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## ***Chlamydia pneumoniae* Stimulates IFN- $\gamma$ Synthesis through MyD88-Dependent, TLR2- and TLR4-Independent Induction of IL-18 Release**

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# *Chlamydia pneumoniae* Stimulates IFN- $\gamma$ Synthesis through MyD88-Dependent, TLR2- and TLR4-Independent Induction of IL-18 Release<sup>1</sup>

Mihai G. Netea,<sup>2\*‡</sup> Bart Jan Kullberg,<sup>\*‡</sup> Liesbeth E. H. Jacobs,<sup>\*‡</sup> Trees J. G. Verver-Jansen,<sup>\*‡</sup> Johanna van der Ven-Jongekrijg,<sup>\*‡</sup> Jochem M. D. Galama,<sup>†‡</sup> Anton F. H. Stalenhoef,<sup>\*</sup> Charles A. Dinarello,<sup>§</sup> and Jos W. M. Van der Meer<sup>\*</sup>

Recent studies suggest that inflammation plays a central role in the pathogenesis of atherosclerosis, and IFN- $\gamma$  is a prominent proinflammatory mediator in this context. However, it is unclear what stimuli are responsible for initial stimulation of IFN- $\gamma$  synthesis in the vessel wall. In the present study, we demonstrate that *Chlamydia pneumoniae* is an important stimulus for IFN- $\gamma$  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-1 $\beta$  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- $\gamma$  production, in addition to the proinflammatory cytokines TNF and IL-1 $\beta$ , 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. *The Journal of Immunology*, 2004, 173: 1477–1482.

There is increasing evidence that immune mechanisms play a central role in the pathogenesis of atherosclerosis, and proinflammatory cytokines are important mediators of these processes. Recent studies have shown that especially IFN- $\gamma$  plays a central role in the development of atherosclerotic lesions. IFN- $\gamma$  mRNA and protein has been shown to be present in atherosclerotic plaques (1), and administration of IFN- $\gamma$  to apoE<sup>-/-</sup> mice promotes atherosclerosis (2). Conversely, deficiency of the IFN- $\gamma$ R leads to a profound decrease in the atherosclerotic lesions of apoE<sup>-/-</sup> mice (3). The secretion of IFN- $\gamma$  is promoted by proinflammatory mediators such as IL-18 and IL-12 released from the monocytes and macrophages. Recent data put IL-18 in a central role in the inflammatory processes leading to atherosclerosis, with expression of IL-18 on macrophages and IL-18Rs on human vascular endothelial cells, smooth muscle cells, and macrophages (4). IL-18 signaling evokes actions implicated in the pathogenesis of atherosclerosis, such as cytokine and chemokine induction and adhesion molecule expression (4), and expression of IL-18 in the human atherosclerotic plaques seems to correlate with plaque instability (5).

It is not known what factors induce IFN- $\gamma$  and IL-18 production in the vessel wall. In recent years, infections caused by the obligate intracellular Gram-negative bacterium *Chlamydia pneumoniae*

have been connected to coronary heart disease and atherosclerosis (6, 7). *C. pneumoniae* has been detected in atheromatous lesions of patients (8), and in recent animal studies infection with *C. pneumoniae* induced and accelerated atherosclerosis (9). There are several mechanisms through which *C. pneumoniae* could induce atherosclerosis, including production of proinflammatory cytokines and chemokines (10), induction of adhesion molecules expression (11), and transendothelial migration of inflammatory cells (12). We hypothesized that an additional proatherogenic mechanism triggered by *C. pneumoniae* is the stimulation of IL-18 and, secondarily, IFN- $\gamma$  release.

