J. Cell. Mol. Med. Vol 13, No 1, 2009 pp. 103-113

Pro-atherogenic lung and oral pathogens induce an inflammatory response in human and mouse mast cells

Anna Oksaharju [a], Jani Lappalainen [a], Anita M. Tuomainen [b], Pirkko J. Pussinen [b], Mirja Puolakkainen [c], Petri T. Kovanen [a], Ken A. Lindstedt [a, *]

[a] Wihuri Research Institute, Kalliolinnantie, Helsinki, Finland
[b] Institute of Dentistry, University of Helsinki, and Department of Oral and Maxillofacial Diseases, Helsinki University Central Hospital, Helsinki, Finland
[c] Haartman Institute, University of Helsinki, University of Helsinki and National Public Health Institute, Mannerheimintie, Helsinki, Finland

Received: November 2, 2007; Accepted: February 18, 2008

Abstract

A broad variety of microbes are present in atherosclerotic plaques and chronic bacterial infection increases the risk of atherosclerosis by mechanisms that have remained vague. One possible mechanism is that bacteria or bacterial products activate plaque mast cells that are known to participate in the pathogenesis of atherosclerosis. Here, we show by real-time PCR analysis and ELISA that *Chlamydia pneumoniae* (Cpn) and a periodontal pathogen, *Aggregatibacter actinomycetemcomitans* (Aa), both induce a time and concentration-dependent expression and secretion of interleukin 8 (IL-8), tumour necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) by cultured human peripheral blood-derived mast cells, but not anti-inflammatory molecules, such as IL-10 or transforming growth factor β1 (TGF-β1). The IL-8 and MCP-1 responses were immediate, whereas the onset of TNF-α secretion was delayed. The Cpn-mediated pro-inflammatory effect was attenuated when the bacteria were inactivated by UV-treatment. Human monocyte-derived macrophages that were pre-infected with Cpn also induced a significant pro-inflammatory response in human mast cells, both in cocultures and when preconditioned media from Cpn-infected macrophages were used. Intranasal and intravenous administration of live Cpn and Aa, respectively, induced an accumulation of activated mast cells in the aortic sinus of apolipoprotein E-deficient mice, however, with varying responses in the systemic levels of lipopolysaccharide (LPS) and TNF-α. Pro-atherogenic Cpn and Aa induce a pro-inflammatory response in cultured human connective tissue-type mast cells and activation of mouse aortic mast cells *in vivo*.

Keywords: mast cell • atherosclerosis • *Chlamydia pneumoniae* • *Aggregatibacter actinomycetemcomitans* • inflammation, macrophage

Introduction

High-serum cholesterol levels, hypertension and smoking are classical risk factors for atherosclerosis and coronary heart disease (CHD). In addition, age, sex, obesity, diabetes, high-serum triglyceride and low- high-density lipoprotein (HDL) levels predispose to CHD. Accumulating evidence also suggests that acute and chronic infections of bacterial origin, including respiratory pathogens, for example *Chlamydia pneumoniae* (Cpn) and periodontal pathogens, for example *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* (Aa), are associated with an increased risk for cardiovascular disease [1–6]. Indeed, recent studies have shown a diverse bacterial signature with DNA from over 50 different bacterial species present in atherosclerotic lesions of patients with CHD, further suggesting a role for bacterial infection in the pathogenesis of atherosclerosis [7–9]. Moreover, the vulnerability of the arterial wall to bacterial infections is augmented in the presence of other cardiovascular risk factors, such as hypercholesterolaemia, suggesting that atherosclerosis *per se* may predispose to the development of chronic infections [10]. In particular, a dysfunctional 'leaky' endothelium with increased expression of leucocyte adhesion molecules may actively promote the infiltration of bacteria-containing monocytes from the circulation into the atherosclerotic lesions [8].

*Correspondence to: Ken A. LINDSTEDT,
Wihuri Research Institute, Kalliolinnantie 4, FI-00140 Helsinki, Finland.
Tel.: +358-9-681 411
Fax: +358-9-637 476
E-mail: ken.lindstedt@wri.fi

doi:10.1111/j.1582-4934.2008.00285.x

Alternatively, the atheromas may simply act as mechanical sieves that collect free bacteria [7] or bacterial products associated with pro-atherogenic serum lipoproteins, such as VLDL and low-density lipoprotein (LDL) [11], from the circulation. However, the precise mechanisms whereby an increase in the local vascular infectious burden, that is accumulation of bacteria or bacterial products in the vascular wall, either alone or in synergy with other risk factors, could contribute to the pathogenesis of atherosclerosis have remained elusive.

Mast cells are sentinel cells that play a pivotal role in allergic inflammation and in co-ordination of the early phases of certain autoimmune disorders [12, 13]. They are typically localized at host-environment interfaces, such as in perivascular areas where they encounter both antigens and invading pathogens, which allows them to exert a unique sentinel role in host defence [14]. Accordingly, mast cells have been shown to recognize and respond to many different pathogens and their products in various diseases. *Staphylococcus aureus* -derived protein A, which is capable of inducing acute peritonitis, was the first pathogen-associated immunoglobulin-binding protein that was shown to activate mast cells [15]. Protein L from *Peptostreptococcus magnus* has been shown to activate human cardiac mast cells [15], and mast cells have also been shown to engulf *Escherichia coli* [16], and to protect mice from *Mycoplasma pneumoniae* infection [17]. Moreover, studies in animal models have indicated that mast cells play a protective role in host defence against bacteria by producing tumour necrosis factor α (TNF-α), mainly as a result of toll-like receptor 4 (TLR4)- or CD48-mediated activation [18, 19]. Thus, mast cell activation during acute bacterial infections is clearly a matter of homeostatic inflammation.

Mast cells are also present in increased numbers in atherosclerotic plaques and they have been shown to participate both in early and late events in the pathogenesis of atherosclerosis and myocardial infarction [20–23]. Since plaque mast cells are an ample source of multi-functional cytokines with inflammatory properties [24, 25], they may also modulate the local immune system by releasing such cytokines in both quantitatively and qualitatively different ways [13]. Despite multiple reports in the literature that show bacteria-induced activation of mast cells in protective host-to-bacteria responses, the effects of pro-atherogenic bacteria on vascular mast cells in the context of atherogenesis have not been studied previously. Thus, in chronic and multi-factorial diseases, such as atherosclerosis, ongoing activation of lesional mast cells by an increased resident bacterial burden in the form of live bacteria, their specific components or as infected macrophages may contribute to atherogenesis and its clinical consequences by sustaining local pathological inflammation.

Here, we studied the direct effects of two pro-atherogenic bacteria, Cpn and Aa on peripheral blood-derived cultured human mast cells, and the indirect effects mediated by Cpn- and/or Aa-infected macrophages. Moreover, to evaluate the *in vivo* significance of our *in vitro* results we studied the effects of chronic Cpn and/or Aa infections on the number of mast cells and their degree of activation in the aortic sinus of apolipoprotein E (apoE)-deficient mice, a commonly used atherosclerosis-prone mouse model [26].

Materials and methods

Mast cell culture

Human peripheral blood was supplied by the Finnish Red Cross Blood Transfusion Service. Mononuclear cells were isolated from heparinized blood by Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) gradient centrifugation. $CD34^+$ progenitor cells were separated by positive immunomagnetic selection using a $CD34^+$ separation Kit and MACS® affinity columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After positive selection, the cells were cultured in serum-free StemSpan™ cell culture medium (Stem Cell Technologies, Vancouver, Canada) supplemented with penicillin and streptomycin (GIBCO BRL Grand Island, NY, USA), human recombinant stem cell factor (SCF, PeproTech, Rocky Hill, NJ, USA), human LDL and different cytokines as previously described [27].

