Acellular components of Chlamydia pneumoniae stimulate cytokine production in human blood mononuclear cells

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Accumulating evidence suggest that infection with Chlamydia pneumoniae is associated with atherosclerosis, but the mechanisms involved remain unclear. Inflammation is important in the initial phase of atherogenesis, and cytokines are important in the initiation and progression of inflammation. The aim of this study was to assess the capacity of acellular components of C. pneumoniae to stimulate the production of pro-inflammatory cytokines and chemokines. Peripheral blood mononuclear cells were stimulated in vitro with sonicated C. pneumoniae. Significant amounts of TNF-α, IL-1, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) were produced. Inhibition of endotoxin using polymyxin B revealed that chlamydial endotoxin plays a minor role in the cytokine induction. Neutralization of TNF by TNF-binding protein and blockade of IL-1 receptors by IL-1 receptor antagonist revealed that TNF, IL-1 and IL-6 production was independent from each other, whereas IL-8 synthesis was strongly dependent on endogenous TNF and IL-1. In contrast, synthesis of MCP-1 and MIP-1α was dependent on endogenous TNF, but not IL-1. In conclusion, acellular components of C. pneumoniae are a potent stimulus for cytokine production, and this mechanism may have an important role in the inflammatory aspects of atherogenesis.

Key words: Chlamydia pneumoniae / Atherosclerosis / Cytokine / Chemokine

1 Introduction

The importance of infectious episodes for the process of atherogenesis has been proposed for several decades, but evidence for this concept has only recently been reported [1, 2]. Chlamydia pneumoniae is a gram-negative obligate intracellular pathogen which causes respiratory tract infections [3, 4]. Initial studies were reported 10 years ago which showed that patients with acute myocardial infarction and established coronary artery disease had significantly higher titers of antibodies against Chlamydia compared to healthy controls [5], whereas patients with elevated IgA titers against C. pneumoniae had a twofold increased risk to suffer from a major cardiac event in the following 6 months [6]. Since then, several studies have generally confirmed this connection between serological evidence of C. pneumoniae infection and coronary heart disease (for review see [7]). In addition, C. pneumoniae has been detected in atheromatous lesions from patients [8–10]. These serological and pathological studies have been joined by two small trials showing that treatment with macrolides, antibiotics with anti-chlamydial potency, results in diminished chance of major cardiac events in patients with unstable angina [11] or after a prior myocardial infarction [12]. Recently, the interim report of the ACADEMIC study has described that treatment of coronary artery disease patients with azithromycin improved markers of...
inflammation, but did not influence clinical events after the initial 6 months of follow-up [13]. Larger clinical studies with a longer follow-up are under way to confirm or disprove these initial reports. The possibility that C. pneumoniae has a role in the pathogenesis of atherosclerosis has been strengthened by recent animal studies in which infection with C. pneumoniae induced or accelerated atherosclerosis [14, 15].

The pathophysiological mechanisms by which C. pneumoniae may contribute to the development of atherosclerosis are not yet fully understood. In vitro studies have shown that C. pneumoniae can infect monocytes/macrophages and smooth muscle cells [16], and can induce macrophage foam cell formation [17, 18]. The multiplication of Chlamydia inside monocytes or macrophages triggers the production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 [19, 20], pro-inflammatory cytokines found in atherosclerotic plaques which play an important role in the inflammatory processes involved in atherogenesis [21]. In addition, infection of endothelial cells by C. pneumoniae induces expression of adhesion molecules such as endothelial-leukocyte adhesion molecule 1, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule [22], and stimulates transendothelial migration of neutrophils and monocytes [23].

However, several important questions remain unanswered to date. It is not known whether infection of human cells with live bacteria is needed for cytokine stimulation, or whether stimulation can be induced by chlamydial antigens, LPS, major outer membrane protein (MOMP) or heat shock proteins. In this respect, it has been suggested that chlamydial heat shock protein 60 is able to induce production of TNF by murine macrophages [24] and activate human vascular endothelium, smooth muscle cells and macrophages [25]. In addition, no information is available regarding the capacity of C. pneumoniae to stimulate human mononuclear cells for the production of chemokines. The latter are essential for monocyte and lymphocyte extravasation into the arterial wall, a crucial event in the early phase of atherogenesis. The precise mechanism of cytokine stimulation by C. pneumoniae is also undefined, and the proximal regulators of Chlamydia-induced mononuclear cell activation are unknown.

