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Production of interleukin-8 *in vitro* by mononuclear cells isolated from human periapical lesions

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Lukić A, Vojvodic D, Majstorović I, Čolić M. Production of interleukin-8 in vitro *by mononuclear cells isolated from human periapical lesions.*
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Introduction: Interleukin-8 (IL-8) is an important mediator of inflammation. However, little is known about its production in chronic dental periapical lesions and this was the main aim of this work.
Methods: Inflammatory cells were isolated from clinically different periapical lesions and analyzed by morphological criteria. The mononuclear cells were isolated, phenotypically analyzed by immunocytochemistry and cultivated *in vitro*. IL-8 was measured in culture supernatants of these periapical lesion mononuclear cells (PL-MNC) using a microbeads fluorescence assay.
Results: We found a relatively high production of IL-8 in 19 out of 21 periapical lesions included in the study. The level of IL-8 and the proportion of neutrophil granulocytes were significantly higher in the group of symptomatic lesions, compared to the asymptomatic lesions, but there was no statistically significant correlation between these parameters. According to the predominance of $CD3^+$ T cells and $Ig^+/CD19^+$ B cells and plasma cells, lesions were divided into T-type and B-type lesions, respectively. The levels of IL-8 were significantly higher in the culture supernatants of PL-MNC in the T-type lesions and were positively correlated with the proportion of macrophages/dendritic cells ($CD11c^+$ cells) and $CD4^+$ T cells. Such a correlation was not shown in B-type lesions.
Conclusion: These results suggest that PL-MNC are a significant source of IL-8, which is probably an important chemokine for the migration and function of different cell types at the site of chronic inflammation.

Key words: culture; human periapical lesions; inflammatory cells; interleukin-8; phenotype

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Periapical lesions, such as granulomas, cysts and scars, develop in periapical tissue in response to chronic stimulation caused, predominantly, by anaerobic bacterial infection, usually from the root canal, that invades and destroys the dental pulp (5, 23). These lesions are characterized histologically by fibrous and granulation tissue, infiltrated by different inflammatory cells (24, 27, 28). The composition of the infiltrating cells depends on the type of immune response to the microbial antigens, the duration of the inflammatory immune processes, bone destruction and the immune competence of the host (27, 28).

Both non-specific and specific (cellular and humoral) immune responses are activated within periapical lesions. It is believed that the T helper type 1 (Th1) immune response, mediated by interferon-γ and interleukin-2 (IL-2), is involved in the progression of lesions and bone destruction, whereas immunosuppressive mechanisms mediated by transforming growth factor-β and Th2 cytokines (IL-4, IL-5, IL-10) are of importance in healing processes and in the restriction of the inflammatory/immune mechanisms (2, 10, 15). However, little is known about how this fine cytokine network is regulated at different levels of development and maintenance of chronic inflammatory processes in periapical lesions.

IL-8 is a proinflammatory cytokine with potent chemoattractive activity not only

for granulocytes but also for other inflammatory cells (9). Levels of this cytokine, which is produced by different hematopoietic and stromal cells in chronic inflammatory lesions, are elevated in many periodontal diseases (4, 20, 25, 26). IL-8 has also been identified in periapical granulomas by immunohistology (17). However, little is known about its production by inflammatory cells *in vitro*, isolated from periapical tissues.

In this work we studied the production of IL-8 by mononuclear cells isolated from human periapical lesions (PL-MNC), and correlated the level of IL-8 with the clinical characteristics of the lesions, the cellular composition of inflammatory cells and the phenotypic characteristics of PL-MNC.

Materials and methods

Tissue

Twenty-one periapical lesions were collected from patients at the Department for Oral Surgery, Institute for Stomatology, Military Medical Academy, Belgrade and at the Department of Endodontics, Faculty of Stomatology, University of Belgrade, at the time of apical surgery. For each specimen, a valid informed consent was obtained from the patients. The specimens were divided into symptomatic and asymptomatic, according to the presence or absence of the main clinical features such as pain, swelling and other symptoms associated with acute infection. The tissue was immediately placed into transport medium consisting of RPMI-1640 medium (Sigma, Munich, Germany) and antibiotics/antimycotics.