Macrophage culture

Human monocytes were isolated from buffy coats, which were supplied by the Finnish Red Cross Transfusion service. *In vitro* differentiation of monocytes into macrophages was performed as described previously [28]. Briefly, mononuclear cells were isolated by density gradient centrifugation using the Ficoll-Paque™ Plus (GE Healthcare) method. The fraction containing mononuclear cells was isolated and re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Basel, Switzerland) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were seeded in 24-well plates, (1.5×10^6 cells per well) and allowed to adhere for 1 hr, after which the non-adherent cells were washed off and Macrophage-SFM medium (GIBGO BRL) containing 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, BioSite, San Diego, CA, USA), penicillin and streptomycin (concentrations as above) was added. The monocytes were cultured for 1 week to induce their differentiation into macrophages.

Bacterial cultures

Cpn isolate Kajaani 6 was propagated in human lung epithelial cell Ime (HL) cells. At 72 hrs after inoculation, the infected cells were harvested and disrupted by ultrasonication, and the released bacteria were purified through a gradient of meglumine diatrizoate [29]. Purified bacteria were stored in aliquots in sucrose-phosphate-glutamate (SPG) solution at −70°C. The infectivity titre (inclusion forming units [IFU]/ml) of the bacteria was determined in cycloheximide-treated HL cells. A clinical strain with fimbriae, AT445b, and a laboratory strain without fimbriae, JP2, both representing Aa serotype b, were grown in tryptic soy-serum-bacitracin-vancomycin medium in 5% CO_2 for 3–4 days [30]. After cultivation, the bacteria were washed three times with phosphate buffered saline (PBS), and their number was estimated spectrophotometrically at A_{492nm}, with an optical density (OD) of 0.43 corresponding to approximately 1×10^9 CFU/ml. When indicated, bacteria were inactivated with UV-light ($2 \times$ UV-dosage of 0.120 joules/cm^2; Crosslinker CL-508, Techne).

Infection of mast cells with bacteria

Cultured mast cells were collected by centrifugation at $200 \times g$ for 5 min. and re-suspended in fresh Stem Span™ medium containing antibiotics

and SCF, as described above. Next, 0.5×10^6 cells were incubated with 0.5×10^6, 2.5×10^6 or 5×10^6 of Cpn or Aa (in a cell-to-bacteria ratio of 1:1, 1:5 or 1:10, respectively) at 37°C in 5% CO_2 for the specified times. After incubation, the suspensions were collected and the mast cells were separated from the media by centrifugation at $200 \times g$ for 5 min., and the media were aliquoted and stored at −20°C until used. The isolated mast cells were washed once with PBS, lysed and homogenized in RLT buffer (Qiagen, Valencia, CA, USA), and stored at −70°C until RNA isolation was performed.

Infection of macrophages with Cpn

Differentiated macrophages were washed once with PBS, re-suspended in fresh culture media and then inoculated with Cpn in a cell-to-bacteria ratio of 1:10 at 37°C in 5% CO_2 for 6 hrs. After incubation, the macrophages were washed three times with PBS (lacking both Ca^{2+} and Mg^{2+}) in order to remove extracellular bacteria. The macrophages were cocultured with human mast cells in a cell-to-cell ratio of 1:1 at 37°C in 5% CO_2 for 16 hrs. The cocultured mast cells were isolated from the media as described above. In a separate experiment, macrophages were incubated alone in fresh culture media at 37°C in 5% CO_2 for 16 hrs, after which the macrophage-preconditioned media were collected and incubated with mast cells at 37°C in 5% CO_2 for 6 hrs. Finally, the mast cells were collected as described above and subjected to RNA analysis.

Real-time PCR

Total RNA was extracted from the cell lysates using RNeasy Mini Kit (Qiagen) and was then treated with heparinase I (Sigma, St Louis, MO, USA) to avoid unwanted inhibition of the reversed transcriptase and polymerase reactions. The RNA was reverse transcribed to cDNA using random hexamers (Invitrogen, Paisley, UK) and moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was applied to determine mRNA expressions for IL-8, MCP-1, IL-10 and TNF-α. The samples were amplified on ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan™ Universal PCR Master Mix (Applied Biosystems) with uracyl-N-glycosylase treatment, gene-specific primers and 6-carboxyfluorescein (FAM)-labelled fluorogenic probes. For detection of IL-8, monocyte chemoattractant protein-1 (MCP-1) and IL-10 mRNA, commercially available primers and minor groove binding (MGB)-probes were obtained from Applied Biosystems. For TNF-α, the annotated mRNA sequences were retrieved from the GenBank database (National Institutes of Health) and BLAST searches were performed to identify unique stretches of the nucleotide sequence. To avoid amplification of genomic DNA, an exon-spanning assay was designed: sense primer: 5′-GCTGCACTTTGGAGTGATCG-3′; antisense: 5′-GTTTGCTACAACATGGGTACAG-3′; probe: 5′-FAM-CCCAGGCAGTCAGATCATCTTCTCGA-BHQ1-3′. Samples were run in triplicate using the following program: 2 min. incubation at 50°C, 10 min. pre-incubation at 95°C, followed by 40 cycles of 15 sec. at 95°C and 60 sec. at 60°C. β-actin was included as an endogenous normalization control. Relative quantification was determined by standard $2^{-\Delta\Delta CT}$ calculations [31]. Data were presented in relation to the median value of uninfected samples of each experiment, which were set to 1.

Determination of cytokine and chemokine levels

Cytokines and chemokines were measured in cell culture media and sera using commercial ELISAs according to manufacturer's protocols. The amounts of human IL-8 and IL-10 and mouse TNF-α were determined with Endogen ELISA kits (Pierce Biotechnology, Rockford, IL, USA) and human MCP-1, TGF-β1 and TNF-α were determined with R&D Quantikine® assays (R&D Systems, Minneapolis, MN, USA).

Cell viability

Bacteria-induced mast cell cytotoxicity was evaluated by determining the quantity of lactate dehydrogenase (LDH) released into culture medium using a Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany).

Apolipoprotein E-deficient mice

The apoE$^{-/-}$ mouse was used as an animal model of human atherosclerosis. ApoE$^{-/-}$ mice have decreased levels of serum apoE and exhibit lipid abnormalities, and thus, are prone to atherosclerosis even on a low-cholesterol diet [26]. Male apoE$^{-/-}$ mice (Charles River Laboratories, Wilmington, MA, USA) were fed a regular mouse chow and kept in germ-free environment. The experiments were conducted in conformity with the Finnish regulations, and the protocols were approved by The Animal Care and Use Committee of National Public Health Institute, Helsinki, Finland.