The aims of the present study were to investigate several key aspects of cytokine stimulation by C. pneumoniae. Is infection with live C. pneumoniae required for stimulation of cytokine production by PBMC, or are molecular components from dead C. pneumoniae also cell activators? Is C. pneumoniae able to induce production of chemokines such as IL-8, monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α from human mononuclear cells, and which cytokines act as regulators of the cytokine network activated by C. pneumoniae? Is the LPS component of C. pneumoniae involved in the cytokine stimulation? Information regarding the stimulatory pathways induced by C. pneumoniae may have important consequences for understanding the mechanisms through which this microorganism promotes early atherosclerosis, and for finding effective strategies in countering its deleterious effects.

2 Results

2.1 Stimulation of pro-inflammatory cytokines and chemokines by sonicated C. pneumoniae

Stimulation of human PBMC for 24 h with sonicated C. pneumoniae from a culture containing 10⁴ inclusion-forming units (IFU)/ml resulted in significant stimulation of the production of TNF, IL-1β and IL-6, compared with the stimulation with the conditioned medium from uninfected Hep2 cells on which the microorganism was grown (Fig. 1 A). Whereas TNF and IL-1β were significantly increased over control (five- and twofold, respectively), IL-6 production was increased 40-fold. Since IL-6 is a growth factor for vascular smooth muscle cells, the particular induction of IL-6 in these cultures suggests that Chlamydia infection in vascular tissue may accel-
ate the proliferation lesion. In addition, we compared cytokine induction by C. pneumoniae with that of 10 ng/ml Escherichia coli LPS. Induction of pro-inflammatory cytokines by sonicated C. pneumoniae was lower when compared with stimulation by endotoxin (TNF: 1140 ± 345 vs. 2005 ± 543 pg/ml, p < 0.05, IL-1β: 247 ± 88 vs. 4431 ± 1238 pg/ml, p < 0.01; IL-6: 3154 ± 1453 vs. 16578 ± 8769 pg/ml, p < 0.01). However, induction of pro-inflammatory cytokines by the acellular components of C. pneumoniae was as strong as that induced by infection of PBMC with corresponding numbers of live chlamydial microorganisms (TNF: 970 ± 233 vs. 817 ± 338 pg/ml, p < 0.05; IL-1β: 334 ± 171 vs. 405 ± 112 pg/ml, p > 0.05).

In addition, C. pneumoniae strongly induced production of the chemokines IL-8, MCP-1 and MIP-1α in these same cultures (Fig. 1 B). The induction of IL-8 by C. pneumoniae was dose dependent (38134 ± 11543 pg/ml by 10^4 IFU/ml; 10112 ± 3438 pg/ml by 10^3 IFU/ml; 3567 ± 1987 pg/ml by 10^2 IFU/ml; 345 ± 80 pg/ml by 10 IFU/ml).

As shown in Table 1, IFN-γ was also induced by C. pneumoniae sonicates derived from 1 × 10^4 IFU/ml. Control sonicates from uninfected cultures induced small but measurable IFN-γ in some donors. However, in PBMC and purified lymphocyte cultures, the fold increase in IFN-γ production ranged from 4 to 18 using chlamydial sonicates. The amounts of IFN-γ induced in PBMC and in purified lymphocyte cultures was modestly greater in PBMC, suggesting that the presence of monocytes may contribute to IFN-γ production induced by chlamydial products.