Preparation of total and mononuclear inlammatory cells

Inflammatory cells were isolated from periapical lesions using a universally accepted procedure that was previously optimized in our laboratory. Briefly, periapical tissue was placed in a Petri dish containing 1 ml RPMI-1640 medium and minced, using a scalpel, into pieces 2–3 mm in diameter. The tissue was then digested for 15 min with 0.05% collagenase type IV (Sigma) and 0.02% DNase (Sigma) in 10 ml RPMI-1640 medium at 37°C. After that, soft tissue was pressed through a stainless-steel mesh using a syringe plunger, filtered through a nylon gauze to remove tissue fragments and resuspended in 10 ml fresh RPMI-1640 medium containing 1 mM EDTA. Cells were washed twice by centrifugation in RPMI medium containing 0.5 mM EDTA at room temperature (400 **g** for 7 min) and counted. Cell viability, as determined by Trypan Blue dye exclusion, was usually between 90 and 95%. Using this method <5% of the non-stromal cells was retained within the rest of the tissue (Lukić A, Vasilijić S and Čolić M, unpublished data). A cell suspension of total inflammatory cells (4 ml) was layered over 3 ml Lymphoprep gradient (Nycomed, Oslo, Norway) and centrifuged at 800 **g** for 20 min. Mononuclear cells were collected from the interphase zone, washed twice in RPMI-1640 medium containing 2% heat-inactivated fetal calf serum and counted. The cell viability was usually >97%. From each sample of total inflammatory cells and PL-MNC, cytospins were prepared using a cytocentrifuge (MPW-350, Poland) on poly-l-lysine-coated glass slides. Cytospins were stained with May–Grünwald–Giemsa. Cytospins from PL-MNC samples were processed for immunocytochemistry.

Cell cultures

PL-MNC were cultivated in 96-well, round-bottomed plates (ICN, Costa Mesa, CA) (1×10^5 cells/well, 200 µl) in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (ICN). After 24 h, cell supernatants were collected, centrifuged and frozen at −70°C until the levels of IL-8 were determined. The viability of cells in cultures after 24 h was 80–90%, as determined by Trypan blue dye exclusion or propidium iodide staining.

Detection of IL-8 in culture supernatants

Levels of IL-8 cell culture supernatants were determined using a fluorescent bead immunoassay (Bender MedSystems, Vienna, Austria) and flow cytometry (EPICS XL-MCL flow cytometer, Coulter, Krefeld, Germany) following exactly the manufacturer's instructions. Concentrations of IL-8 in the investigated samples were obtained by comparing the mean fluorescence intensity of samples and known concentrations of cytokines from standards using the commercial flow cytomix software (FF software, BMS-FFS, Bender MedSystems).

Monoclonal antibodies

For immunostaining, anti-CD3, -CD4, -CD8, -CD19 and -CD11c unconjugated monoclonal antibodies (mAbs) were obtained from Serotec, Oxford, UK. Polyclonal rabbit anti-human immunoglobulin antibody conjugated with alkaline phosphatase, rabbit anti-mouse immunoglobulin unconjugated antibody, and alkaline phosphatase anti-alkaline phosphatase (APAAP) complex were purchased from DAKO, Copenhagen, Denmark.

Immunocytochemistry

Cytospins were fixed with 2% pararosaniline for 2 min at room temperature, washed with phosphate-buffered saline for 10 min and incubated with 20% rabbit serum diluted in Tris-buffered saline (TBS), pH 7.6 and 0.05% Tween-20 for 20 min. After washing in TBS/0.5% bovine serum albumin/0.05% Tween-20, slides were incubated with the mAbs for 60 min at room temperature in a humidified slide chamber. Cytospins were incubated with rabbit anti-mouse immunoglobulin containing 10% human AB serum, previously inactivated at 56°C for 45 min, followed by APAAP solution. After each incubation step, slides were washed with TBS/bovine serum albumin/Tween-20 solution for 10 min. The AP reaction was visualized using Fast Red as substrate. Finally, slides were lightly counterstained with hematoxylin, mounted in Keiser gel and examined by light microscopy.

For identification of B cells and plasma cells, cytospins were stained with anti-CD19 mAb as described for other mAbs, followed by anti-human immunoglobulin conjugated with streptavidin–alkaline phosphatase and Fast Red. Positive cells were designated $Ig^+/CD19^+$.

Cytospins were analyzed by light microscopy. At least 500 cells were counted in each sample; the percentages of positive cells were determined on the basis of total counted cells. A similar light microscopical analysis was performed for morphological identification of total inflammatory cells.

Statistical analysis

Statistical analysis was performed using Student's *t*-test and a Pearson correlation test. Values of $P < 0.05$ were considered to be significant.