Administration of bacteria

A single colony of the fresh clinical strain of Aa AT445b (serotype b, fimbriated, aggregative phenotype) was grown and the density of the culture was determined as described above. The Cpn isolate Kajaani 7 (K7), a Finnish epidemic strain, was produced as previously described [32]. The experiments were performed according to the design presented in the study by Tuomainen *et al.* [11]. Briefly, at the age of 9 weeks, the mice were divided into six groups. Group 1 ($n = 10$) received a weekly intravenous dose of live Aa (10^7 CFU/50 μl/mouse) for 10 weeks starting at the age of 14 weeks. Group 2 ($n = 10$) was inoculated intranasally with Cpn (6×10^6 IFU/mouse) three times at 2-week intervals under inhaled methoxyflurane (Medical Developments, Australia) starting at the age of 9 weeks. Group 3 ($n = 10$) was inoculated intranasally with Cpn (6×10^6 IFU/mouse) three times at 2-week intervals under inhaled methoxyflurane (Medical Developments) starting at the age of 9 weeks, followed by 10 weekly intravenous doses of live Aa. Control mice (group 4, $n = 9$) received SPG (sucrose-phosphate-glutamic acid) buffer [32] intranasally three times at 2-week intervals, followed by 10 weekly intravenous doses of 50 μl 0.9% NaCl. All these mice were killed 1 week after the last injection at the age of 24 weeks. Group 5 ($n = 7$) was inoculated intranasally with Cpn (6×10^6 IFU/mouse) three times at 2-week intervals under inhaled methoxyflurane starting at the age of 9 weeks, while group 6 (control group, $n = 6$) received SPG buffer [32] intranasally three times at 2-week intervals. The animals in group 5 and group 6 were killed 1 week after the last inoculation at the age of 14 weeks.

Serum collection and LPS measurement

Blood was collected from mice by cardiac punction, after which it was allowed to stand for 30 min. and then centrifuged at $2500 \times g$ for 20 min. The serum fraction was separated into a new tube and the remaining

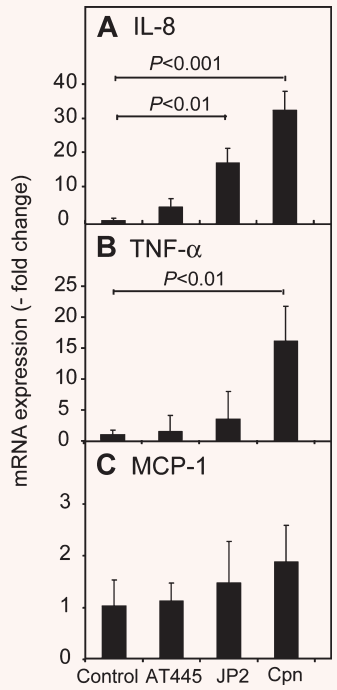

Fig. 1 Effect of pro-atherogenic bacteria on human mast cell cytokine expression. Cultured human mast cells were infected with *Aggregatibacter actinomycetemcomitans* (Aa) strains AT445b and JP2 or *Chlamydia pneumoniae* (Cpn) in a cell-to-bacteria ratio of 1:10 and incubated for 6 hrs. mRNA expressions for IL-8 (**A**), TNF-α (**B**) and monocyte chemoattractant protein-1 (MCP-1) (**C**) were analysed by real-time PCR using TaqMan chemistry. Data are normalized to β-actin and presented as means ± SD.

blood cells were centrifuged once more, after which the two serum fractions were pooled. The serum samples were stored at +4°C overnight and then transferred to −70°C. Serum LPS was measured by limulus amebocyte lysate (LAL) chromogenic end-point assay (HyCult Biotechnology, Uden, Netherlands) with a 1:5 dilution according to manufacturer's instructions.

Histology

Frozen sections (10 μm) of mice aortic sinus were fixed in methanol for 10 min. followed by blocking of the endogenous peroxidase activity with 2% H_2O_2 in methanol for 20 min. After washing with PBS, the mast cells were detected with the naphthol AS-D chloroacetate esterase method (CAE) that gives a pinkish-red reaction product. Briefly, the cryosections were incubated in staining solution containing 4% New Fuchsin in 2 N HCl, 4% sodium nitrite, phosphate buffer and 5% naphtol AS-D chloroacetate in N-N dimethyl formamide for 20 min. followed by two washes in aqua. The slides were counterstained with Mayer's haematoxylin (Merck, Whitehouse Station, NJ, USA) and mounted with aqueous mounting media (Dako, Glastrop, Denmark).

Statistics

Data were reported as means ± standard deviation (SD). Differences between two groups were analysed using Student's two-tailed t-test or Mann-Whitney *U*-test. The null hypothesis was that the means of the uninfected and infected results do not differ. Values of $P < 0.05$ were considered statistically significant.

Results

To test the ability of infectious pro-atherogenic pathogens, Cpn and two strains (AT445b and JP2) of Aa, to induce an inflammatory response in cultured human mast cells, the mast cells were infected with the different bacteria for 6 hrs at 37°C in 5% CO_2, after which two selected atherosclerosis-related inflammatory cytokines (TNF-α and IL-8) and one chemokine (MCP-1) were analysed. Cpn induced a significant expression of both IL-8 ($P = 0.00007$) and TNF-α ($P = 0.0026$) mRNA, whereas the expression of MCP-1 mRNA was only slightly increased (statistically not significant) (Fig. 1). Of the two Aa strains, only the JP2 strain induced a significant expression of IL-8 ($P = 0.0039$) mRNA after 6 hrs of incubation (Fig. 1A), whereas the expression of TNF-α and MCP-1 mRNA was only slightly increased (statistically not significant). The AT445b strain did not induce a statistically significant mRNA expression of the tested cytokines after 6 hrs of incubation. Following inoculation with Cpn, IL-8 and MCP-1 mRNA rapidly increased (within 1 hr), and also rapidly returned to the basal level (Fig. 2, left side). In sharp contrast to this rapid fluctuation, the expression of TNF-α mRNA was increased only after 3 hrs of bacterial inoculation, and was further increased at 24 hrs of incubation.

Next, we determined the concentrations of the above indicated pro-inflammatory molecules, and also of two anti-inflammatory cytokines, IL-10 and TGF-β1, which are considered to be atheroprotective, in the culture media derived from the Cpn-inoculated mast cells. As shown in Fig. 2 (right side), under the basal cell culture conditions, human peripheral blood-derived mast cells secreted no IL-8 or TNF-α and only very low amounts of MCP-1. After inoculation of the cells with Cpn, the secretion of MCP-1 increased in a linear fashion. The secretion of IL-8 was also rapidly initiated after inoculation. In contrast, the secretion of TNF-α was delayed after bacterial inoculation and was found to be increased only after 6 hrs of incubation, corresponding to the delay in the mRNA response to Cpn. There was no measurable secretion of IL-10 or TGF-β1 (data not shown). The responses of mRNA and protein secretion to the inoculation of Aa strains were also studied and were found to be similar to those found for Cpn (data not shown). The bacterial infection also induced a release of histamine indicating mast cell degranulation, but it did not induce mast cell death, when measured as release of LDH (data not shown).

