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Production of immunoregulatory cytokines in clinically asymptomatic periapical lesions depends on the lesions size

Zavisnost produkcije imunoregulatornih citokina u klinički asimptomatskim periapeksnim lezijama od veličine lezije

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Abstract

Bacground/Aim. Development of periapical lesions (PLs) involves a complex cross-talk between pathogenic microorganisms from the root canal and host immune mechanisms. In these processes proinflammatory cytokines are involved in stimulation of inflammation and osteodestructive mechanisms, whereas anti-inflammatory cytokines, with the immunoregulatory functions, have the opposite effects. How this balance is controlled is still the subject of numerous studies. The aim of this study was to examine whether the local production of interleukin (IL)-10, IL-27 and transforming growth factor (TGF)-β in human asymptomatic PLs depends on the lesion size and how levels of these immunoregulatory cytokines correlate with the cellular composition of PLs. **Methods.** The study was conducted on 30 PLs which were collected at the Clinic for Stomatology of the Military Medical Academy, Belgrade, Serbia. The PLs were divided according to their size into small- and large-size lesions (n = 12 and n = 18, respectively). The inflammatory cells (PL-ICs) were isolated from PLs and cultivated for 24 hours in culture medium supplemented with phorbol myristate acetate and Ca^{2+} ionophore. Cytospins were processed for immunocytology by using monoclonal antibodies to cell subsets. The levels of cytokines in culture supernatants were determined by the ELISA method. Statistical analysis was performed using the Student *t*-test and the Spearman's correlation test. The values of $p < 0.05$ were considered to be significant. **Results.** The levels of IL-10 and TGF-β were significantly higher in the PL-ICs cultures of large-size lesions than in small ones ($p < 0.01$ and $p < 0.05$, respectively). In contrast, the levels of IL-27 were higher in the cultures of small-size lesions than in small ones ($p < 0.05$). Although the total number of PL-ICs and the proportion of mononuclear phagocytes were higher in the large-size PLs ($p < 0.01$ and $p < 0.05$, respectively), their main composition was not significantly different between the groups. The proportions of B cells/plasma cells ($CD19/CD38^+$ cells), $CD8^+$ T cells and $CD14^+$ cells were significantly higher in the large-size PLs ($p < 0.005$; $p < 0.05$; $p < 0.05$, respectively). In contrast, the proportion of total T cells ($CD3^+$ cells) was higher in the small-size lesions ($p < 0.05$). No statistically significant correlation was found between the levels of these cytokines and the composition/phenotype of PL-ICs. **Conclusion.** This study suggests that IL-10, IL-27 and TGF-β may play different roles in suppression of unwanted immune/inflammatory reactions in asymptomatic PLs, depending on the extension of pathological process as judged by the size of lesions.

Key words:
periapical diseases; cytokines; microbiota; immunologic factors; inflammation.

Apstrakt

Uvod/Cilj. Razvoj periapeksnih lezija (PLs) prolazi kroz složenu interakciju između patogenih mikroorganizama iz kanala zuba i imunskih mehanizama domaćina. U ovim procesima proinflamacijski citokini stimulišu zapaljenske reakcije i destrukciju kostiju dok anti-inflamacijski citokini sa imunoregulacijskim svojstvima imaju suprotan efekat. Način kako je taj balans kontrolisan je i dalje predmet brojnih studija. Cilj ovog rada bio je da se ispita da li lokalna produkcija interleukina (IL)-10, IL-27 i transformišućeg faktora rasta beta (TGF-β) u humanim asimptomatskim PLs zavisi od njihove veličine i kako nivo ovih citokina korelira sa ćelijskim sastavom PLs. **Metode.** Istraživanje je sprovedeno na