### Table 1. IFN-γ production of human PBMC or lymphocytes from 4 healthy volunteers after stimulation with sonicated C. pneumoniae

<table>
<thead>
<tr>
<th></th>
<th>PBMC</th>
<th>Lymphocytes&lt;sup&gt;a)&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>IFN-γ (pg/ml)&lt;sup&gt;b)&lt;/sup&gt;</td>
<td>Conditioned medium</td>
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<tr>
<td>Donor 1</td>
<td>40</td>
<td>215 (5)&lt;sup&gt;c)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Donor 2</td>
<td>&lt;8</td>
<td>120 (15)</td>
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<tr>
<td>Donor 3</td>
<td>130</td>
<td>2340 (18)</td>
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<tr>
<td>Donor 4</td>
<td>&lt;8</td>
<td>125 (15)</td>
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<sup>a</sup> Separated by anti-CD4/CD8 magnetic beads (see Sect. 4.3); 5 ± 10^5 lymphocytes/ml.
<sup>b</sup> IFN-γ was measured after 72 h of culture.
<sup>c</sup> Numbers in parentheses indicate fold-increase over control.

### 2.2 The role of endogenous cytokines in the C. pneumoniae-induced cytokine production

Previous studies have shown that endogenous TNF and/or IL-1 are important factors in the downstream synthesis of other cytokines [26, 27]. To test whether this is also the case in C. pneumoniae-stimulated cytokine production, we stimulated cells with Chlamydia sonicates in the presence of TNF-binding protein (TNFbp) or IL-1 receptor antagonist (IL-1Ra), both at a final concentration of 10 µg/ml.

As shown in Fig. 2 A, neutralization of endogenous TNF by TNFbp resulted in a significant reduction in the production of IL-8 (77 % reduction, p < 0.01), MCP-1 (44 %, p < 0.05) and MIP-1α (70 %, p < 0.01). Blockade of IL-1R by IL-1Ra, and the subsequent neutralization of endogenous IL-1 action, significantly inhibited production of IL-8 (53 %, p < 0.05), but not of MCP-1 and MIP-1α (Fig. 2 B). In contrast, neither TNFbp (Fig. 3 A) nor IL-1Ra (Fig. 3 B) reduced the production of IL-1β, TNF or IL-6. In this respect, it appears that stimulation of the synthesis of TNF, IL-1β and IL-6 by Chlamydia is independent of each other. Using E. coli LPS in human PBMC, IL-6 production is reduced by TNFbp or IL-1Ra [28].

To test whether stimulation of IFN-γ synthesis by C. pneumoniae sonicates is mediated by endogenous IL-18 (IFN-γ-inducing factor) [29], the cells were separately co-incubated with either an IL-1β converting enzyme (ICE) inhibitor (final concentration 10 µM) or IL-1Ra (10 µg/ml). The ICE inhibitor blocks the processing of both pro-IL-18 and pro-IL-1β, whereas IL-1Ra blocks the action of the endogenously released IL-1(α and β). The addition of IL-1Ra had no effect on IFN-γ production (not shown). In contrast, inhibition of ICE significantly reduced the stimulation of IFN-γ/synthesis by sonicates of C. pneumoniae by 54 % (771 ± 324 vs. 1683 ± 467 pg/
ml, p < 0.05). This inhibition suggests an important role of endogenous IL-18 for stimulation of IFN-γ by chlamydial acellular components.

2.3 LPS does not play a major role in the stimulation of cytokine production by sonicates of C. pneumoniae

C. pneumoniae is a gram-negative microorganism, and the LPS component of gram-negative bacteria cell wall is one of the most potent stimuli of cytokine production. To test the hypothesis that chlamydial LPS may account for the induction of cytokines, before being added to PBMC sonicated C. pneumoniae was preincubated for 2 h at 37 °C with 2 μg/ml polymyxin B, an antibiotic which binds and neutralizes LPS derived from most gram-negative bacteria. As shown in Fig. 4, polymyxin B inhibited the TNF production induced by 10 ng/ml E. coli LPS (> 95 % inhibition). However, preincubation with polymyxin B did not influence the C. pneumoniae-stimulated TNF synthesis to further test the involvement of chlamydial-derived LPS, the cytokine induction capacity of heat-killed (30 min, 100 °C) Chlamydia-containing supernatants was compared with that of sonicated Chlamydia. Sonicated Chlamydia supernatants induced 1821 ± 434 pg/ml TNF, whereas following heat treatment this was reduced to 636 ± 302 pg/ml (65 % reduction, p < 0.02). This suggests that a heat-labile epitope, rather than the heat-resistant LPS, is largely responsible for cytokine synthesis after stimulation of human PBMC with C. pneumoniae.