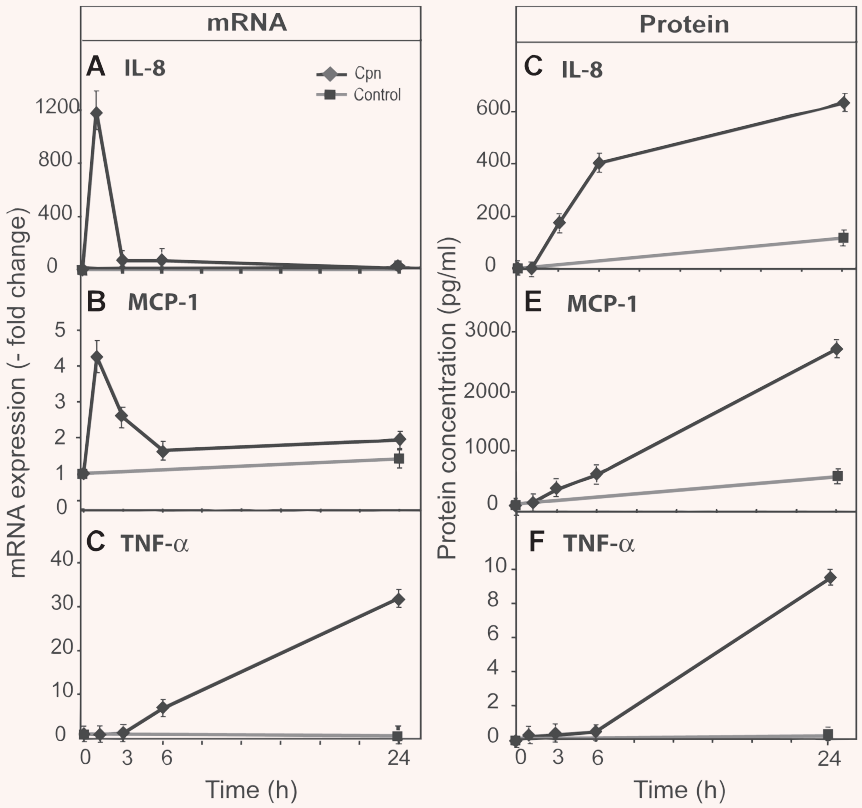

Fig. 2 Temporal induction of pro-inflammatory cytokines and chemokines. Cultured human mast cells were infected with Cpn in a cell-to-bacteria ratio of 1:10 and incubated for 0, 1, 3, 6 and 24 hrs. Quantitative analysis of interleukin 8 (IL-8) (**A**), MCP-1 (**B**) and tumour necrosis factor-α (TNF-α) (**C**) mRNA expression was performed with real-time PCR and the concentrations of these cytokines in the incubation media were detected by ELISA (IL-8 [**D**], MCP-1 [**E**] and TNF-α [**F**]). Data are means ± SD and the results shown are representative of four independent experiments.

To estimate the concentration of bacteria required to trigger cytokine release by human mast cells in culture, increasing amounts of bacteria were used to inoculate the cells. Mast cells cocultured with Cpn in a 1:1 cell-to-bacterium ratio already induced a significant expression of TNF-α ($P = 0.049$), IL-8 ($P = 0.020$) and MCP-1 ($P = 0.035$) mRNA, and the corresponding ratios of 1:5 and 1:10 still further increased the expression levels of the investigated genes (Fig. 3). To test whether the induction of cytokine mRNA expression and protein secretion by mast cells was dependent on the viability of the bacteria, the pathogens were treated with UV radiation before inoculation, as described in Materials and Methods. Indeed, UV treatment of Cpn inhibited the induction of IL-8, both at the mRNA ($P = 0.002$) and protein ($P = 0.0015$) level, revealing that the IL-8 response was dependent on the viability of the bacteria. Similarly, both the synthesis and secretion of TNF-α were significantly reduced ($P = 0.017$ and $P = 0.0001$, respectively) when the mast cells were inoculated with UV-treated Cpn, as compared to live bacteria (Fig. 4). In contrast, inactivation of Cpn failed to reduce the expression and secretion of MCP-1. Finally, UV-treatment of either strain of Aa had only minor effects on the expression and secretion of cytokines (Fig. 4).

Since monocyte-derived macrophages is an abundant cell type in atherosclerotic lesions, and also, since a likely route for certain bacteria, such as Cpn, to reach atherosclerotic lesions is *via* infected circulating monocytes, we next studied the ability of inoculated human monocyte-derived macrophages to activate cocultured human mast cells. As shown in Fig. 5 (parts A and B), Cpn-infected macrophages induced a significant expression of IL-8 ($P = 0.0039$) and TNF-α $P = 0.050$) mRNA in cocultured human mast cells. To test whether cell-to-cell contact between the two types of cultured cell was necessary for the observed Cpn-dependent induction of cytokines, we next incubated mast cells with preconditioned media obtained from macrophages inoculated with Cpn. Interestingly, mast cells treated with the preconditioned media from Cpn-infected macrophages responded by increasing the expression of IL-8 ($P = 0.0040$) and TNF-α ($P = 0.0037$) (parts C and D). Thus, inoculation of macrophages with Cpn must have resulted in secretion of soluble substances which were capable of inducing cytokine expression in mast cells without direct cell-to-cell contact.

Next, it was consequential to explore whether Cpn and Aa were able to activate mast cells *in vivo*. ApoE$^{-/-}$ mice that are widely used as a model of human atherosclerosis, were infected with Aa, Cpn or both Aa and Cpn as described in the Material and Methods, and vehicle-treated mice were used as a control. The aortas of the mice were cryoconserved and sections were cut from the aortic sinus and stained for mast cells using a histochemical method. The number of degranulated aortic mast cells was significantly increased in the

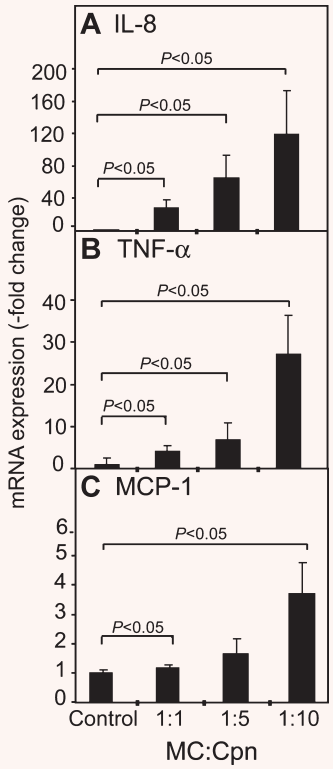

Fig. 3 Effect of different Cpn concentrations on mast cell cytokine expression. Cultured human mast cells (MC) were infected with Cpn in cell-to-bacteria ratios 1:1, 1:5 and 1:10 and then incubated for 6 hrs. IL-8 (**A**), TNF-α (**B**) and MCP-1 (**C**) mRNA levels were quantified with real-time PCR. Data are means ± SD and the results shown are representative of three independent experiments.

Aa ($P = 0.0007$), Cpn ($P = 0.0002$) and Aa + Cpn ($P = 0.0007$) groups as compared to control mice at 24 weeks, as well as in the Cpn group ($P = 0.00008$) as compared to control mice at 14 weeks (Fig. 6). Moreover, in the 24-week-old Cpn-inoculated mice, there was a slight increase in the total number of mast cells as compared to control mice.

Since previous results obtained by us [11] have shown the presence of bacterial LPS in the pro-atherogenic lipoprotein fraction of the Aa-infected mice, we analysed for the presence of LPS in the sera from the Cpn- and Aa-infected animals. As shown in Fig. 7A, LPS was found to be slightly increased in the 14-week-old Cpn-infected mice, but not in the 24-week-old mice. Furthermore, the 24-week-old Aa-infected mice showed a significant increase in the serum LPS levels, whereas the double-infected mice showed a slight but non-significant increase in serum LPS. To further analyse the level of systemic inflammation in the infected animals, we measured the concentration of TNF-α in their sera. As shown in Fig. 7B, the level of TNF-α was significantly increased only in the 24-week-old Aa-infected mice, whereas the 14-week-old Cpn-infected showed decreased levels of serum TNF-α.

Discussion

In the present study we show that two species of pro-atherogenic bacteria, Cpn and Aa, induce an inflammatory response in cultured human mast cells, which was characterized by expression and secretion of the pro-inflammatory cytokines IL-8, TNF-α and MCP-1, but not of IL-10 and TGF-β1, that is two anti-inflammatory cytokines also known to be secreted by activated mast cells. The observed pro-inflammatory response was both concentration- and time-dependent and the Cpn-mediated response was inhibited by treating the Cpn with UV. Furthermore, Cpn-infected human macrophages were capable of inducing a pro-inflammatory response in the cocultured mast cells, and a similar stimulatory effect was observed when preconditioned media from the infected macrophages were added to the cultured mast cells. Moreover, the pro-atherogenic bacteria were shown to increase the number of activated mast cell in the aortic sinus of infected apoE-deficient mice. In addition, the LPS and TNF-α levels in serum was found to be significantly increased only in the 24-week-old Aa-infected animals.