## Materials and Methods

### Reagents

*Escherichia coli* LPS (serotype 055:B5), peptidoglycan, and cycloheximide were obtained from Sigma-Aldrich (St. Louis, MO). rIL-18 binding protein (BP)<sup>3</sup> (recombinant human IL-18BP expressed in Chinese hamster ovary cells with carboxyl His<sup>6</sup>) was produced as previously described (13). Recombinant human IL-1R agonist (IL-1ra) was a kind gift of Dr. D. Tracey (Upjohn, Kalamazoo, MI). Recombinant human TNF $\alpha$  (4 domain p55 soluble TNFR) was kindly provided by Dr. C. Edwards (Amgen, Boulder, CO). The monoclonal mouse anti-human IL-12 Ab was purchased from R&D Systems (Minneapolis, MN). The monoclonal anti-TLR4 HTA125 Ab was a kind gift of Dr. K. Miyake (Saga Medical School, Saga, Japan). The monoclonal anti-TLR2 Ab was kindly provided by Dr. D. Golenbock (Boston University, Boston, MA). The monoclonal anti-CD14 WT14 Ab was a kind gift of Dr. W. Tax (University Medical Center, Nijmegen, The Netherlands). RPMI 1640 culture medium (Life Technologies, Grand Island, NY) was supplemented with 10 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Life Technologies). An irrelevant mouse anti-human IgG Ab was used as a negative control, and did not influence cytokine release.

### Animals

Specific pathogen-free TLR4-defective (ScCr) mice (14) and age- and weight-matched wild-type (C57BL/10) mice (20–25 g, 6–8 wk old) were

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<sup>3</sup> Abbreviations used in this paper: BP, binding protein; ICE, IL-1 $\beta$  converting enzyme; IFU, inclusion-forming units; IL-1ra, IL-1R agonist.

used for the experiments. TLR2-deficient (TLR2<sup>-/-</sup>) and MyD88-deficient (MyD88<sup>-/-</sup>) 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<sup>+/+</sup> 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 elementary bodies released from the cells was collected and pooled. The cell debris was separated by low speed centrifugation (10 min, 500 g). The infectivity of the *Chlamydia*-containing supernatants (inclusion-forming units (IFU)) was further tested in Hep2 cells and adjusted to 10<sup>5</sup> 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 terms of 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 Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMC were washed twice in saline and suspended in culture medium (RPMI 1640 Dutch modification) supplemented with gentamicin 10  $\mu$ g/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  $\times$  10<sup>6</sup> cells/ml.

A total of 5  $\times$  10<sup>5</sup> PBMC in a 100- $\mu$ l volume were added to round-bottom 96-well plates (Greiner Bioscience, Alphen a/d Rijn, The Netherlands), and incubated with either 100  $\mu$ l of conditioned medium from uninfected Hep2 cells, sonicated *C. pneumoniae* (10<sup>4</sup> IFU/ml, unless otherwise indicated), heat-killed *C. albicans* (10<sup>7</sup> microorganisms/ml), heat-killed *S. aureus* (10<sup>7</sup> microorganisms/ml), LPS (10 ng/ml), or peptidoglycan (10  $\mu$ g/ml). In a pilot experiment, we have measured IL-18 concentrations after 1, 4, 8, and 24 h, and found that IL-18 reaches a plateau after 8 h and remains unchanged at 24 h. Twenty-four-hour stimulation has been chosen as the stimulation period for the rest of the experiments.

In blocking studies, the cytokine inhibitors IL-18BP (125 ng/ml), anti-IL-12 (5  $\mu$ g/ml), IL-1ra (10  $\mu$ g/ml), and TNFBP (10  $\mu$ g/ml) were added to the cells. These concentrations block the bioactivity of the respective cytokines, as shown in separate experiments (data not shown). In additional experiments aimed to assess the role of CD14 and TLRs for stimulation of cytokines, PBMC were preincubated (1 h, 37°C) with the various mAbs (anti-TLR4, anti-TLR2, or anti-CD14; 20  $\mu$ g/ml) before the stimulation with *C. pneumoniae*. As a heparan sulfate-like glycosaminoglycan has been shown to be a *C. pneumoniae* receptor on epithelial cells, the effect of blocking this potential chlamydial receptor with 500  $\mu$ g/ml heparin was investigated, as previously reported (15). To investigate the effects of de novo protein synthesis on IL-18 release, PBMC were stimulated with *C. pneumoniae* in the presence of 10  $\mu$ g/ml cycloheximide (16). In a separate experiment, we have investigated the effect of IL-1 $\beta$  converting enzyme (ICE) inhibition by a specific inhibitor (10  $\mu$ M Ac-Tyr-Val-Ala-Asp-chloromethylketone; Bachem, Bubendorf, Switzerland) on the release of IL-18. After 24 or 48 h of incubation at 37°C, the supernatants were collected and stored at -70°C until assay.

