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Commensal oral bacteria antigens prime human dendritic cells to induce Th1, Th2 or T_{reg} differentiation

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In various immunopathologic conditions, bacterial flora induce an immune response which results in inflammatory manifestations, e.g. periapical granuloma. Dendritic cells provide the main orchestration of specific immune responses. The aim of our study was to test the capacity of distinct oral bacterial antigens (prepared from *Streptococcus mitis, Propionibacterium acnes*, and *Bacteroides* spp.) to prime human dendritic cells for stimulation of the T-lymphocyte response. To assess the T-lymphocyte response, the expression of CD25, CD69, intracellular interferon γ (cIFN-γ), and intracellular interleukin 4 (cIL-4) was determined. Dendritic cells were prepared from leukocyte buffy coat from healthy blood donors. Monocytes were stimulated with IL-4 and GM-CSF and dendritic cells activated with bacterial lysates. Cell suspensions contained up to 90% dendritic cells, which represented 2–12% of the initial number of mononuclear cells. Lymphocyte subsets that developed in lymphocyte cultures after 1 week of stimulation were analyzed by flow cytometry. Dendritic cells, primed with antigens of *Bacteroides fragilis* have shown significantly higher activation and expression of intercellular IFN-γ by T lymphocytes compared to negative controls. The dendritic cells primed with antigens of *P. acnes* had no effect on T-lymphocyte activation or cytokine production; instead they induced differentiation of T lymphocytes into CD25bright cells (regulatory T cells) with a potentially inhibitory effect on immune response. Dendritic cells primed with antigens of *S. mitis* induced increased expression of cIL-4. We conclude that commensal oral bacteria antigens prepared from *B. fragilis, S. mitis*, and *P. acnes* prime human dendritic cells to induce Th1, Th2, and T_{reg} differentiation, respectively. This may advance our understanding of immunopathologic manifestations in the oral cavity and offer new possibilities for redirecting immune responses in mucosal vaccination.

Key words: cytokines; dendritic cells; oral bacteria; Th1 cells; Th2 cells

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Bacterial flora in the oral cavity consists of different anaerobic and microaerophilic bacteria which are part of the indigenous oral flora. All of these bacteria are present in 'healthy' oral cavities. In various immunopathologic conditions, the oral flora may induce an immune response resulting in inflammatory manifestations, e.g. periapical granuloma. Human periapical granuloma develops as a result of a pathologic immune response to stimuli from infected root canals. T cells play an important role in the development of these lesions (14), but the pathogenesis of that common pathology is still controversial. The presence of bacteria in periapical granulomas was confirmed (22) and inflammatory cells have been identified immunohistochemically (17), but the role of distinct inflammatory cells and cytokines is not clear.

Many studies have indicated that dendritic cells play a critical role in antigen presentation *in vivo* (8, 16). Dendritic cells exist in two stages of maturation according to their capacity to stimulate T cells, as nonactivated (immature) and as activated (mature) dendritic cells (8). As immature cells, dendritic cells are scattered throughout the body in nonlymphoid organs, where they are specialized for antigen

capture and processing. A number of microbial and inflammatory products, as well as cytokines, activate dendritic cells, leading to the increased expression of major histocompatibility complex (MHC)-II and costimulatory molecules. Activated dendritic cells migrate to draining lymph nodes, where they are able to stimulate T cells. Mature dendritic cells display increased levels of the cell surface costimulatory molecules CD40, CD80, and CD86, as well as HLA-DR. Furthermore, mature dendritic cells express CD83 and secrete increased amounts of various cytokines and chemokines that aid in T-cell activation (21).