2.4 The cellular source of cytokine production

To investigate which cell population in the PBMC is responsible for the induction of cytokines after stimulation with sonicates of C. pneumoniae, identical numbers of PBMC and lymphocytes from the same volunteers were stimulated with sonicated Chlamydia. TNF and IL-8 induced by stimulation with C. pneumoniae were almost exclusively synthesized in unfractionated PBMC, but not...
in purified lymphocytes (Fig. 5). These results suggest that the monocytes in PBMC are the source of TNF and IL-8 when stimulated by Chlamydia sonicates. In contrast, IFN-γ from purified lymphocytes and unfractionated PBMC from the same donors was synthesized in equal amounts (Table 1).

3 Discussion

The present study demonstrates that stimulation of human PBMC with sonicates of *C. pneumoniae* induces the production of the chemokines IL-8, MCP-1 and MIP-1α, as well as the pro-inflammatory cytokines TNF, IL-1β and IL-6. These sonicates preferentially stimulated IL-6 and IL-8 production, which may have specific pathological implications. In addition, IFN-γ production was also demonstrated in both PBMC and purified lymphocytes cultures. Whereas *C. pneumoniae* induced production of TNF, IL-1 and IL-6 independent of each other, the production of IL-8, MCP-1 and MIP-1α is dependent on endogenous TNF. In addition, the production of IL-8, but not MCP-1 or MIP-1α, is also partially dependent on endogenous IL-1. These results are different from those observed following stimulation of PBMC with *E. coli* LPS, where MIP-1α was suppressed by IL-1 blockade more than 70% [30].

The role of chronic low-grade infection of the arterial wall with *C. pneumoniae* in the pathogenesis of atherosclerosis has been indirectly suggested by a series of epidemiological and pathological studies reported during the last decade (for review see [7]). The bacteria may either infect an intact vessel wall, leading to inflammation and atherogenesis, or infect an established atherosclerotic plaque, leading to instability and rupture. There are several potential mechanisms by which *C. pneumoniae* could influence atherogenesis. Several studies have demonstrated the expression of pro-inflammatory cytokines such as TNF, IL-1 and IL-6 in atheromatous plaques [31, 32]. TNF and IL-1 increase the expression of endothelial adhesion molecules [33], enhance the uptake of oxidized low density lipoprotein (LDL) through increased expression of macrophage scavenger receptors [34], regulate the plaque stability [35], and induce production of endogenous growth factors which regulate cell proliferation in the arterial cell wall [36], each potentially important mechanisms in the process of atherosclerosis. Therefore, induction of these inflammatory mediators by *Chlamydia*, as shown in the present report, may be of central importance for the pro-atherogenic affect of *C. pneumoniae* infection. Moreover, we have also shown in the present study that heat-labile antigen-like products in sonicated/killed *C. pneumoniae*, and not only infection of monocytes with live bacteria ([19, 20] and the present study), are able to induce release of pro-inflammatory cytokines. The acellular components of *C. pneumoniae* are as potent as the live microorganisms themselves in stimulating the production of cytokines. This may be of particular importance, since dead *Chlamydia* present in the atheromatous lesion may be a source of antigen which would activate cells in a paracrine and autocrine manner, and recent data suggest that at late stages only chlamydial antigens, and not live bacteria, persist and maintain the chronic inflammation [37]. A similar case has been made for reactive arthritis, where disease progresses despite antibiotic therapy for the *C. trachomatis*.