### Cytokine production by murine peritoneal macrophages

Resident peritoneal macrophages from either ScCr and C57BL/10 mice, or TLR2<sup>-/-</sup> 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  $\mu$ g/ml gentamicin, and 2% fresh mouse plasma.

Cells were cultured in 96-well microtiter plates (Greiner Bioscience) at 1  $\times$  10<sup>5</sup> cells/well, in a volume of 100  $\mu$ l. The cells were stimulated with 100  $\mu$ l of conditioned medium from uninfected Hep2 cells, or sonicated *C. pneumoniae* (final concentration, 1  $\times$  10<sup>4</sup> 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- $\alpha$  and IL-1 $\beta$  concentrations were determined by specific RIAs, with a detection limit of 20 pg/ml. IFN- $\gamma$  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-1 $\beta$  and TNF- $\alpha$  were determined by specific RIAs (detection limit, 20 pg/ml), as previously described (17).

### Statistical analysis

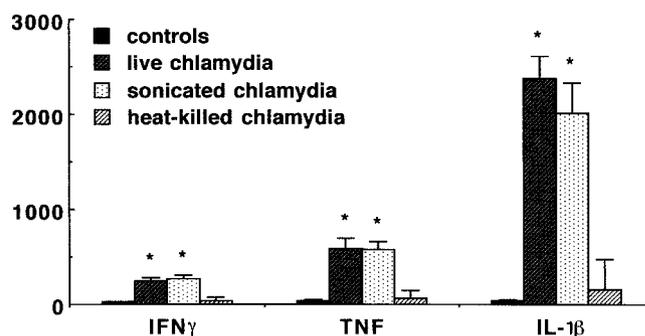
The human experiments were performed in triplicate in a total of seven volunteers, and all data were pooled. The mouse experiments were performed twice in five mice per group, and the data are presented as cumulative results of all experiments performed. The differences between groups were analyzed by 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  $\pm$  SEM.

## Results

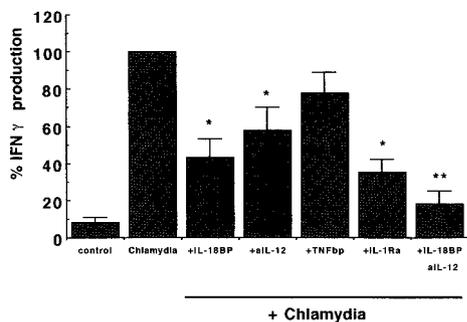
### *C. pneumoniae* stimulates IFN- $\gamma$ production

Stimulation of human PBMC for 24 h with either live or sonicated *C. pneumoniae* from a culture containing 10<sup>4</sup> IFU/ml resulted in significant production of TNF and IL-1 $\beta$  (Fig. 1). In addition, IFN- $\gamma$  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- $\gamma$  (240  $\pm$  20 pg/ml) compared with LPS (720  $\pm$  140 pg/ml), *C. albicans* (1760  $\pm$  430 pg/ml), or *S. aureus* (5800  $\pm$  1230 pg/ml). Conditioned medium from uninfected cultures induced small but measurable IFN- $\gamma$  in some donors. However, when PBMC were stimulated with either live *Chlamydia* or chlamydial sonicates, a 4- to 14-fold-increase in IFN- $\gamma$  occurred (*p* < 0.01). In contrast, heat-killed *C. pneumoniae* induced only 10–25% of the cytokine production stimulated by live microorganisms (Fig. 1).