Results

IL-8 was detected in 19 of 21 PL-MNC culture samples. Mean values were 3080 ± 1835 pg/ml. The levels of IL-8 were higher in the symptomatic lesions (4292 ± 1337 pg/ml) than the asymptomatic lesions (2334 ± 1792 pg/ml;

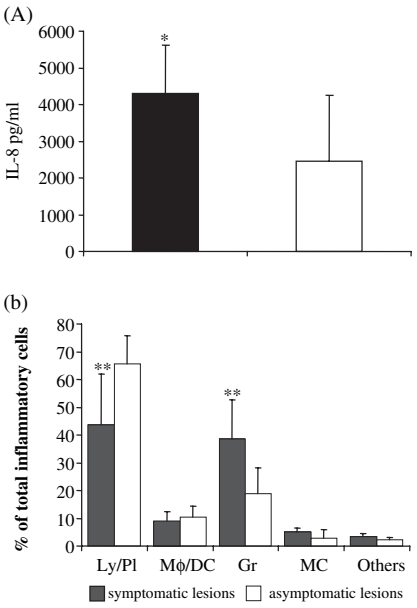

Fig. 1. Production of IL-8 *in vitro* by mononuclear cells isolated from clinically symptomatic and asymptomatic periapical lesions (A) and composition of lesional inflammatory cells (B). The levels of IL-8 in culture supernatants of PL-MNC was determined using a microbeads fluorescence assay. Values are given as pg/ml (mean ± SD) for $n = 8$ (symptomatic) and $n = 13$ (asymptomatic) lesions. The percentages of different types of inflammatory cells were determined by clear morphological criteria, on the basis of at least 500 cells counted on each cytospin. Ly/Pl = lymphocytes, plasma cells, lymphoblastoid and plasmablastoid cells; Mϕ/DC = macrophages, dendritic cells and monocyte-like cells; Gr = granulocytes; MC = mast cells. $*P < 0.05$; $**P < 0.01$ compared with corresponding values in asymptomatic lesions.

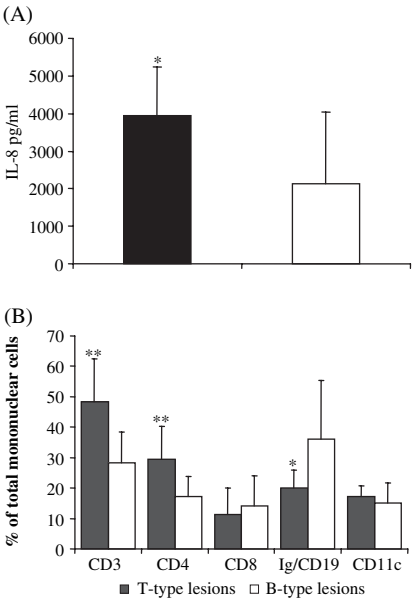

Fig. 2. Production of IL-8 *in vitro* by mononuclear cells isolated from T-type and B-type periapical lesions (A) and composition of PL-MNC (B). The levels of IL-8 in culture supernatants of PL-MNC were determined using a microbeads fluorescence assay. Values are given as pg/ml (mean ± SD) for $n = 11$ (T-type) and $n = 9$ (B-type) lesions. The percentages of different PL-MNC subsets were determined by immunocytochemistry as described in the Materials and methods, on the basis of at least 500 cells counted on each cytospin. $*P < 0.05$; $**P < 0.01$ compared with corresponding values in B-type lesions.

$P < 0.05$) (Fig. 1A). Since symptomatic lesions were characterized by higher percentages of granulocytes among the isolated inflammatory cells than in asymptomatic lesions (Fig. 1B), we correlated the relationship between the levels of IL-8 and the composition of inflammatory cells. No significant correlation was found between the concentrations of IL-8 and the proportion of any type of inflammatory cells, either in the whole group or in clinically different types of lesions (data not shown).