In our previous studies, we analyzed immunologic differences between periapical granulomas with predominantly *Streptococcus* ssp. isolates (Strep. group) and predominantly strictly anaerobic bacteria isolates (Anaer. group) (6). Significantly increased percentages of activated T lymphocytes expressing IL-2 receptors (CD25) and ICAM-1 molecules were detected in periapical granuloma tissues compared to peripheral blood. Increased percentages of $CD8^+$ T lymphocytes expressing HLA-DR molecules were also detected in the periapical granuloma tissues of the Anaer. group granulomas compared to Strep. group. We also performed an *in vitro* study using lymphocyte cultures stimulated by antigens of oral streptococci (*Streptococcus salivarius, Streptococcus mitis, Streptococcus oralis*) and antigens of strict anaerobes (*Bacteroides stercoris, Bacteroides eggerthii*, and *Bacteroides fragilis*) (5, 19). Our results revealed a significantly increased expression of IFN-γ molecule by $CD3^+$ cells stimulated with antigens of strict anaerobes compared to negative controls. Conversely, the antigen preparations of oral streptococci increased the expression of IL-4 by $CD3^+$ cells and the expression of HLA-DR molecules by $CD8^+$ T cells (19).

To clarify the differences in the activation of T lymphocytes by oral bacteria *in vivo* and *in vitro* we included dendritic cells to establish an *in vitro* model of oral bacteria antigen presentation in periapical granulomas. Dendritic cells are the most efficient antigen-presenting cells that ingest and present antigen to naive T cells. Depending on different microbial molecules and local tissue signals, they promote the development of a particular type of immune response (e.g. Th1, Th2, mixed Th1/Th2, T_{reg} response) (16).

In the present study we therefore evaluated the role of antigen presentation by dendritic cells primed with distinct oral bacterial species. We prepared dendritic cells incubated with bacterial antigens in order to stimulate T-lymphocyte activation and differentiation. Antigenic preparations of the three representative bacterial species (*S. mitis, B. fragilis*, and *Propionibacterium acnes*) isolated most frequently from a series of 30 periapical granulomas were used. After stimulation by dendritic cells, lymphocyte subsets were analyzed by flow cytometry for expression of the activation marker (CD69), T_{reg} cells marker CD25, and intracellular cytokine production (IFN-γ, IL-4), since these molecules are important determinants of specific cellular immune response.

Material and methods

Bacterial species selection

Thirty periapical granulomas were collected aseptically during surgical procedures for apicectomy. The periapical lesion was exposed by the use of gingival margin and vertical relieving incisions. The granuloma was removed from alveolar bone with an excavator and transferred to a bottle containing 1.0 ml of sterile anaerobe broth. Each tissue sample was desegregated by using sterile pincers and then gently agitated in a tube containing 1 ml of sterile thioglycolate broth. Of the 30 periapical granulomas (gained from 28 patients) analyzed, 90% yielded a positive bacterial growth when homogenized and cultured. In 33% of samples, *Streptococcus* ssp. was the predominant isolate, in 16.7% *P. acnes* ssp., and in 10% *Bacteroides* ssp. In one or two samples we also isolated *Eubacterium, Lactococcus* ssp., *Bacillus* ssp., *Escherichia coli, Staphylococcus* ssp. or enterococci. Ten percent of the samples were sterile. Because of the predominance of *S. mitis*, *B. fragilis*, and *P. acnes* found in the periapical granulomas, representative strains of these bacteria were selected for bacterial antigen preparation.

Mononuclear cells

Mononuclear cells from healthy blood donors were isolated on Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden), resuspended in RPMI-10% FSC and 0.8 mM L-glutamine, 1 mM penicillin and streptomycin. Isolated mononuclear cells were split into two parts: one used for dendritic cell preparation and one frozen in liquid nitrogen until used for preparation of responding (autologous) mononuclear cells. Ethical approval was obtained for this study.

Bacterial antigens

The bacterial strains from granuloma tissue were initially categorized by their atmospheric requirements, gram staining, and catalase reaction. Facultative streptococci were identified by means of the API 20 Strep. System (API, BioMerieux, Marcy-l'Etoile, France). Strict anaerobes were identified by means of the API 20 A system. Oral bacteria used in the *in vitro* study were incubated at 37 °C in aerobic or anaerobic conditions (5% carbon dioxide, 10% hydrogen, and 85% nitrogen) according to their atmospheric requirements. Bacteria were sonicated to antigens with the Misonix Sonicator LX (Misonix, Farmingdale, NY). Suspensions of bacterial antigens were filtered through a 0.45-μm membrane filter and stored at − 20 °C until used. The concentration of total proteins was determined by Bio-Rad (Richmond, CA) protein assay.