Previous studies have demonstrated that mature IL-18 is required for the LPS-induced production of IFN- $\gamma$ , even when IL-12 is present (18). To investigate the role of endogenous IL-18 for the *C. pneumoniae*-induced IFN- $\gamma$  synthesis, IL-18BP was added to the PBMC cultures. IL-18BP reduced *C. pneumoniae*-induced IFN- $\gamma$  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 conditioned medium (■), live (▨), sonicated (▩), or heat-killed (▧) 10<sup>4</sup> IFU/ml *C. pneumoniae*. TNF and IL-1 $\beta$  (after 24-h stimulation), or IFN- $\gamma$  (after 48-h stimulation) concentrations were measured by RIA or ELISA. Donors, *n* = 7; \*, *p* < 0.01 when compared with stimulation by conditioned medium.



**FIGURE 2.** The effect of IL-18BP, anti-IL-12 Abs, TNFBP, and IL-1ra on *C. pneumoniae*-stimulated production of IFN- $\gamma$ . PBMC were incubated with *C. pneumoniae* sonicates in the absence or presence of IL-18BP (125 ng/ml), anti-IL-12 Abs (5  $\mu$ g/ml), TNFBP (10  $\mu$ g/ml), IL-1ra (10  $\mu$ g/ml), or a combination of IL-18BP and anti-IL-12 Abs. IFN- $\gamma$  concentrations were measured after 48-h stimulation. One hundred percent IFN- $\gamma$  production represents 353 pg/ml. 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.

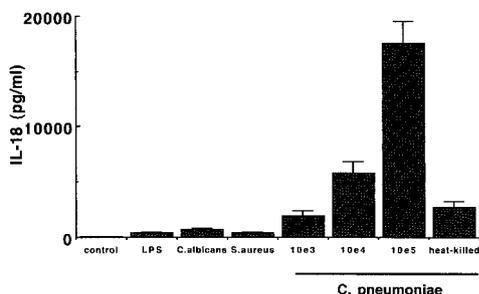
IL-12 Abs or IL-1ra to the PBMC stimulated by *Chlamydia* reduced the production of IFN- $\gamma$  by 42 ( $p < 0.05$ ) and 65% ( $p < 0.01$ ), respectively (Fig. 2). In contrast, no significant effect of TNFBP on *C. pneumoniae*-induced IFN- $\gamma$  could be observed. TNFBP significantly decreased LPS-induced IL-8 release by 54%, demonstrating that it is biologically active (data not shown). A combination of IL-18BP and anti-IL-12 Abs reduced *C. pneumoniae*-induced IFN- $\gamma$  production by 82% ( $p < 0.01$ , Fig. 2).

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

The marked inhibitory effect of IL-18BP on *C. pneumoniae*-induced IFN- $\gamma$  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. albicans* (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-1 $\beta$ , and IL-18 by *C. pneu-*



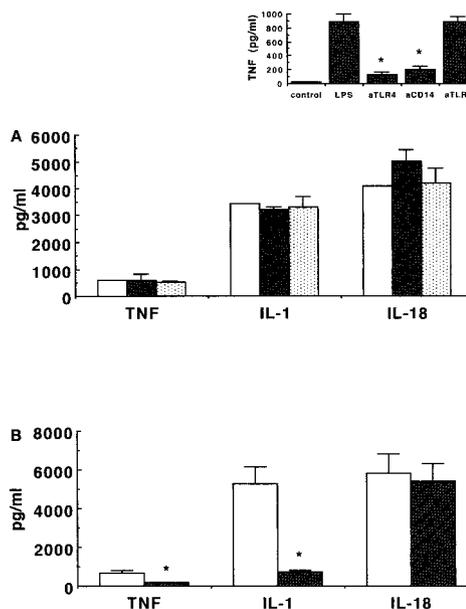
**FIGURE 3.** Stimulation of IL-18 production by *C. pneumoniae*. Human PBMC were stimulated for 24 h with culture medium, LPS (10 ng/ml), heat-killed 10<sup>7</sup> CFU/ml *C. albicans* or 10<sup>7</sup> CFU/ml *S. aureus*, or heat-killed *C. pneumoniae* or sonicated *Chlamydia* at three different concentrations: 10<sup>3</sup>, 10<sup>4</sup>, or 10<sup>5</sup> 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.