PL-MNC were phenotypically analyzed by an APAAP method. Based on the proportion of $CD3^+$ and $Ig^+/CD19^+$ cells and their ratios, lesions were divided into T-type ($n = 11$) and B-type ($n = 10$) lesions, respectively. As shown in (Fig. 2A), levels of IL-8 in PL-MNC cultures were statistically higher in the T-type lesions (3346 ± 1304 pg/ml) compared to B-type lesions (2127 ± 1916 pg/ml; $P < 0.05$). T-type lesions were characterized by a higher proportion of $CD3^+$ and $CD4^+$ cells and a lower proportion of $Ig^+/CD19^+$ cells compared to B-type cells, whereas no significant differences were identified between the lesion types in the proportions of $CD8^+$ and $CD11c^+$ cells (Fig. 2B). Table 1 shows the positive correlation between levels of IL-8 and the proportion of $CD4^+$ and $CD11c^+$ cells in T-type lesions. No other significantly relevant correlations were found in either T- or B-type lesions (Table 1) nor in the whole group of lesions (data not shown).

Discussion

The initiation of an immune process in the periapical tissue, as a result of infection from the root canal, is triggered by microbial antigens and different inflammatory mediators. IL-8 is one of the first cytokines secreted at the site of inflammation that causes migration of neutrophil granulocytes (9). Numerous studies have shown increased production of IL-8 in gingivitis and periodontal diseases and its level has been correlated with the severity of inflammation (4, 20, 25, 26). IL-8 was also detected in periapical granulomas by immunohistochemistry. Its expression was mainly restricted to Malassez epithelial cells. In addition, finely dispersed IL-8 positivity was also observed in the extracellular matrix (17) but the authors did not show the positivity of infiltrating cells.

This is the first report showing the capability of mononuclear cells isolated from periapical lesions to produce IL-8 *in vitro*. Such an approach has some advantages over immunohistochemistry because it allows the quantification of cytokine production by different cell populations and the modulation of its production using various experimental conditions *in vitro*. We believe that the composition of isolated PL-MNC represents its *in vivo* counterpart. This hypothesis is based on our accompanying study, in which part of a periapical lesion was studied by immunohistochemistry and the remainder of the same sample was used for *ex vivo* cell preparation (Lukić et al. manuscript submitted). The proportion of inflammatory cells and their phenotypic properties (CD3, CD4, CD8, HLA-DR, CD14, CD19 and CD38 staining) were studied *in situ* and correlated strongly with the findings of cytospins *in vitro*.

Table 1. Correlation between the levels of IL-8 and the percentages of PL-MNC subsets*

Group of lesions	IL-8 (Pg/ml)	Cell type (%)	n	r	statistical significance
T - type lesions	IL-8	CD3	11	0.51	p > 0,05
	IL-8	CD4	11	0.77	**P < 0,05**
	IL-8	CD8	10	0.38	p > 0,05
	IL-8	Ig/CD19	11	0.16	p > 0,05
	IL-8	CD11c	10	0.72	**p < 0,05**
B - type lesions	IL-8	CD3	9	0.19	p > 0,05
	IL-8	CD4	9	0.41	p > 0,05
	IL-8	CD8	8	0.44	p > 0,05
	IL-8	Ig/CD19	9	0.29	p > 0,05
	IL-8	CD11c	9	0.48	p > 0,05

*The analysis was performed using Pearson test of correlation.

We detected IL-8 in the culture supernatants of most PL-MNC. In only two samples of asymptomatic lesions were levels of this cytokine undetectable. Periapical lesions were clinically and histologically very heterogeneous in terms of composition of the cellular infiltrate, number of infiltrating cells, phenotypic characteristics of PL-MNC and the presence or absence of symptoms. Such differences may reflect different stages of the development and progression of chronic inflammation. Our group of clinically symptomatic lesions was characterized by a higher number of infiltrating granulocytes. This finding suggests a recent reinfection and disturbance of an already established chronic inflammatory lesion. Significantly higher levels of IL-8 in symptomatic lesions are in agreement with the well-known effects of IL-8, which is a key chemoattractant for neutrophil granulocytes (9). In addition, IL-8 also activates neutrophil functions, such as release of lysosomal enzymes (21) and generation of superoxide anions (22), and increases the expression of adhesion molecules on neutrophils (19). Tissue damage induced by activated neutrophils may cause some of these patients' clinical symptoms. However, we did not show any significant correlation between levels of IL-8 and percentages of granulocytes. These results could be explained by the finding that activated human neutrophils produce IL-1 receptor antagonist and tumor necrosis factor-α soluble receptors that cause down-regulation of IL-8 production (7).