Dendritic cell preparation

Monocytes ($CD45^+$ and $CD14^+$ cells) were differentiated into dendritic cells using recombinant human GMC-SF and recombinant human IL-4. Mononuclear cells were isolated on Ficoll-Paque, resuspended in RPMI-10% FSC and 0.8 mM L-glutamine, 1 mM penicillin and streptomycin, and allowed to adhere to tissue culture dishes 100 × 20 mm in diameter (Corning Glass Works, Corning, NY). After 2 h at 37 °C the nonadherent cells were removed and adherent cells were detached by incubation with Mg^{2+}- and Ca^{2+}-free phosphate buffered saline (PBS) containing 0.5 mM EDTA at 0 °C for 5 min and than removed by cell scraper (Costar Corporation, Cambridge, MA). Adherent cells were cultured at 4×10^5/ml in RPMI − 10% FSC supplemented with 50 ng/ml GM-CSF, 200 U/ml IL-4 for 5 days. Dendritic cell were activated with bacterial lysates for 48 h. The cells were analyzed by flow cytometry and characterized as $CD80^+$, $CD83^+$, $CD86^+$, $HLA\text{-}DR^+$ and CD14 cells.

In vitro lymphocyte activation by dendritic cells and mononuclear cells

The 1×10^6 mononuclear cells (concentration 5×10^5/ml) were washed with cultured medium and stimulated for five

consecutive days with 1 μg/ml of bacterial antigens. As a control, the population of unstimulated mononuclear cells was analyzed.

The 1×10^6 dendritic cells (concentration 5×10^5/ml) were incubated for 2 days (48 h) with 1 μg/ml of bacterial antigens The cells were subsequently washed, resuspended in medium and added to 1×10^6 mononuclear cells. The cells were then cultured at 37 °C for 5 days. Activation of the mononuclear cells was followed by determination of the proportion of cells expressing CD25 or CD69, or producing IFN-γ or IL-4 by $CD3^+$ cells. As a control we used mononuclear cells cocultured with dendritic cells not exposed to bacterial antigens.

Flow cytometry

Flow cytometry analysis was performed using a fluorescence cell counter (FACSCalibur Becton Dickinson, Mountain View, CA). A tree-parameter analysis was performed for determination of CD25, CD69, IFN-γ and IL-4 expression by CD3-positive cells and for determination of CD80 (FITC), CD83 (PE), and CD86 (PE) expression by dendritic cells. Cells were labeled with monoclonal antibodies and analyzed with CELL QUEST software (Becton Dickinson). Histograms of fluorescence distribution were generated by plotting the numbers of cells (*y* axis) vs. fluorescence intensities (log scale; *x* axis). Two parameters were considered: first, the median fluorescence intensity, which reflects the overall level of expression of the epitope specifically recognized by MAb, and second, the percentage of cells expressing higher fluorescence compared to cells labeled by isotype control (MAb^+ cells). A control of viable cells (LIVE/DEAD kit, Molecular Probes, Oregon USA) were included in the analysis. For the detection of intracellular IL-4 and IFN-γ the inhibitor of exocytose breferdin A 5 M (Sigma, St Louis, MO) was used. At least 1000 gated cells were analyzed for each test and signals from the two light scatters and the four fluorescence parameters were analyzed.

Statistical analysis

Standard methods were used for descriptive analysis. The Student-Newman-Keuls Test of one-way ANOVA was used for comparison of the chosen immune parameters. $P < 0.05$ was accepted as a significant difference.

Results

Expression of surface markers by dendritic cells, prepared from peripheral blood mononuclear cells

To obtain dendritic cells, adherent mononuclear cells were treated with GM-CSF and IL-4 for 5 days. Dendritic cells were then incubated with bacterial lysates for 48 h. The expression of dendritic cell surface antigen was analyzed by flow cytometry. The results in Fig. 1 demonstrate enhanced expression of CD83, CD80, HLA-DR and decreased expression of CD14 by dendritic cells compared to monocytes at the beginning of the experiment.