*moniae* (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-1 $\beta$ , 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-1 $\beta$ , 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-1 $\beta$  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- $\gamma$  by 40–65% (from 459  $\pm$  154 to 243  $\pm$  99 pg/ml,  $p < 0.05$ ). This effect is most likely mediated through initial inhibition of endogenous IL-1 release, as >90% of IL-1 $\beta$  release was inhibited by the anti-TLR2 Ab, and IL-1 has proved essential for effective *Chlamydia*-induced IFN- $\gamma$  production (see above).



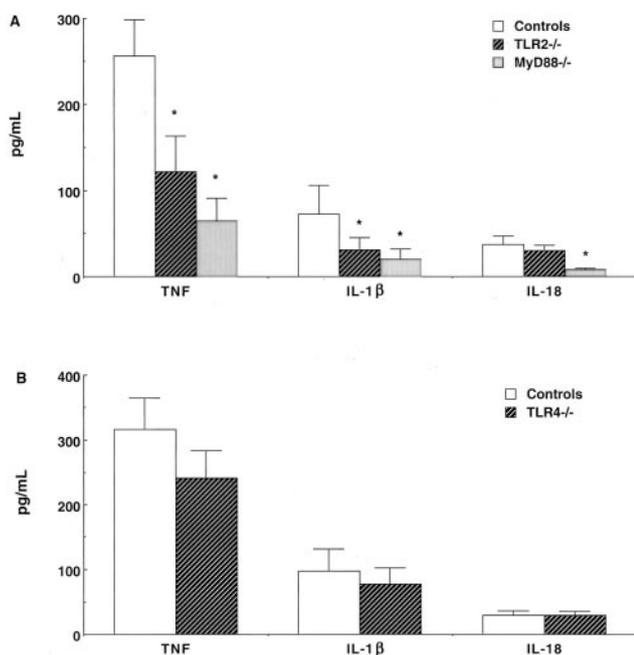
**FIGURE 4.** The effect of TLR2, TLR4, and CD14 on *C. pneumoniae*-induced stimulation of cytokines in human PBMC. A, Freshly isolated PBMC were stimulated with *C. pneumoniae* ( $\square$ ). Anti-TLR4 ( $\text{hatched}$ ) or anti-CD14 ( $\text{stippled}$ ) neutralizing Abs (20  $\mu$ g/ml) had no effect on cytokine production. B, Freshly isolated PBMC were stimulated with *C. pneumoniae* ( $\square$ ) and the addition of anti-TLR2 neutralizing Abs (20  $\mu$ g/ml;  $\text{hatched}$ ) strongly diminished TNF and IL-1 $\beta$  production. Inset, The effect of anti-TLR4, anti-TLR2, or anti-CD14 Abs on TNF production upon stimulation with LPS (1 ng/ml). Cytokines were measured after 24 h of incubation. Data represent mean  $\pm$  SEM of six volunteers. \*,  $p < 0.05$ .

*Induction of proinflammatory cytokines by C. pneumoniae in peritoneal macrophages of MyD88<sup>-/-</sup>, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice*