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30 PLs koje su ekstrahovane u Klinici za stomatologiju VMA. Lezije su podeljene na grupu malih lezija (n = 12) i grupu velikih lezija (n = 18). Inflamatorne ćelije izolovane iz lezija (PL-ICs) kultivisane su u toku 24 časa u medijumu za ćelijske kulture sa dodatkom forbol miristat acetata i Ca^{2+} jonofora. Citospin preparati obojeni su pomoću imunocitohemijskih metoda korišćenjem monoklonskih antitela prema ćelijskim subpopulacijama. Nivo citokina u supernatantima ćelijskih kultura određen je ELISA metodom. Za statističku obradu podataka korišćen je Studentov *t*-test i Spearman-ov test korelacije. Vrednosti razlika $p < 0,05$ smatrane su statistički značajnim. **Rezultati.** Nivoi IL-10 i TGF-β su bili statistički značajno viši u supernatantima PL-ICs velikih lezija ($p < 0,01$, odnosno $p < 0,05$), za razliku od IL-27 čiji su nivoi bili veći u kulturama malih lezija ($p < 0,05$). Iako je ukupan broj PL-ICs i procenat mononuklearnih fagocita bio viši u velikim lezijama ($p < 0,01$, odnosno $p < 0,05$), njihov osnovni ćelijski sastav nije se bitnije razlikovao između grupa. Procenat B ćelija/plazma ćelija (CD19/CD38$^+$ćelija), CD8$^+$ T ćelija i CD14$^+$ ćelija je bio veći u velikim lezijama ($p < 0,005$; $p < 0,05$; $p < 0,05$), za razliku od procenta ukupnih CD3$^+$T ćelija koji bio je veći u malim lezijama ($p < 0,05$). Nisu nađene korelacije između nivoa ispitivanih citokina i ćelijskog sastava/fenotipa PL-ICs. **Zaključak.** Ova studija ukazuje na to da IL-10, IL-27 i TGF-β najverovatnije imaju različitu ulogu u suzbijanju neželjenih imunskih/inflamacijskih reakcija u asimptomatskim PLs, zavisno od ekstezivnosti patološkog procesa, procenjivanog na osnovu veličine lezije.

Ključne reči:
periapeksne bolesti; citokini; mikroorganizmi; imunski faktori; zapaljenje.

Introduction

Periapical lesions (PLs), common chronical pathological processes of the oral cavity, are triggered by bacterial infection of pulpal and endodontic environment [1]. Their pathogenesis involves a complex host immune/inflammatory response to the bacteria and their products in order to eliminate the invading microorganisms. However, the same mechanisms may also result in the tissue injury, followed by the destruction of soft and mineralized tissues surrounding the root apex [2]. The breakdown of tissues is triggered by different host mediators, which independently or cooperatively stimulate proteolysis and bone resorption processes [1–5]. Simultaneously, the regulatory mechanisms are triggered with the aim to suppress the inflammation and tissue destruction and to restrict the PL development [3, 4].

PLs consist of granulation tissue, proliferating epithelium or cyst infiltrated by different inflammatory cells (ICs) [5]. The compostion of ICs as well as functional and phenotypic properties of both infiltrating and stromal cells depend on the activation status of PLs which is under control of a series of cytokines. Cytokines play a major role in the modulation of immune/inflammatory reactions within PLs, and are critical determinants of lesions outcome [2, 6]. In this context, it is believed that the T-helper 1 (Th1) immune response, mediated by interferon-γ (IFN-γ) is involved in the progression of PLs and bone destruction, whereas T-helper 2 (Th2) cytokines, such as interleukin 4 (IL-4), IL-5, IL-10 and IL-33, are described to be important for the humoral immune response and to limit or attenuate the tissue damage [2]. IL-17 may play a role in exacerbating inflammation[7]and osteolytic processes [8] within PLs. On the other hand, the Foxp3$^+$CD4$^+$ CD25$^+$ subset of T regulatory cells (Tregs) and Tr1 cells exert suppressive effects on inflammatory osteolysis, in which cytokines Transforming growth factor beta (TGF-β) and IL-10 seem to play a crucial role [9–11]. The functions of Th17 and Tregs cytokines are interconnected and the Th17/Tregs archetype was suggested to influence the PLs outcome [12, 13] through the balance between the production of the osteoclastogenic factor named a receptor activator of nuclear factor kappa-B ligand (RANKL) and its antagonist osteoprotegerin (OPG) [6, 14]. Our previous results showed that IL-27 may have both pro-inflammatory and immunoregulatory functions in PLs [15].

Pro-inflammatory cytokines prevail in symptomatic PLs, whereas the production of immunoregulatory cytokines characterizes predominantly asymptomatic PLs [3, 6, 16]. Since restriction of inflammation within PLs may occur at different stages of their development, the aim of this study was to examine whether the local production of three immunoregulatory cytokines (IL-10, IL-27 and TGF-β) in human asymptomatic PLs depends on the lesion size and how the levels of these cytokines correlate with the cellular composition/phenotype of such PLs. Similar data have not been published yet.