Bacterial antigens differently primed dendritic cells to stimulate expression of CD69, CD25 IL-4 and IFN-γ molecules by T lymphocytes

We investigated the capability of different oral bacterial antigens prepared from *S. mitis*, *P. acnes*, and *B. fragilis* to prime human dendritic cells to stimulate T-cell response. For a negative control we used mononuclear cells incubated with dendritic cells that were not activated with oral bacterial antigens. Each result in Table 1 represents a mean value of 10 results from 10 independent experiments.

As demonstrated in Table 1, dendritic cells incubated with antigens of *B. fragilis* increased the expression of CD25 and CD69 molecules by T lymphocytes compared to the negative control. The production of intercellular IFN-γ was also significantly higher. Dendritic cells incubated with antigens of *S. mitis* increased the expression of CD25 and cIL-4 molecules by T lymphocytes but dendritic cells incubated with antigens of *P. acnes* increased only the expression of CD25 molecules by T lymphocytes compared to the negative control.

In Table 2 we present the MFI of CD25 and CD69 molecules by $CD3^+$ lymphocytes after 5 days of *in vitro* culture of activated dendritic cells and primary blood lymphocytes (PBL). The median fluorescence intensities were measured after labeling with fluorescent monoclonal antibodies and flow cytometric analysis of fluorescence light emission. According to our results, dendritic cells incubated with the *P. acnes* preparation induced differentiation of T lymphocytes into CD25bright cells (Fig. 2).

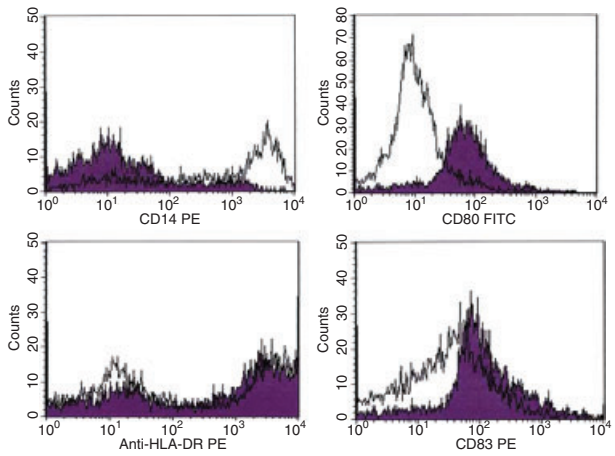

Fig. 1. Dendritic cells incubated with bacterial antigens exhibit enhanced expression of CD83, CD80, HLA-DR and decreased expression of CD14 (shaded histogram) compared to monocytes at the beginning of the experiment (white histogram).

Table 1. Mean percentages (with standard deviations) of distinct T-lymphocyte phenotypes obtained after 5 days of *in vitro* culture of activated dendritic cells and PBL. Each number represents the results of 9 independent experiments

	K- (mononuclear cells + dendritic cells)	*B. fragilis* + dendritic cells	*P. acnes* + dendritic cells	*S. mitis* + dendritic cells
$CD25^+$ $CD3^+$	7.5 ± 2.1	12.7 ± 5.1**	9.1 ± 0.8 *	11.0 ± 2.6 **
$CD69^+$ $CD3^+$	6.6 ± 2.2	10.3 ± 4.6*	7.0 ± 1.6	9.8 ± 4.9
cIFN-γ $^+CD3^+$	3.5 ± 1.8	5.7 ± 2.1*	4.6 ± 2.7	4.7 ± 0.7
cIL-4 $^+CD3^+$	3.1 ± 1.3	4.4 ± 1.7	4.3 ± 1.8	5.1 ± 1.6**

*$P = 0.05$. **$P = 0.01$.

