-18 production by 35–50% (p < 0.02).

Discussion

In the present study we demonstrate that C. pneumoniae stimulates production of IFN-γ, in addition to the proinflammatory cytokines IL-1β, TNF, and IL-12. Endogenous IL-18, IL-12, and IL-1β are crucial for the induction of IFN-γ, as the cytokine antagonists IL-18BP, anti-IL-12 Abs, and IL-1ra strongly reduced IFN-γ synthesis stimulated by Chlamydia. Whereas stimulation of TNF and IL-1β synthesis was TLR2 dependent, the release of IL-18 by C. pneumoniae appeared to be mediated by posttranscriptional mechanisms independent of de novo protein synthesis, through TLR2-, TLR4- and CD14-independent pathways.

Experimental studies have shown that exogenous IFN-γ promotes plaque development in apoE<sup>-/-</sup> mice (2), whereas the absence of IFN-γR strongly inhibits atherogenesis (3). Our studies demonstrate that both live C. pneumoniae and acellular chlamydial components not only induce TNF, IL-1β, and IL-6 (19), but are also a strong stimulus of IFN-γ production, and this may be a crucial step in the atherogenic role of Chlamydia. These results are supported by other studies reporting IFN-γ production by C. pneumoniae infection (20, 21). However, the finding that even acellular chlamydial components strongly stimulate IFN-γ production is important in the view of the reports suggesting that mainly the chlamydial Ags and not the live microorganisms are present in the atheromatous plaques in humans (22, 23). IFN-γ may induce atherogenesis by enhancing expression of adhesion molecules and MHC molecules on vascular cells, and by inhibition of collagen synthesis by smooth muscle cells (24). Additional atherogenic effects of C. pneumoniae consist of induction of macrophage foam cell formation (25, 26), oxidation of low density lipoproteins (27), induction of transmigration of leukocytes (12), and blockade of apoptosis of inflammatory cells in the vessel wall (28).

In view of the importance of IFN-γ for the inflammatory reactions in atherosclerosis, it is important to decipher the regulatory mechanism responsible for its release. In the context of stimulation by bacterial components, the monocyte products, IL-18 and IL-12, are responsible for the IFN-γ release from T cells (29). This appears to be true also for stimulation by C. pneumoniae, as the release of IFN-γ was strongly down-regulated by the cytokine antagonists IL-18BP and anti-IL-12 Abs. This is in line with the inhibitory effects of ICE-inhibition on IL-18 release. Similar effects have been reported for C. trachomatis-induced release of IFN-γ, although with this stimulus, the relative role of IL-12 seems to be more important (16).

![Figure 5](http://jimmunol.org/Download/010412/002_010412_002_f05_p1480-f05_p1480.png)

**FIGURE 5.** Stimulation of proinflammatory cytokines by C. pneumoniae in MyD88<sup>-/-</sup>, and TLR2- and TLR4-deficient mice. Peritoneal macrophages from MyD88<sup>-/-</sup> and TLR2<sup>-/-</sup> (A) or TLR4<sup>-/-</sup> (B) knockout mice were stimulated with C. pneumoniae (10<sup>5</sup> IFU/ml) for 24 h at 37°C. Cytokine measurement was performed in the supernatants, and the data represent mean ± SEM of seven mice. *, p < 0.05.

![Figure 6](http://jimmunol.org/Download/010412/002_010412_002_f06_p1480-f06_p1480.png)

**FIGURE 6.** Effect of de novo protein synthesis on cytokine stimulation by C. pneumoniae. Freshly isolated human PBMC were stimulated with 10<sup>5</sup> IFU/ml C. pneumoniae (□), and the effect of de novo protein synthesis on cytokine stimulation was investigated by adding 10 µg/ml cycloheximide during the stimulation (●). Cytokines were measured in the supernatant after 24-h incubation at 37°C, and the data represent mean ± SEM of five volunteers. *, p < 0.05.
We found that *C. pneumoniae* was a stronger stimulus for IL-18 production than other microbial components (such as LPS, heat-killed *C. albicans*, or *S. aureus*). From the experiments using IL-18BP, it can be concluded that the immunoreactive IL-18 is bioactive. The IL-18 and IFN-γ production induced by *C. pneumoniae* may be very relevant for atherogenesis. First, IL-18 has been shown to be expressed on macrophages in atherosclerotic plaques (4), its presence has been correlated with an unstable phenotype (5), and it has been shown to be a predictor of cardiovascular death in stable and unstable angina (30). Second, gene transfer of a plasmid encoding IL-18BP has been shown to modulate plaque development and stability (31), whereas administration of exogenous IL-18 enhanced atherosclerosis in apoE-/- mice (32). Finally, it is important to note that these effects of IL-18 are dependent on IFN-γ release (32).

In our experiments, endogenous IL-1 was involved in the regulation of IFN-γ synthesis, as IL-1ra also strongly decreased *Chlamydia*-induced IFN-γ production, although in earlier studies, IL-1ra failed to show significant inhibitory activity (19). Although IL-1β by itself cannot stimulate production of IFN-γ, recent data indicate that addition of IL-1β to IL-12 potentiates the induction of IFN-γ (33). Our results are supported by Hunter et al. (34), who reported inhibition of IFN-γ by anti-IL-1β Abs when stimulation was induced by microbial stimuli such as LPS, heat-killed *Toxoplasma gondii*, *Salmonella typhimurium*, *Legionella pneumophila*, and *Yersinia pseudotuberculosis*. This role of IL-1β may be explained by structure similarities between the receptor complexes for IL-1α and IL-1β, which consist of a binding chain (IL-1R type I and IL-1Rα) and a signaling chain (IL-1R accessory protein and IL-1Rβ)) respectively (29). In addition to IL-1β, IL-12, and IL-1, Rothfuchs and colleagues (35) have also suggested IFN-α/β mediated induction of IFN-γ by *C. pneumoniae* infection of bone marrow-derived macrophages, and this mechanism may also be present in our system.

A second aim of the present study was to investigate the mechanisms responsible for the stimulation of proinflammatory cytokines by *C. pneumoniae*. TLRs are pattern-recognition receptors believed to have a central role in the innate immunity to pathogens. Ten human TLRS have been identified to date, some of which have crucial roles in the recognition of pathogen-associated molecular patterns, such as TLR4 for LPS and lipoteichoic acid, TLR2 for crucial roles in the recognition of pathogen-associated molecular patterns, such as TLR4 for LPS and lipoteichoic acid, TLR2 for...