Interestingly, although mast cells isolated from different human donors showed similar qualitative responses to bacteria, variable differences in the intensity of the Cpn- and Aa-mediated responses were observed (data not shown). This may be due either to genetically determined differences in the isolated mast cell precursor cells between the different donors, or to variations in their mature phenotype. Furthermore, there were no clear differences in the inflammatory responses to the two Aa strains AT445b and JP2, the former being endowed, and the latter not endowed with fimbriae. Although the Aa strains represent the same serotype, they may differ from each other regarding other virulence factors than fimbriae, and thus, no definite conclusions can be drawn from these results.

Our observation that infection with viable Cpn induced a higher inflammatory response in the cultured mast cells compared to UV-inactivated Cpn, is supported by a recent study showing an increased Th1 response in THP-1 cells with viable Cpn compared to heat-inactivated Cpn [33]. Interestingly, but not surprisingly, UV-treatment did not abolish the capacity of Aa to induce cytokine expression by mast cells. This clearly indicates that Aa does not have to be viable to activate mast cells, that is the virulence factors that it poses may be sufficient for the induction. The difference between Cpn and Aa in the ability to induce mast cell activation after UV-treatment may relate to the fact that Cpn is an obligatory intracellular bacterium and cannot infect cells when inactivated, whereas Aa is not an intracellular bacterium and may bind to cell surface receptors both in an intact and inactivated form. However, the precise mechanism behind this observation needs to be explored further.

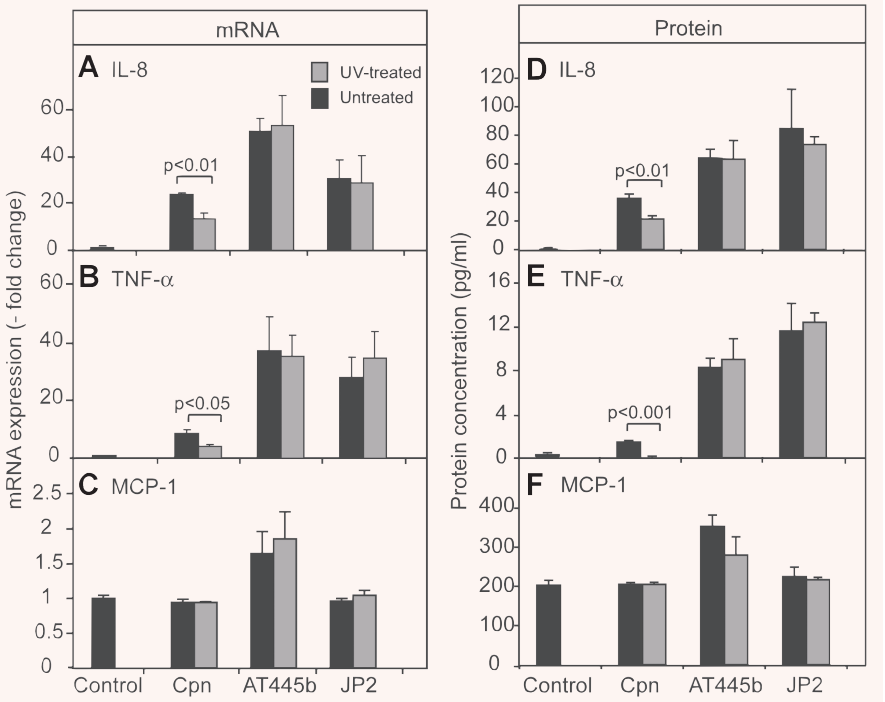

Fig. 4 Effect of bacterial infectivity on mast cell cytokine expression. Cpn and 2 strains (AT445b and JP2) of Aa were treated with UV-light (2 × dosage of 0.120 J/cm^2). Cultured human mast cells were inoculated with UV-treated or untreated bacteria in a cell-to-bacteria ratio of 1:10 for 6 hrs. Quantitative analysis of IL-8 (**A**), TNF-α (**B**) and MCP-1 (**C**) mRNA expression was performed with real-time PCR and the concentrations of these cytokines in the incubation media were detected by ELISA (IL-8 [**D**]), TNF-α [**E**] and MCP-1 [**F**]). Data are means ± SD and the results shown are representative of three independent experiments.

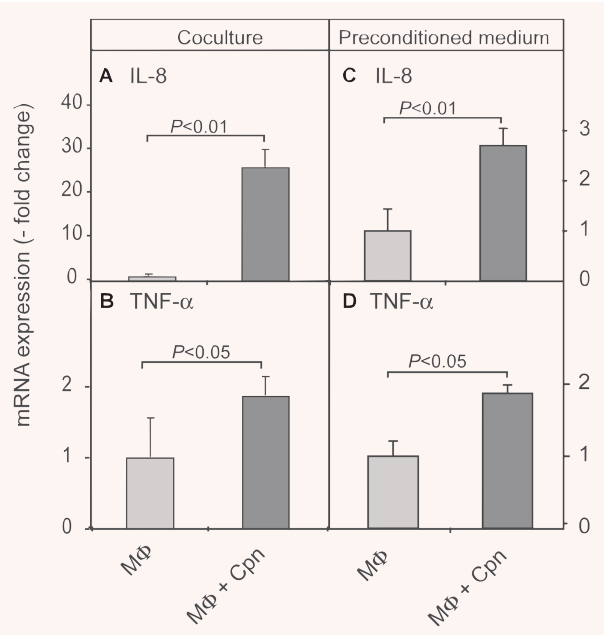

Fig. 5 Infected human macrophages induce cytokine expression in cocultured mast cells. Human monocyte-derived macrophages were inoculated with Cpn in a cell-to-bacteria ratio of 1:10 and incubated for 6 hrs. After washing the macrophages, human mast cells were added to the macrophage culture and cocultured with the infected macrophages in a ratio of 1:2 for 16 hrs (left side). Mast cells were also cultured in preconditioned medium obtained from Cpn-infected macrophages for 6 hrs (right side). IL-8 (**A**, **C**) and TNF-α (**B**, **D**) mRNA levels in mast cells were quantified with real-time PCR. Data are means ± SD and the results shown are representative of three independent experiments.

Recent observations have indicated that several types of bacteria are present in human atherosclerotic lesions [8] and that most of them originate from peripheral tissues, such as the lungs, the gastrointestinal tract and the oral cavity. How do bacteria or bacterial products from the periphery find their way into the atherosclerotic plaques? Since LPS from Cpn is present in serum of patients with acute coronary syndrome (ACS) [34] and have been found associated with pro-atherogenic lipoproteins in a mouse model [35], LPS, a well-known activator of mast cells, may enter the atherosclerotic plaques while bound to the plaque-infiltrating lipoproteins, and then activate plaque mast cells. Indeed, our observation that LPS is present in serum from infected mice supports such an interpretation.