Table 2. Mean Fluorescence Intensity (MFI) with standard deviations of CD25bright CD3 cells obtained after 5 days of *in vitro* culture of activated dendritic cells and PBL. T_{reg} were determined according to the level of CD25 expression. CD3 cells with a CD25 expression above the level of CD3- cells were considered T_{reg}. Each number represent the results of nine independent experiments

Expression marker	Mononuclear cells + dendritic cells MFI ± SD	*B. fragilis* + dendritic cells MFI ± SD	*P. acnes* + dendritic cells MFI ± SD	*S. mitis* + dendritic cells MFI ± SD
CD25	19.1 ± 8.2	24.6 ± 11.2	31.6 ± 11.2 **	24.1 ± 11.6
CD69	11.8 ± 2.7	16.7 ± 11.5	18.3 ± 10.2	15.7 ± 7.9

*$P = 0.05$. **$P = 0.01$.

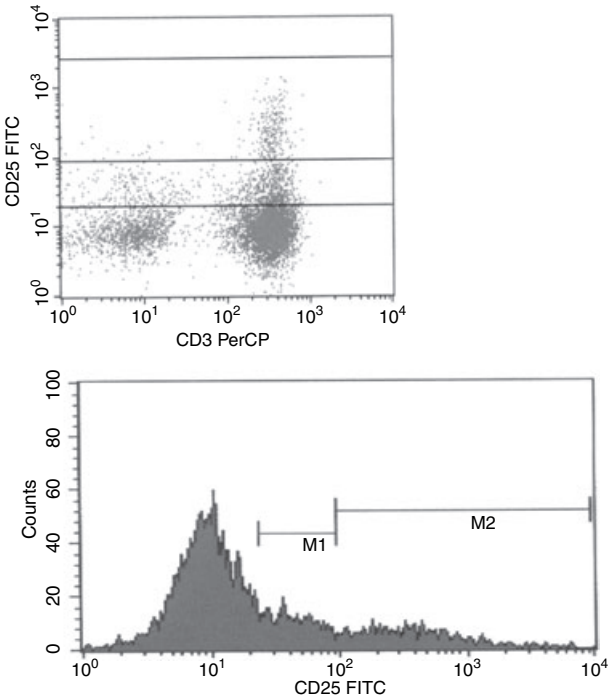

Fig. 2. CD25bright T cells are enriched in the cultures of dendritic cells primed with *P. acnes* antigens compared to cultures with nonprimed dendritic cells. Representative FACS plot and histogram of cultured T cells expressing a low and a high density (T_{reg}) of CD25 molecules. T_{reg} were determined according to the level of CD25 expression. CD3 cells with a CD25 expression above that of CD3 cells were considered to be T_{reg}.

Discussion

Periapical granuloma is a chronic inflammatory granulation tissue developed as a response to an infected dental root canal, and represent a possible beginning stage of radicular cysts, which are the most common osteolytic lesions in human pathology. The role of periapical granulomas as a chronic source for spreading bacterial infections is also important. The etiopathogenesis of periapical granuloma involves inflammatory cells, the T cells being most numerous in the lesions (12). Their activation and function is not known yet, as many regulatory circuits are activated not only within the immune system but also in interactions with other cells, e.g. oral epithelium and oral bacteria may play both activating and inhibitory roles (3).

Our previous studies investigated the immunomodulatory properties of distinct oral bacteria species in periapical granulomas, *in vitro* lymphocyte cultures, and in the mouse model of bacterial infection. After the correlation of the causative bacteria and the form of immune response in periapical granuloma tissue was confirmed (6), in an *in vitro* study in experimental models we were able to demonstrate significant differences in the inflammatory process induced by oral streptococci or oral anaerobic bacteria such as *Bacteroides* spp. as in lymphocyte cultures (19) and in the mouse model (5). The periapical granulomas colonized by anaerobic bacteria exerted significantly increased levels of cell infiltration, cytotoxic cell activation, and IFN-γ secretion. To analyze our previous results we carried out an *in vitro* study using dendritic cells as antigen presenting cells. Because of their predominance among bacterial species found in periapical granulomas, representatives strains of *S. mitis*, *B. fragilis*, and *P. acnes* were selected for bacterial antigen preparation. Similar bacterial species in periapical granulomas were also obtained by Iwu and coworkers in 1990 (7) and Vigil and coworkers in 1997 (20).