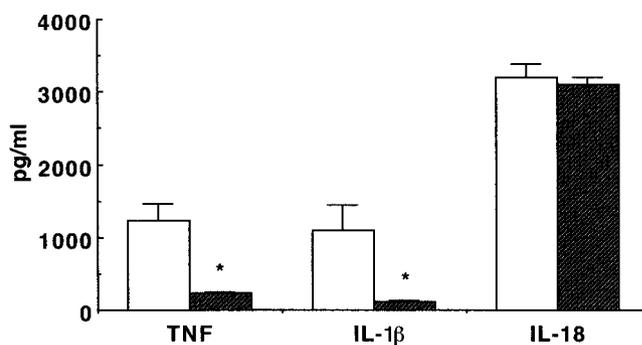
Peritoneal macrophages from all the mouse strains tested did not release detectable proinflammatory cytokines after incubation with culture medium alone. In addition, peritoneal macrophages isolated from TLR2<sup>-/-</sup> mice produced 60–75% less TNF and IL-1 $\beta$  compared with cells isolated from 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  $\pm$  10% of control TNF production, and 89  $\pm$  15% of IL-1 $\beta$  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-1 $\beta$ , 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% ( $p < 0.02$ ).



**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) knock-out mice were stimulated with *C. pneumoniae* ( $10^5$  IFU/ml) for 24 h at 37°C. Cytokine measurement was performed in the supernatants, and the data represent mean  $\pm$  SEM of seven mice. \*,  $p < 0.05$ .



**FIGURE 6.** Effect of de novo protein synthesis on cytokine stimulation by *C. pneumoniae*. Freshly isolated human PBMC were stimulated with  $10^5$  IFU/ml *C. pneumoniae* (□), and the effect of de novo protein synthesis on cytokine stimulation was investigated by adding 10  $\mu$ g/ml cycloheximide during the stimulation (▨). Cytokines were measured in the supernatant after 24-h incubation at 37°C, and the data represent mean  $\pm$  SEM of five volunteers. \*,  $p < 0.05$ .

## Discussion

In the present study we demonstrate that *C. pneumoniae* stimulates production of IFN- $\gamma$ , in addition to the proinflammatory cytokines IL-18, TNF, and IL-1 $\beta$ . Endogenous IL-18, IL-12, and IL-1 $\beta$  are crucial for the induction of IFN- $\gamma$ , as the cytokine antagonists IL-18BP, anti-IL-12 Abs, and IL-1ra strongly reduced IFN- $\gamma$  synthesis stimulated by *Chlamydia*. Whereas stimulation of TNF and IL-1 $\beta$  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- $\gamma$  promotes plaque development in apoE<sup>-/-</sup> mice (2), whereas the absence of IFN- $\gamma$ R strongly inhibits atherogenesis (3). Our studies demonstrate that both live *C. pneumoniae* and acellular chlamydial components not only induce TNF, IL-1 $\beta$ , and IL-6 (19), but are also a strong stimulus of IFN- $\gamma$  production, and this may be a crucial step in the atherogenic role of *Chlamydia*. These results are supported by other studies reporting IFN- $\gamma$  production by *C. pneumoniae* infection (20, 21). However, the finding that even acellular chlamydial components strongly stimulate IFN- $\gamma$  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- $\gamma$  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- $\gamma$  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- $\gamma$  release from T cells (29). This appears to be true also for stimulation by *C. pneumoniae*, as the release of IFN- $\gamma$  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- $\gamma$ , although with this stimulus, the relative role of IL-12 seems to be more important (16).

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- $\gamma$  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<sup>-/-</sup> mice (32). Finally, it is important to note that these effects of IL-18 are dependent on IFN- $\gamma$  release (32).