Methods

Patients

The study was conducted on human PLs (n = 30) at the Military Medical Academy (MMA), Belgrade, Serbia after an approval from the Ethics Committee of MMA and obtaining an informed consent from the patients.The average age of the patients was 31 years (range: 19–59 years). The patients had no malignant or autoimmune diseases and did not use immunosuppressive /immunomodulatory drugs. In addition, the patients had not been treated with antibiotics one month before the PLs excision. The clinical part of the study was performed at the Department for Oral Surgery, Clinic for Stomatology, MMA, at the time of teeth extraction or apicoectomy. The immunological part of the study was performed at the Institute for Medical Research, MMA. PLs were radiographically diagnosed using the standard equipment for intraoral (Carestream CS 2200 rentgenaparature) and extra-oral (ortopantomography and Dental Cone Beam Computed Tomography – CBCT) radiography of the maxillofacial region. Intraoral radiographs were obtained using the Digora imaging system (Soredex Corporation, Helsinki, Finland), size 2 photostimulable storage phosphor (PSP) plate sensors (40.0 mm × 30.0 mm) and the Scanora software. Extraoral tomographs were made by the Vatech aparature in the

OrtoDent digital diagnostic center, Belgrade, following the rules for patients positioning according to the manufacturer's instructions. The gained ortopantomography shots were calibrated by the Vatech program software for panoramic pictures (Ez dent-i), while the 3D CBCT analyses were done in the Ez 3D plus and Ez 3D-i Vatech program software. The size of radiolucent PLs on the radiographs and tomographs was analyzed by the above mentioned softwares and the smallest and largest diameters were measured. All PLs were clinically asymptomatic.

The lesions were divided according to their size into the small and large PLs. The small lesions (n = 12) were those whose greatest diameter was less than 3.5 mm. The lesions whose smallest diameter was higher than 4.5 mm were classified as the large lesions (n = 18). No further distinctions between specimens were made regarding sex, age, and etiology or tooth type. The examples of radiographs/tomographs of one small- and one large-size PLs are given on Figures 1 and 2. After extraction, PLs were immediately placed in a medium consisting of RPMI-1640 (Sigma, Munich, Germany) and antibiotics/antimycotics, and transported to the laboratory.

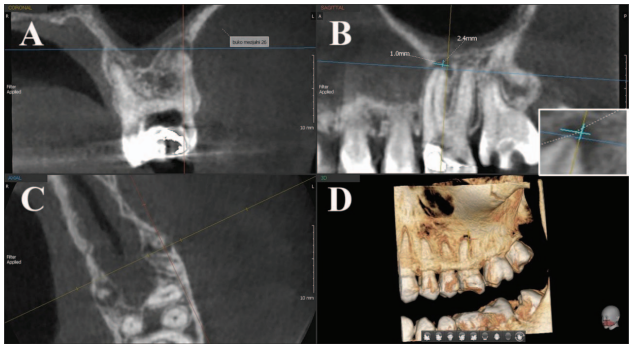

Fig. 1 – The images of one representative small-size periapical lesion (teeth 26): A) Sagittal cross-section (bucomesial and palatinal root); B) Coronary cross-section (bucomesial root); C) Axial cross-section (rout apex); D) 3D reconstruction – Dental Cone Beam Computed Tomography – CBCT. The insert represents a higher magnification of the measured lesion size.

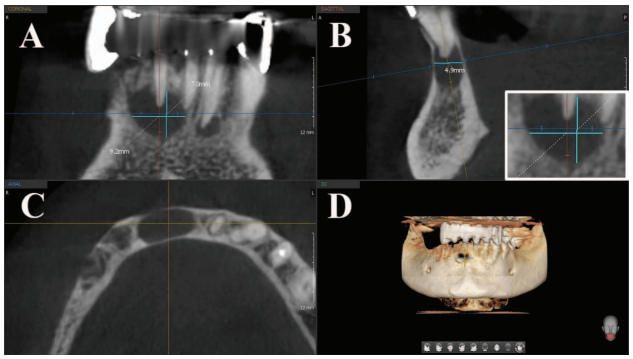

Fig. 2 – The images of one representative large-size periapical lesion (teeth 41): A) Coronary cross-section of the root; B) Sagittal cross-section of the root; C) Axial cross-section (largest bucomesial size); D) 3D reconstruction (Dental Cone Beam Computed Tomography – CBCT). The insert represents a higher magnification of the measured lesion size.