Our present findings demonstrated that dendritic cells loaded with *B. fragilis* antigens strongly activated and increased proliferation of T lymphocytes as well as IFN-γ production. Antigen presentation alone can stimulate pathogen-specific T-cell clones, but is not sufficient to trigger efficient T-cell expansion and differentiation, which requires an additional signal delivered by costimulatory molecules such as CD80 and CD86 (1). According to our results, dendritic cells incubated with *B. fragilis* preparation strongly differentiate naive T-cells into Th1 subsets. Similar observations were published by Hou and colleagues in 2000, as they established a model of anaerobic infection in immunocompromised mice (4).

Dendritic cells incubated with antigens of *S. mitis* increased expression of CD25 and differentiated T-helper cells into Th2 cells. This result may be in accordance with the study of Kettering and coworkers (10) that found a relatively low index of IgG vs. IgA antistreptococcal response in patients with symptoms of chronic periapical lesions. Similarly, our previous measurements of cytokine production in clinical '*ex vivo*' samples provided evidence that Th1-type cytokine production of $CD4^+$ T cells is increased in periapical lesions predominantly colonized by anaerobic bacteria compared to periapical lesions predominantly colonised by streptoccoci (6). As oral streptococci are a part of the commensal oral flora highly adapted to the host, they probably developed immunoregulatory molecules for induction-free inflammation and tissue damage in periapical lesions compared to anaerobic bacteria (18). Similarly, a relatively mild immune response in infection with *Streptococcus mutans* compared to anaerobic infection with *Porphyromonas endodontalis* was reported by Kim & Lim (11) using an experimental rat model.

Dendritic cells incubated with *P. acnes* antigens did not significantly increase IFN-γ or IL-4 production compared to negative controls. Instead they induced

differentiation of T lymphocytes into CD25bright cells (Table 2) with a potentially inhibitory (regulatory) effect on the immune response. In our experiments we demonstrated that different bacteria are able to stimulate different T-cell responses that trigger different cytokine production. This may be important in the pathogenesis of oral diseases, e.g. in the developement of inflammatory odontogenic cysts, where it is still not known which type of inflammation or immune reaction may cause the transition from a periapical inflammation to inflammatory odontogenic cyst formation (2, 23).

Our results confirm that dendritic cells primed with *B. fragilis* antigens induced significantly higher IFN-γ expression compared to dendritic cells primed with *S. mitis* antigens. We conclude, with Kabashima et al. (9), that Th1 cells may play an important role in the pathologic process of local inflammation such as periapical granulomas. We also came to the conclusion that dendritic cells primed with antigens of *P. acnes* induced differentiation of T lymphocytes into regulatory T cells with CD25bright expression. Our present results demonstrate that dendritic cells stimulated and loaded with distinct oral bacterial preparations were able to influence *in vitro* T-lymphocyte differentiation similarly as observed in *in vivo* inflammatory lesions infected by same bacterial species (6). It is therefore probable that activated dendritic cells alone are able to influence a distinct phenotypic differentiation of T-cell response.

The knowledge of the pathogenesis of periapical granuloma could open some new possibilities to prevent radicular cyst formation and tooth loss. To modulate the repair process of a chronic lesion, it is necessary to identify both healing and destructive mediators that determine the clinical course of the lesion. We therefore conclude that distinct commensal oral bacteria species prime human dendritic cells for inducing different inflammatory responses; this may have important consequences in understanding various immunopathologic manifestations in the oral cavity as well as in developing better immunization and immuno-intervention procedures in the oral cavity (13, 15).