Levels of IL-8 were significantly higher in the culture supernatants of T-type lesions than B-type lesions. The lesions differed in their proportions of T cells and B cells/plasma cells, whereas the proportions of $CD11c^+$ cells (monocyte-like cells/macrophages/dendritic cells) were almost equal in the two lesion types. Since both macrophages and lymphocytes produce IL-8 (9) the differences in IL-8 levels could be related to different numbers of T cells as possible producers of IL-8. Hitomi et al. (8) recently demonstrated that Th1 cells, but not Th2 cells, produce IL-8 *in vitro* after stimulation with anti-CD3 and IL-18, indicating that IL-8 in combination with other proinflammatory mediators and cytokines, could be an important factor contributing to the severity of inflammation. Our finding that the level of IL-8 being positively correlated with the proportions of $CD4^+$ and $CD11c^+$ cells in T-type lesions is in accordance with the previous finding that the Th1 immune response predominates in periapical lesions (15). Activated $CD4^+$ Th1 cells produce interferon-γ, which is a key activator of macrophages (1). It is believed that T cells and macrophages, as well as other antigen-presenting cells, play an important role in the complex events of tissue destruction and repair taking place in the periapical region (10, 16). It remains to be tested in our further experiments which type of T cells (activated naive T cells or activated memory T cells) in periapical lesions produce IL-8 and how the process is regulated.

Which cells could be the target of IL-8 in periapical lesions where granulocytes are a minor cell subset, as was the case with many asymptomatic lesions in our study? One possibility is the cells of the monocyte–macrophage system. IL-8 has been shown to cause the rapid conversion of initially rolling monocytes on monolayers of endothelial cells to firmly adherent cells, by activating leukocyte integrins (6). A similar function of IL-8 was postulated in the accumulation of monocytes into atherosclerotic lesions (29).

IL-8 could also induce the infiltration of T cells into periapical lesions, as judged by previous *in vivo* and *in vitro* experiments showing the chemoattractive activity of IL-8 for rodent and human lymphocytes (3, 11, 13, 14). In a model of delayed type hypersensitivity reaction, anti-IL-8 mAb reduced the infiltration of both neutrophils and lymphocytes (14). Kudo et al. (13) found that depletion of neutrophils inhibited the IL-8-induced *in vivo* migration of $CD4^+$ T cells, suggesting that migration of these cells promoted by IL-8 partly depends on prior infiltration by neutrophils.

Another target of IL-8 in periapical lesions could be endothelial cells. This hypothesis is based on the results that IL-8, secreted by inflamed tissues, is internalized and transported through endothelial cells. The C-terminal end of this cytokine is essential for internalization, as well as for heparin binding (18). In addition, IL-8 induced angiogenesis in rat cornea without inducing leukocyte infiltration (12).

In conclusion, our results suggest that PL-MNC are a significant source of IL-8. The chemokine could be a significant mediator contributing to the development and progression of inflammation by acting on different cells in the periapical tissue.

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References

1. Abbas KM, Murphy KM, Sher A. A functional diversity of helper T lymphocytes. Nature 1996: **383**: 787–793.
2. Akamine A, Hashiguchi I, Toriya Y, Maeda K. Immunological examination on the localization of macrophages and plasma cells in induced rat periapical lesions. Endod Dent Traumatol 1994: **10**: 121–128.
3. Bacon KB, Camp RDR. Interleukin (IL)-8 induced *in vitro* human lymphocyte migration is inhibited by cholera and pertussis toxins and inhibitors of protein kinase C. Biochem Biophys Res Commun 1990: **169**: 1099–1104.
4. Gamonal J, Acevedo A, Bascones A, Jorge O, Silva A. Characterization of cellular infiltrate, detection of chemokine receptor CCR5 and interleukin-8 and RANTES chemokines in adult periodontitis. J Periodontal Res 2001: **36**: 194–203.
5. Gemmell E, Carter CL, Seymour GJ. Mast cells in human periodontal disease. J Dent Res 2004: **83**: 384–387.
6. Gerszten RE, Garcia-Zepeda EA, Lim YC et al. MPC-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 1999: **398**: 718–723.
7. Hattar K, Fink L, Fietzner K et al. Cell density regulates neutrophil IL-8 synthesis: role of IL-1 receptor antagonist and soluble TNF receptor. J Immunol 2001: **166**: 6287–6293.
8. Hitomi H, Yoshimoto T, Hayashi N, Hada T, Nakanishi K. IL-18 together with anti-CD3 antibody induces human Th1 cells to produce Th1- and Th2 cytokines and IL-8. Int Immunol 2004: **16**: 1733–1739.
9. Iizasa H, Matsushima K. IL-8. In: Oppenheim JJ, Feldmann M, ed. Cytokine reference. San Diego, CA: Academic Press, 2001: 1061–1067.
10. Kawashima N, Stashenko P. Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. Arch Oral Biol 1999: **44**: 55–66.
11. Koch AE, Kunkel SL, Pearce WH et al. Enhanced production of the chemotactic cytokines interleukin-8 and monocyte chemoattractant protein-1 in human abdominal aortic aneurysms. Am J Pathol 1993: **142**: 1423–1431.
12. Koch AE, Polverini PJ, Kunkel SL et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 1992: **258**: 1798–1801.
13. Kudo C, Araki A, Matsushima K, Sendo F. Inhibition of IL-8 induced $W3/25^+$ ($CD4^+$) T lymphocyte recruitment into subcutaneous tissues of rats by selective depletion of *in vivo* neutrophils with a monoclonal antibody. J Immunol 1991: **147**: 2196–2201.
14. Larsen CG, Anderson AO, Appella J, Oppenhaim J, Matsushima K. The neutrophil-activating protein (NAP-1) is also