In contrast, it seems unlikely that circulating live bacteria would selectively enter atherosclerotic plaques. However, monocytes that have been infected by bacteria in peripheral tissues, as non-infected monocytes, may selectively invade atherosclerotic

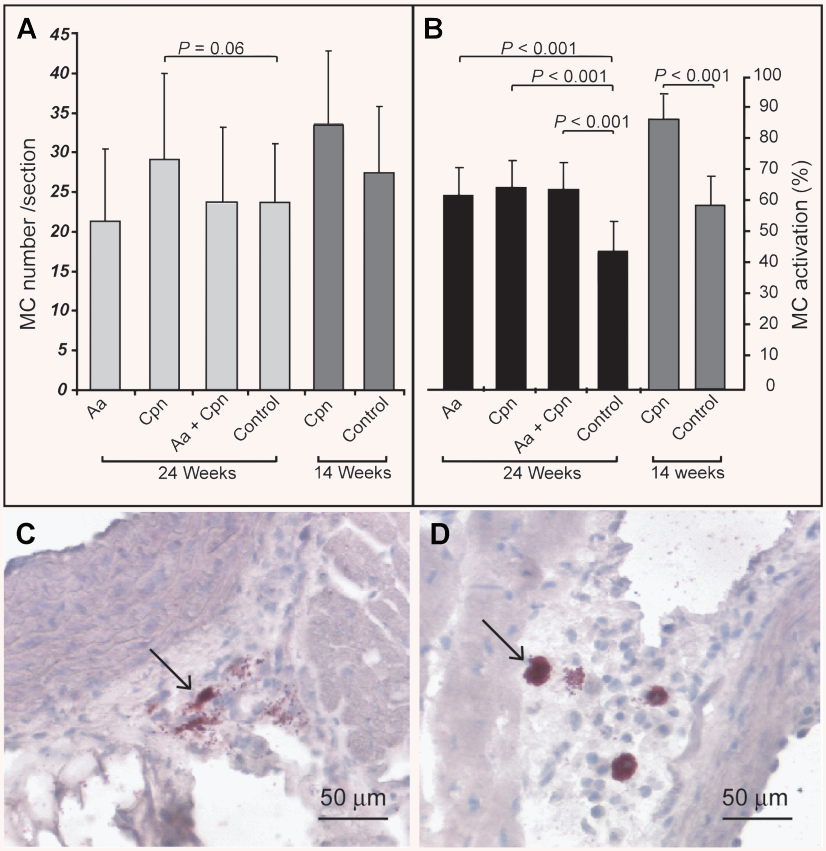

Fig. 6 Effects of pro-atherogenic bacteria on mouse mast cells *in vivo*. Cryosections of aortas of ApoE$^{-/-}$ mice infected with Aa (n = 10), Cpn (n = 10) or both (Aa + Cpn, n = 10), and control mice (n = 8), which were killed at the age of 24 weeks, as well as Cpn-infected (n = 7) and control (n = 6) mice killed at 14 weeks, were stained for mast cells with naphthol AS-D chloroacetate esterase method (CAE). Amount of total (**A**) and degranulated (**B**) mast cells were counted in each group and presented as means ± SD. Degranulated mast cells in Cpn and Aa infected mouse (**C**) and resting mast cells in control mouse (**D**), 20× magnification. Bar = 50 µm.

lesions due to an increased expression of leucocyte adhesion molecules on the surface of the dysfunctional endothelium covering the atherosclerotic plaque. Infected monocytes that have infiltrated the atherosclerotic plaques may then affect other cells present in the lesions, such as the mast cells. Our present observation that Cpn-infected monocyte-macrophages can either directly or indirectly, by their secreted soluble mediators, induce an inflammatory response in mast cells, supports this hypothesis. Furthermore, the infected monocytes-macrophages may also directly release bacteria or bacterial products that are capable of activating mast cell in the intima. In contrast to what have been reported for human lesions, previous data by Aalto-Setälä *et al.* [36] and our present study in which apoE$^{-/-}$ mice were infected intranasally and intravenously with Cpn and Aa, respectively, do not support the idea of infiltration of live bacteria into the aortic sinus of the infected mice. Thus, according to our present results, the presence of bacterial LPS in the pro-atherogenic lipoproteins, VLDL and LDL, is the most likely mechanism by which a systemic bacterial infection may activate mast cells in the aortic sinus of mice. However, presently we cannot exclude the presence of also other potential mechanisms.

In the normal coronary intima, an infection induced by bacteria most likely leads to a rapid activation of mast cells followed by an acute pro-inflammatory response with subsequent events leading to attempts to remove the bacteria and terminate the inflammatory process. However, in an atherosclerotic coronary intima a repeated exposure to bacteria [8] may lead to repeated activation of mast cells with ongoing stimulation of inflammatory processes and progression of atherosclerosis. This is supported by our findings that mast cells in aortic sinus show signs of activation for several weeks after the bacterial inoculations, although the circulatory levels of TNF-α between these animals varied substantially. In fact, the animals that were killed 10 weeks after the last Cpn-inoculation had still activated mast cells in their aortic sinus, although their circulatory levels of TNF-α were similar to those in the control animals. This finding suggests that local mast cell-activating effects persisted in the aortic sinus despite the absence of signs of systemic inflammation in these animals. Surprisingly, however, the systemic levels of inflammation were reduced in the 14-week-old Cpn-infected mice as compared to control mice.

Several interventional trials designed to inhibit Cpn-induced plaque infection with macrolide antibiotics after an ACS have suggested a reduction in risk for cardiovascular complications and restenosis [5, 37]. However, large randomized controlled studies (*e.g.* WIZARD, PROVE-IT, ROXIS and ACES) have failed to show a clear benefit for specific antibiotics such as roxithromycin or

azithromycin in the treatment of CHD [38–42]. One explanation may be that the used antibiotic regimens do not effectively act on the slowly or non-dividing bacteria inside infected cells, and thus, are not able to completely remove the bacterial burden from the lesions [43]. According to our present results, Cpn-infected macrophages could activate cocultured mast cells to synthesize and secrete pro-inflammatory cytokines. Therefore, another pharmaceutical approach to stabilize Cpn-infected vulnerable plaques susceptible to erosion or rupture would be to directly inhibit the activation of plaque mast cells.

What then may be the molecular mechanisms by which bacteria or bacterial products induce the pro-inflammatory response? Several reports suggest that TLR ligation and signalling mediate a pro-atherogenic effect, and that mice lacking TLR4 [44] and TLR2 [45] develop smaller neointimal lesions after vascular injury. A defect in the TLR-associated signal transduction molecule, MyD88, reduces the level of atherosclerosis in apoE knockout mice [46]. Furthermore, since downstream molecules in the TLR signalling pathway, such as mitogen-activated protein kinase (MAPKs) are responsible for the dynamic regulation of both pro- and anti-inflammatory cytokines in innate immune responses [47], a repeated chronic activation of TLRs may disturb the local balance of the antagonizing cytokines. Since human mast cells contain both TLR2 [48] and TLR4 [49] that are capable of recognizing bacterial components, such as LPS, and capable of inducing mast cell activation, these receptors may also be responsible for the effects observed in the present work. Indeed, Tiirola *et al.* have recently shown that Cpn-derived LPS is liberated from the damaged tissue into the circulation during ACS events in patients persistently infected with Cpn [34], so rendering it possible that TLR-bearing plaque mast cells become activated *via* this route.

Thus, the strategic location of mast cells beneath the endothelium of atherosclerotic plaques makes them crucial as sentinels during the invasion of bacterial pathogens into the inflamed lesions [19]. However, repeated activation of plaque mast cells by an increasing bacterial burden may overrun their protective role and instead support adverse pathological effects that contribute to the clinical consequences of atherosclerosis. As the mechanisms of action of infectious agents in atherosclerotic plaques become clarified, effective therapeutic and preventive measures may emerge, one such potential measure being the stabilization of mast cells in atherosclerotic plaques.