One of the important steps in the early phase of atherogenesis is migration of monocytes and lymphocytes into the arterial vessel wall. Leukocyte migration across the endothelium is under control of chemokines, such as IL-8, MCP-1 and MIP-1α [38]. Such chemokines are produced after cholesterol loading of macrophages [39], and have been detected in human atheromatous plaques [32, 40]. A recent study has demonstrated that a leukocyte homologue of the IL-8R mediates the accumulation of macrophages in the atherosclerotic lesions of LDL receptor-deficient mice [41], demonstrating the central role of the chemokines in the development of atherosclerosis. In the present study we have demonstrated that *C. pneumoniae* induces production of large amounts...
of IL-8, MCP-1 and MIP-1α after stimulation of human PBMC. Assuming that this takes place in the vessel wall, the result could be accumulation of monocytes and lymphocytes in a developing plaque with subsequent progression of atherogenesis. The relevance of this issue increases as it has been shown that the spread of chlamydial infection in the vessel wall is mediated through infected monocytes [42]. The results of our study are supported by the study of Molestina et al. [43] who showed that a strain of C. pneumoniae isolated from human atheromas induces production of MCP-1, IL-8 and soluble (s) ICAM-1 from human umbilical endothelial cells and stimulates transendothelial migration of neutrophils and monocytes [23].

We tested the hypothesis that TNF and IL-1 are proximal mediators of C. pneumoniae-stimulated cytokine production, by blocking these cytokines with TNFbp or IL-1Ra, and found that chlamydial stimulation of chemokines is largely modulated by endogenous TNF. The production of IL-8, but not MCP-1 and MIP-1α, is also dependent on endogenous IL-1, as also previously shown for C. trachomatis and C. psittaci-stimulated production of IL-8 [44]. This is not surprising, as TNF/IL-1-driven induction of IL-8 has been reported for other stimuli as well [27, 45, 46]. Elucidation of the mechanism of the cytokine cascade induced by C. pneumoniae may prove particularly useful in finding therapeutic strategies to counteract its deleterious effects.

Monocytes are the major source of TNF and IL-8 in the present experiments, whereas lymphocytes respond with significant production of IFN-γ after stimulation with C. pneumoniae. The lymphocyte populations, however, contained 2–5% contaminating monocytes and NK cells. It was therefore considered that induction of IFN-γ in lymphocyte preparations is modulated through initial induction of IL-12 and IL-18 from monocytes and/or NK cells [29]. Indeed, inhibition of pro-IL-18 and pro-IL-1α processing by an ICE inhibitor reduced the stimulation of IFN-γ by C. pneumoniae by more than 50%. As IL-1Ra had no effect of IFN-γ synthesis, this demonstrates that endogenous IL-18 is involved in Chlamydia stimulation of IFN-γ. The remaining IFN-γ synthesis is probably mediated by endogenous IL-12. A possible correlation may exist between the production of IFN-γ and the state of immunity to C. pneumoniae, and we are currently performing a larger study investigating this aspect.

Despite the accumulating data suggesting induction of cytokine production by C. pneumoniae and the potential role of these molecules in the development of atherosclerosis, very little is known about the chlamydial antigens responsible for cytokine stimulation. Since LPS from the related species C. trachomatis has been shown to induce cytokine production [47], one may expect that C. pneumoniae LPS is also involved in the induction of pro-inflammatory cytokines. However, the inability of polymyxin B to inhibit the stimulation of cytokines by C. pneumoniae argues against a major role of LPS in the induction of cytokines by C. pneumoniae. Further, the reduction of cytokine-inducing property following heating of the C. pneumoniae preparation suggests that a heat-labile chlamydial component, and not the heat-resistant LPS, is mainly responsible for cytokine stimulation. Similar inhibition after heat inactivation was also observed when endothelial cells were stimulated with C. pneumoniae [43]. In contrast, the study of Ingalls et al. [48] performed with LPS extracted from C. trachomatis suggests that chlamydial endotoxin can stimulate cytokine production [47]. Differences in structure between LPS of various Chlamydia species may partly account for this discrepancy. In addition, Ingalls et al. [48] have shown that C. trachomatis LPS has a low biological activity, and it should be noted that they isolated LPS from 10^5 IFU/ml, which is five orders of magnitude higher than our antigenic preparation. Of note, heat killing of C. pneumoniae in our study did not lead to a complete loss of cytokine induction, suggesting that chlamydial LPS may play a limited role in the stimulation of cytokines. Other indirect evidence suggests that LPS-like products are not the major components responsible for the cytokine-inducing property of the Chlamydia sonicates. For example, T cell production of IFN-γ was similar in purified lymphocytes preparations and monocyte-rich unfractionated PBMC. Taken together, the data support a non-LPS, cytokine-inducing product in the acellular Chlamydia sonicates. It has been recently reported that chlamydial heat shock protein 60 is able to induce production of TNF and matrix metalloproteinases by macrophages [24, 25], whereas the MOMP of the related species C. trachomatis is also able to stimulate production of IL-1 [48], indicating these two chlamydial antigens as possible candidates responsible for cytokine stimulation.