References

1. Erridge C, Pridmore A, Eley A, Stewart J, Poxton IR. Lipopolysaccharides of *Bacteroides fragilis, Chlamidia trahoatis* and *Pseudomonas aeroginosa* signal via Toll-like receptor 2. J Med Microbiol 2004: **53**: 735–740.
2. Formigli L, Orlandini SZ, Tonelli P, Giannelli M, Martini M, Brandi ML, et al. Osteolytic process in human radicular cysts: morphological and biochemical results. J Oral Pathol Med 1995: **24**: 216–220.
3. Han DC, Huang GTJ, Lin LM, Warner NA, Gim JS, Jewett A. Expression of MHC Class II, CD70, CD80, CD86 and pro-inflammatory cytokines is differentially regulated in oral epithelial cells following bacterial challenge. Oral Microbiol Immunol 2003: **18**: 350–358.
4. Hou L, Sasaki H, Stashenko P. Toll-like receptor 4-deficient mice have reduced bone destruction following mixed anaerobic infection. Infect Immun 2000: **68**: 4681–4687.
5. Hudler P, Gubina M, Ihan Hren N, Seme K, Malovrh T, Gale N, et al. A mouse model of chronic bacterial lesions (a cotton trap) for studying oral bacteria – lymphocyte interactions. Eur J Physiol 2000: **440**: 91–93.
6. Ihan Hren N, Gubina M, Ihan A. Cytotoxic T lymphocytes in periapical granulomas. Anaerobe 1999: **5**: 247–249.
7. Iwu C, Macfarlane TW, MacKenzie D, Stenhouse D. The microbiology of periapical granulomas. Oral Surg Oral Med Oral Pathol 1990: **69**: 502–505.
8. Jankovic D, Liu Z, William CG. Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways. Trends Immunol 2001: **22**: 450–457.
9. Kabashima H, Yoneda M, Nakamuta H, Nagata K, Isobe R, Motooka N, et al. Presence of CXCR3-positive cells and IFN-gamma-producing cells in human periapical granulomas. J Endod 2004: **30**: 634–637.
10. Kettering JD, Torabinejad M, Jones SL. Specificity of antibodies present in human periapical lesions. J Endod 1991: **17**: 213–216.
11. Kim SA, Lim SS. T lymphocyte subpopulations and interleukin-2, interferon-gamma, and interleukin-4 in rat pulpitis experimentally induced by specific bacteria. J Endod 2002: **28**: 202–205.
12. Liapatas S, Nakou M, Rontogianni D. Inflammatory infiltrate of chronic periradicular lesions: an immunohistochemical study. Int Endod J 2003: **36**: 464–471.
13. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. Nat Immunol 2001: **2**: 816–822.
14. Matsuo T, Ebisu S, Shimabukuro Y, Ohtake T, Okada H. Quantitative analysis of immunocompetent cells in human periapical lesions: correlations with clinical findings of the involved teeth. J Endod 1992: **18**: 497–500.
15. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995: **155**: 1151–1164.
16. Smits HH, van Beelen AJ, Hessle C, Westland R, de Jong E, Soeteman E, et al. Commensal Gram-negative bacteria prime human dendritic cells for enhanced IL-23 and IL-27 expression and enhanced Th1 development. Eur J Immunol 2004: **34**: 1371–1380.
17. Stern MH, Dreizen S, Mackler BF, Levy BM. Isolation and characterization of inflammatory cells from the human periapical granuloma. J Dent Res 1982: **61**: 1408–1412.
18. Torabinejd M, Backland DSS, Loma L. Immunopathogenesis of chronic periapical lesions. Oral Surg 1978: **46**: 685–699.
19. Vardjan N, Kopitar AN, Ihan-Hren N, Malovrh T, Wraber B, Ihan A. Immune response in lymphocyte cultures stimulated by oral bacteria preparations. Eur J Physiol 2000: **5** (Suppl.): R67–R69.
20. Vigil GV, Wayman BE, Dazey SE, Fowler CB, Bradley DV. Identification and antibiotic sensitivity of bacteria isolated from periapical lesions. J Endod 1997: **23**: 110–114.
21. Wassenaar A, Snijders A. Abraham-Inpijn L. Kapsenberg ML, Kievits F. Antigen-presenting properties of gingival fibroblasts in chronic adult periodontitis. Clin Exp Immunol 1997: **110**: 277–284.
22. Wayman BE, Murata SM, Almeida RJ, Fowler CB. A bacteriological and histological evaluation of 58 periapical lesions. J Endod 1992: **18**: 152–155.
23. Zecchi-Orlandini S, Formigli L, Giannelli M, Martini M, Tonelli P, Brandi ML, et al. Radicular cysts are involved in the recruitment of osteoclast precursors. J Oral Pathol Med 1996: **25**: 325–330.