In our experiments, endogenous IL-1 was involved in the regulation of IFN- $\gamma$  synthesis, as IL-1ra also strongly decreased *Chlamydia*-induced IFN- $\gamma$  production, although in earlier studies, IL-1ra failed to show significant inhibitory activity (19). Although IL-1 $\beta$  by itself cannot stimulate production of IFN- $\gamma$ , recent data indicate that addition of IL-1 $\beta$  to IL-12 potentiates the induction of IFN- $\gamma$  (33). Our results are supported by Hunter et al. (34), who reported inhibition of IFN- $\gamma$  by anti-IL-1 $\beta$  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-1 $\beta$  may be explained by structure similarities between the receptor complexes for IL-1 and IL-18, which consist of a binding chain (IL-1R type I and IL-18R $\alpha$ ) and a signaling chain (IL-1R accessory protein and IL-18R $\beta$ ) respectively (29). In addition to IL-18, IL-12, and IL-1, Rothfuchs and colleagues (35) have also suggested IFN- $\alpha\beta$  mediated induction of IFN- $\gamma$  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 peptidoglycans, lipoarabinomannan and bacterial lipoproteins, TLR5 for flagellin, and TLR9 for bacterial DNA (for review see Ref. 36). As we and others have previously shown, *C. pneumoniae* uses TLR2 as signaling receptor to induce production of TNF and IL-1 $\beta$  (37, 38), and we now further substantiated these findings in mice deficient in TLR2 or TLR4. In addition, *C. pneumoniae* induced IFN- $\gamma$  through TLR2-dependent mechanisms, a process in which down-regulation of endogenous IL-1 $\beta$  is likely to be involved. The lack of effect of TLR4 on induction of cytokines by *Chlamydia* is also in line with the fact that heat-killed *Chlamydia* was much less potent for the stimulation of proinflammatory cytokines, suggesting that not (heat-stable) chlamydial LPS, but a heat-labile component is responsible for the stimulatory activity (19).

An important new finding is that induction of IL-18 by *C. pneumoniae* appears to be mediated by a different pathway, because its production is influenced by neither anti-TLR2, nor anti-TLR4-blocking Abs. Blockade of CD14 also did not influence the production of any of the cytokines studied. The lack of effects of the anti-TLR2 and anti-TLR4 Abs could have been due to a different binding site of the putative TLR agonists of *C. pneumoniae*. However, the similar lack of effects on cytokine production in the absence of TLRs in the knockout mice demonstrate that neither

TLR2, nor TLR4, are involved in *Chlamydia*-induced IL-18. In contrast to TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice, macrophages of MyD88<sup>-/-</sup> mice produced significantly less IL-18 than control macrophages, suggesting that other TLR pathways mediate the release of IL-18 by *C. pneumoniae*. Further studies are warranted to determine what pathways play a role here.

The differential pathways used to stimulate TNF and IL-1 $\beta$  on the one hand, and IL-18 on the other hand, do not appear to be a phenomenon restricted to *Chlamydia* stimulation. Whereas TNF and IL-1 $\beta$  induction mainly involves transcription, the blockade of IL-18 release by an ICE inhibitor demonstrates the crucial role of processing mechanisms in the release of IL-18 by *C. pneumoniae*. This is in line with a recent study, in which Lu and colleagues (16) have shown the release of mature IL-18 from epithelial cells infected with *C. trachomatis*, independent of de novo protein synthesis. This release was mediated by posttranscriptional mechanisms dependent on activation of caspase-1 and cleavage of inactive pro-IL-18 into active mature IL-18. Similarly, in our experiments, *C. pneumoniae*-induced production of TNF and IL-1 $\beta$  was completely dependent on transcriptional mechanisms and de novo protein synthesis, whereas the blockade of protein synthesis by cycloheximide did not influence IL-18 release. This suggests that IL-18 release induced by both *C. pneumoniae* and *C. trachomatis* is mediated by similar posttranscriptional events independent of de novo protein synthesis. The precise receptors through which *Chlamydia* induces the release of IL-18 are currently unknown, but recognition of chlamydial components by TLR other than TLR2 and TLR4,  $\beta_{1,2}$  integrins, (39) or heparan sulfate-like glycosaminoglycans (15) may be involved. The role of the latter seems to be excluded by the lack of effect of heparin in our experiments.

In conclusion, *C. pneumoniae* is a potent stimulus of IL-18/IFN- $\gamma$  production, in addition to the proinflammatory cytokines TNF and IL-1 $\beta$ , which may contribute to its proatherogenic effects. Interestingly, *C. pneumoniae* induces TNF and IL-1 $\beta$  synthesis through TLR2-mediated signals, whereas stimulation of IL-18 production is mediated through pathways independent of TLR2 and TLR4.

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