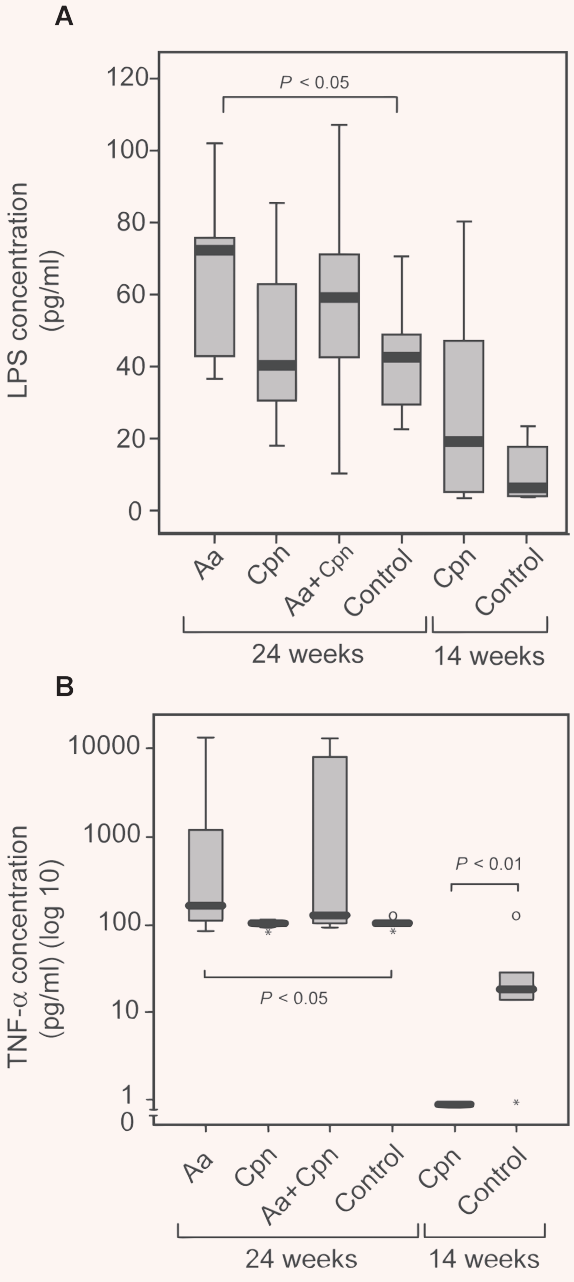

Fig. 7 Serum levels of LPS and TNF-α in infected mice. Serum from ApoE$^{-/-}$ mice infected with Aa (n = 10 mice), Cpn (n = 10) or both (Aa + Cpn, n = 10), and control mice (n = 8), which were killed at the age of 24 weeks, as well as Cpn-infected (n = 7) and control (n = 6) mice killed at 14 weeks, were analysed for the amount of LPS (7**A**) and TNF-α (7**B**). Data are presented as box-and-whisker plots with median values highlighted and outliers marked with * and °.

Acknowledgements

The Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. We acknowledge the excellent technical assistance of Suvi Sokolnicki, Maija Atuegwu, Outi Rautio and Kati Hyvärinen. Pirkko Pussinen was supported by a grant from the Academy of Finland (grant number 118391) and Mirja Puolakkainen was supported by grants from the Academy of Finland (grant number 110340) and ERA-NET PathoGenoMics project ECIBUG (grant 119004).

References

1. **Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR, Jr., Sacco RL, Papapanou PN.** Periodontal microbiota and carotid intima-media thickness: the oral infections and vascular disease epidemiology study (INVEST). *Circulation.* 2005; 111: 576–82.
2. **Spahr A, Klein E, Khuseyinova N, Boeckh C, Muche R, Kunze M, Rothenbacher D, Pezeshki G, Hoffmeister A, Koenig W.** Periodontal infections and coronary heart disease: role of periodontal bacteria and importance of total pathogen burden in the Coronary Event and Periodontal Disease (CORODONT) study. *Arch Intern Med.* 2006; 166: 554–9.
3. **Pussinen PJ, Mattila K.** Periodontal infections and atherosclerosis: mere associations *Curr Opin Lipidol.* 2004; 15: 583–8.
4. **Saikku P, Leinonen M, Tenkanen L, Linnanmaki E, Ekman MR, Manninen V, Manttari M, Frick MH, Huttunen JK.** Chronic Chlamydia pneumoniae infection as a risk factor for coronary heart disease in the Helsinki Heart Study. *Ann Intern Med.* 1992; 116: 273–8.
5. **Etminan M, Carleton B, Delaney JA, Padwal R.** Macrolide therapy for Chlamydia pneumoniae in the secondary prevention of coronary artery disease: a meta-analysis of randomized controlled trials. *Pharmacotherapy.* 2004; 24: 338–43.
6. **Scannapieco FA, Bush RB, Paju S.** Associations between periodontal disease and risk for nosocomial bacterial pneumonia and chronic obstructive pulmonary disease. A systematic review. *Ann Periodontol.* 2003; 8: 54–69.
7. **Lehtiniemi J, Karhunen PJ, Goebeler S, Nikkari S, Nikkari ST.** Identification of different bacterial DNAs in human coronary arteries. *Eur J Clin Invest.* 2005; 35: 13–6.
8. **Ott SJ, El Mokhtari NE, Musfeldt M, Hellmig S, Freitag S, Rehman A, Kuhbacher T, Nikolaus S, Namsolleck P, Blaut M, Hampe J, Sahly H, Reinecke A, Haake N, Gunther R, Kruger D, Lins M, Herrmann G, Folsch UR, Simon R, Schreiber S.** Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. *Circulation.* 2006; 113: 929–37.
9. **Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ.** Identification of periodontal pathogens in atheromatous plaques. *J Periodontol.* 2000; 71: 1554–60.
10. **Engelmann MG, Redl CV, Pelisek J, Barz C, Heesemann J, Nikol S.** Chronic perivascular inoculation with Chlamydophila pneumoniae results in plaque formation *in vivo*. *Lab Invest.* 2006; 86: 467–76.
11. **Tuomainen AM, Jauhiainen M, Kovanen PT, Metso J, Paju S, Pussinen PJ.** Aggregatibacter actinomycetemcomitans induces MMP-9 expression and proatherogenic lipoprotein profile in apoE-deficient mice. *Microb Pathog.* 2008; 44: 111–7.
12. **Galli SJ, Nakae S, Tsai M.** Mast cells in the development of adaptive immune responses. *Nat Immunol.* 2005; 6: 135–42.
13. **Marshall JS.** Mast-cell responses to pathogens. *Nat Rev Immunol.* 2004; 4: 787–99.
14. **Galli SJ, Maurer M, Lantz CS.** Mast cells as sentinels of innate immunity. *Curr Opin Immunol.* 1999; 11: 53–9.
15. **Genovese A, Bouvet JP, Florio G, Lamparter-Schummert B, Bjorck L, Marone G.** Bacterial immunoglobulin superantigen proteins A and L activate human heart mast cells by interacting with immunoglobulin E. *Infect Immun.* 2000; 68: 5517–24.
16. **Shin JS, Gao Z, Abraham SN.** Involvement of cellular caveolae in bacterial entry into mast cells. *Science.* 2000; 289: 785–8.
17. **Xu X, Zhang D, Lyubynska N, Wolters PJ, Killeen NP, Baluk P, McDonald DM, Hawgood S, Caughey GH.** Mast cells protect mice from Mycoplasma pneumonia. *Am J Respir Crit Care Med.* 2006; 173: 219–25.
18. **Echtenacher B, Mannel DN, Hultner L.** Critical protective role of mast cells in a model of acute septic peritonitis. *Nature.* 1996; 381: 75–7.
19. **Malaviya R, Ikeda T, Ross E, Abraham SN.** Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature.* 1996; 381: 77–80.
20. **Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S, Libby P, MacFarlane LA, Mallen-St Clair J, Shi GP.** Mast cells promote atherosclerosis by releasing proinflammatory cytokines. *Nat Med.* 2007; 13: 719–24.
21. **Kaartinen M, Penttila A, Kovanen PT.** Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture. *Circulation.* 1994; 90: 1669–78.
22. **Lindstedt KA, Kovanen PT.** Mast cells in vulnerable coronary plaques: potential mechanisms linking mast cell activation to plaque erosion and rupture. *Curr Opin Lipidol.* 2004; 15: 567–73.
23. **Bot I, de Jager SC, Zernecke A, Lindstedt KA, van Berkel TJ, Weber C, Biessen EA.** Perivascular mast cells promote atherogenesis and induce plaque destabilization in apolipoprotein E-deficient mice. *Circulation.* 2007; 115: 2516–25.
24. **Ali AS, Lax AS, Liljestrom M, Paakkari I, Ashammakhi N, Kovanen PT, Konttinen YT.** Mast cells in atherosclerosis as a source of the cytokine RANKL. *Clin Chem Lab Med.* 2006; 44: 672–4.
25. **Kaartinen M, Penttila A, Kovanen PT.** Mast cells in rupture-prone areas of human coronary atheromas produce and store TNF-alpha. *Circulation.* 1996; 94: 2787–92.
26. **Smith JD, Breslow JL.** The emergence of mouse models of atherosclerosis and their relevance to clinical research. *J Intern Med.* 1997; 242: 99–109.
27. **Lappalainen J, Lindstedt KA, Kovanen PT.** A protocol for generating high numbers of mature and functional human mast cells from peripheral blood. *Clin Exp Allergy.* 2007; 37: 1404–14.
28. **Hakala JK, Oksjoki R, Laine P, Du H, Grabowski GA, Kovanen PT, Pentikainen MO.** Lysosomal enzymes are released from cultured human macrophages, hydrolyze LDL *in vitro*, and are present extracellularly in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* 2003; 23: 1430–6.
29. **Caldwell HD, Kromhout J, Schachter J.** Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. *Infect Immun.* 1981; 31: 1161–76.
30. **Slots J.** Selective medium for isolation of Actinobacillus actinomycetemcomitans. *J Clin Microbiol.* 1982; 15: 606–9.
31. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25: 402–8.
32. **Tormakangas L, Alakarppa H, David DB, Leinonen M, Saikku P.** Telithromycin treatment of chronic Chlamydia pneumoniae infection in C57BL/6J mice. *Antimicrob Agents Chemother.* 2004; 48: 3655–61.
33. **Mamata Y, Hakki A, Newton C, Burdash N, Klein TW, Friedman H.** Differential