4 Materials and methods

4.1 Reagents

E. coli LPS (serotype 055:B5) and polymyxin B were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human TNFbp was a kind gift of Dr. Carl Edwards (Amgen, Boulder, CO). Recombinant human IL-1Ra was kindly provided by Dr. Daniel Tracey (Upjohn, Kalamazoo, MI). ICE inhibitor II (Ac-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Bachem (Bubendorf, Switzerland).
4.2 C. pneumoniae

C. pneumoniae TW-183 was grown in Hep2 cells, cultured in MEM (Hepes buffered) containing 10% FCS, 0.5% (w/v) glucose and 0.5% (w/v) cycloheximide (Flow Laboratories, Irvine, GB). 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 pooled supernatant was further tested in Hep2 cells and adjusted to 10⁴ 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 either by sonic cation (10 min on ice) (Bransonic 2200, Branson, Shelton, CT) or by heat (30 min, 100°C). The same procedure was followed for the conditioned medium obtained from uninfected Hep2 cells.

4.3 Isolation of human PBMC and lymphocytes

Venous blood was drawn from a cubital vein of healthy volunteers into three 10-ml EDTA tubes (Monoject, S-Hertogenbosch, The Netherlands). Isolation of PBMC was performed as described elsewhere [49], with minor modifications. The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). PBMC were washed twice in saline suspension in the culture medium (RPMI 1640 DM) supplemented with 10 μg/ml gentamicin, 10 mM L-glutamine and 10 mM pyruvate. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands) and the number was adjusted to 5 × 10⁶ cells/ml.

In separate experiments, PBMC isolated from the Ficoll-Paque gradients were incubated for 60 min with a 1:3 mixture of anti-CD4 and anti-CD8-bound magnetic beads (Dynal, Oslo, Norway). The incubation was performed at 4°C with both gentle tilting and rotation. The cells were isolated by magnet according to the manufacturer’s recommendations, and the supernatant was discarded. The cells were washed three times in cold PBS. The cells were detached from the magnetic beads by incubation with DETACHaBEADS (10 μl for 100 μl cell suspension) (Dynal) for 45 min at room temperature, under gentle tilting and rotation. The released beads were collected with the magnet, and the lymphocytes in the supernatant were washed three times with cold PBS to remove the magnetic beads. The lymphocyte population obtained was > 95% pure, as tested by flow cytometry analysis (Becton Dickinson, Mountain View, CA). The rest of the cells (2 to 5%) were monocytes and NK cells.

4.4 Stimulation of cytokine production

PBMC (5 × 10⁵ in 100 μl) were added to round-bottom 96-wells plates (Greiner, Alphen a/d Rijn, The Netherlands) followed by 100 μl conditioned medium from uninfected Hep2 cells, or sonicated or heat-killed C. pneumoniae. After 24 h or 72 h incubation at 37°C, the supernatants were collected and stored at −70°C until assay.

4.5 Cytokine measurements

TNF-α and IL-1β concentrations were determined by specific radioimmunoassays as described [50]. IL-6, IL-8 and IFN-γ were measured by commercial ELISA kits (PeliKe Compact, OLB, Amsterdam, The Netherlands), according to the instructions of the manufacturer. MCP-1 and MIP-1α determinations were performed using ELISA kits from R&D (Quantikine, R&D Systems Europe, Abingdon, GB).

4.6 Statistical analyses

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 means ± SEM.

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