chemotactic for T lymphocytes. Science 1989: **243**: 1464–1466.
15. Lukić A. Transforming growth factor-β is a major down-regulatory cytokine in periapical lesions. Balk J Stom 2000: **4**: 157–160.
16. Marton IJ, Kiss C. Characterisation of inflammatory cell infiltrate in dental periapical lesions. Int Endod J 1993: **26**: 131–136.
17. Marton IJ, Rot A, Schwarzinger F et al. Differential in situ distribution of interleukin-8, monocyte chemoattractant protein-1 and Rantes in human chronic periapical granuloma. Oral Microbiol Immunol 2000: **15**: 63–65.
18. Middleton J, Neil S, Wintle J et al. Transcytosis and surface presentation of IL-8 by venular endothelial cells. Cell 1997: **91**: 385–395.
19. Paccaud JP, Schifferli JA, Baggiolini M. NAP-1/IL-8 induces up-regulation of CR1 receptor in human neutrophil leukocytes. Biochem Biophys Res Commun 1990: **166**: 187–192.
20. Payne JB, Reinhart RA, Masada MP, DuBois LM, Allison AC. Gingival crevicular fluid IL-8: correlation with local IL-1β levels and patient estrogenous status. J Periodontol 1993: **31**: 451–453.
21. Peveri P, Walz A, Dewald B, Baggiolini M. A novel neutrophil-activating factor produced by human mononuclear phagocytes. J Exp Med 1988: **167**: 1547–1559.
22. Schroder JM. The monocyte-derived neutrophil activating peptide (NAP/interleukin 8) stimulates human neutrophil arachidonate-5-lipoxygenase, but not release of cellular arachidonate. J Exp Med 1989: **170**: 847–863.
23. Stashenko P. Role of immune cytokines in the pathogenesis of periapical lesions. Endod Dent Traumatol 1990: **6**: 89–96.
24. Tani N, Osada T, Watanabe Y, Umemoto T. Comparative immunohistochemical identification and relative distribution of immunocompetent cells in sections of frozen or formalin-fixed tissue from human periapical inflammatory lesions. Endod Dent Traumatol 1992: **8**: 163–169.
25. Tonetti MS, Imboden MA, Gerber I, Lang NP, Laissue J, Mueller C. Localized expression of mRNA for phagocyte-specific chemotactic cytokines in human periodontal infections. Infect Immun 1994: **62**: 4005–4014.
26. Tsai CC, Ho YP, Chen CC. Levels of interleukin-1 beta and interleukin-8 in gingival crevicular fluids in adult periodontitis. J Periodontol 1995: **66**: 852–859.
27. Wallstrom JB, Torabinejad M, Kettering J, McMillan P. Role of T cells in the pathogenesis of periapical lesions. A preliminary report. Oral Surg Oral Med Oral Pathol 1993: **76**: 213–218.
28. Waterman PA, Jr, Torabinejad M, McMillan PJ, Kettering JD. Development of periradicular lesions in immunosuppressed rats. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998: **85**: 720–725.
29. Yang Y-Y, Hu C-J, Chang S-U, Tai T-Y, Leu S-Y. Aspirin inhibits monocyte chemoattractant protein-1 and interleukin-8 expression in TNF-α stimulated human umbilical vein endothelial cells. Atherosclerosis 2004: **174**: 207–213.