effects of Chlamydia pneumoniae infection on cytokine levels in human T lymphocyte- and monocyte-derived cell cultures. *Int J Med Microbiol.* 2007; 297: 109–15.

34. **Tiirola T, Sinisalo J, Nieminen MS, Silvennoinen-Kassinen S, Paldanius M, Saikku P, Jauhiainen M, Leinonen M.** Chlamydial lipopolysaccharide is present in serum during acute coronary syndrome and correlates with CRP levels. *Atherosclerosis.* 2007; 194: 403–7.
35. **Tuomainen AM, Jauhiainen M, Kovanen PT, Metso J, Paju S, Pussinen PJ.** Aggregatibacter actinomycetemcomitans induces MMP-9 expression and proatherogenic lipoprotein profile in apoE-deficient mice. *Microb Pathog.* 2008; 44: 111–7.
36. **Aalto-Setala K, Laitinen K, Erkkila L, Leinonen M, Jauhiainen M, Ehnholm C, Tamminen M, Puolakkainen M, Penttila I, Saikku P.** Chlamydia pneumoniae does not increase atherosclerosis in the aortic root of apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol.* 2001; 21: 578–84.
37. **Paju S, Pussinen PJ, Sinisalo J, Mattila K, Dogan B, Ahlberg J, Valtonen V, Nieminen MS, Asikainen S.** Clarithromycin reduces recurrent cardiovascular events in subjects without periodontitis. *Atherosclerosis.* 2006; 188: 412–9.
38. **Gupta S, Leatham EW, Carrington D, Mendall MA, Kaski JC, Camm AJ.** Elevated Chlamydia pneumoniae antibodies, cardiovascular events, and azithromycin in male survivors of myocardial infarction. *Circulation.* 1997; 96: 404–7.
39. **Gurfinkel E, Bozovich G, Beck E, Testa E, Livellara B, Mautner B.** Treatment with the antibiotic roxithromycin in patients with acute non-Q-wave coronary syndromes. The final report of the ROXIS Study. *Eur Heart J.* 1999; 20: 121–7.
40. **O'Connor CM, Dunne MW, Pfeffer MA, Muhlestein JB, Yao L, Gupta S, Benner RJ, Fisher MR, Cook TD.** Azithromycin for the secondary prevention of coronary heart disease events: the WIZARD study: a randomized controlled trial. *JAMA.* 2003; 290: 1459–66.
41. **Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, Pfeffer MA, Braunwald E.** C-reactive protein levels and outcomes after statin therapy. *N Engl J Med.* 2005; 352: 20–8.
42. **Grayston JT, Kronmal RA, Jackson LA, Parisi AF, Muhlestein JB, Cohen JD, Rogers WJ, Crouse JR, Borrowdale SL, Schron E, Knirsch C.** Azithromycin for the secondary prevention of coronary events. *N Engl J Med.* 2005; 352: 1637–45.
43. **Paju S, Sinisalo J, Pussinen PJ, Valtonen V, Nieminen MS.** Is periodontal infection behind the failure of antibiotics to prevent coronary events? *Atherosclerosis.* 2007; 193: 193–5.
44. **Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, Quax PH, Lim SK, Borst C, Pasterkamp G, de Kleijn DP.** *In vivo* evidence for a role of toll-like receptor 4 in the development of intimal lesions. *Circulation.* 2002; 106: 1985–90.
45. **Schoneveld AH, Oude Nijhuis MM, van Middelaar B, Laman JD, de Kleijn DP, Pasterkamp G.** Toll-like receptor 2 stimulation induces intimal hyperplasia and atherosclerotic lesion development. *Cardiovasc Res.* 2005; 66: 162–9.
46. **Michelsen KS, Wong MH, Shah PK, Zhang W, Yano J, Doherty TM, Akira S, Rajavashisth TB, Arditi M.** Lack of Toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E. *Proc Natl Acad Sci USA.* 2004; 101: 10679–84.
47. **Chi H, Barry SP, Roth RJ, Wu JJ, Jones EA, Bennett AM, Flavell RA.** Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proc Natl Acad Sci USA.* 2006; 103: 2274–9.
48. **Yoshioka M, Fukuishi N, Iriguchi S, Ohsaki K, Yamanobe H, Inukai A, Kurihara D, Imajo N, Yasui Y, Matsui N, Tsujita T, Ishii A, Seya T, Takahama M, Akagi M.** Lipoteichoic acid downregulates FcepsilonRI expression on human mast cells through Toll-like receptor 2. *J Allergy Clin Immunol.* 2007; 120: 452–61.
49. **Kubo Y, Fukuishi N, Yoshioka M, Kawasoe Y, Iriguchi S, Imajo N, Yasui Y, Matsui N, Akagi M.** Bacterial components regulate the expression of Toll-like receptor 4 on human mast cells. *Inflamm Res.* 2007; 56: 70–5.