Preparation of inflammatory cells

The inflammatory cells from PLs (PL-ICs) were isolated using a procedure previously optimized by our research group [5, 17]. Briefly, periapical tissue was placed in a Petri dish containing 1 mL RPMI-1640 medium and cut into 2–3 mm diameter pieces using a scalpel. 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 in a cell incubator at 37ºC. After that, the tissue was pressed through a stainless-steel mesh using a syringe plunger, filtered and resuspended in RPMI-1640 medium containing 1 mm EDTA. The released cells were washed twice by centrifugation in the RPMI medium containing 0.5 Mm EDTA at room temperature (400 g for 10 min), and counted. The viability of cells, determined by Trypan Blue dye, was between 90% and 95%. After that, cytospins were prepared from each sample of PL-ICs using a cytocentrifuge (MPW-350, Poland) on the poly-l-lysine-coated glass slides.

The cytospins were stained with May-Grünwald-Giemsa or used for immunocytochemistry. The cellular composition was analyzed by using a light microscope (Olympus). The identification of cells was done by the morphological criteria. A minimum of 500 cells was analyzed on each cytospin. The results are given as percentages of cell subsets.

Immunocytochemistry

For immunostaining, anti-CD3, -CD4, -CD8, -CD14, -CD19, - CD38 and -HLA DR unconjugated monoclonal antibodies (mAbs) were obtained from Serotec, Oxford, UK. Rabbit anti-mouse unconjugated- and peroxidase conjugated-Ig, as well asan alkaline phosphatase anti-alkaline phosphatase (APAAP) complex were purchased from DAKO, Copenhagen, Denmark. Cytospins were fixed with 2% pararosaniline (Sigma) for 2 min at room temperature, washed with phosphate-buffered saline (PBS) for 10 min, blocked with rabbit serum for 20 min and washed with PBS. Cytospins were incubated with the primary antibodies for 60 min at room temperature followed by washing with PBS. When the staining was further processed for the immunoperoxidase method, the slides were blocked with 0.3%H_2O_2 in PBS for 20 min and then incubated with appropriate dilution of anti-mouse peroxidase conjugated Ig. The immunoperoxidase reaction was developed with diaminobenzidine. When the immunostaining was performed with the immunoalkaline phosphatase method, the slides were incubated with anti-mouse unconjugated Ig and then with APAAP. The reaction was finished by using Fast Red, as a substrate. Both enzyme substrates were obtained from Sigma. The controls were the samples incubated with an irrelevant mAb, mouse anti-rat CD4 (OX-38), Serotec, non-reactive with human cells. To identify B cells and plasma cells, cytospins were stained with the combination of anti-CD19 mAband anti-CD38 mAb. T cells were identified based on the positivity with anti-CD 3 mAb and subsets of T cells based on the staining with anti-CD4 and anti-CD8 mAbs, respectively. CD14 was a marker of mononuclear phagocytes, whereas HLA-DR was a marker

of antigen-presenting cells. Cytospins were analyzed by light microscopy. On each cytospin at least 500 cells were counted. The percentages of positive cells were determined on the basis of total counted cells.

Cell cultures

PL-ICs were cultivated in 96-wells, with the round-bottomed plates (ICN, Costa Mesa, CA) (1×10^5 cells/well, 200 μL) in the complete culture medium consisted of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Sigma) and the standard culture solutions of antibiotics [17]. Phorbol myristate acetate (PMA) (20 ng/mL) (Sigma) and Ca^{2+} ionophore (A 23187, 1 M) (Sigma) were used for the cell stimulation [18]. After 24 h, the cell supernatants were collected, centrifuged and frozen at -70ºC until the levels of cytokines were determined.

Cytokine assays

The concentrations of IL-10, IL-27 and TGF-β in culture supernatants were detected by using the specific ELISA kits (R&D, Minneapolis, USA) following the instructions of the manufacturer. The levels of cytokines were determined on the basis of standard curve, constructed by known concentrations of these cytokines. The results are presented as pg/mL.

Statistical analysis

Statistical analysis was performed by using the Student *t*-test and Spearman's correlation test. The values of $p < 0.05$ were considered to be significant.

Results

The first aim of this study was to examine the composition of PL-ICs isolated from clinically asymptomatic lesions divided according to their size. As presented in Table 1, the total number of PL-ICs from the small-size lesions was lower compared to the number of PL-ICs isolated from the large size lesions ($p < 0.01$). However, their main composition was not significantly different, except that the large-size lesions contained a higher proportion of mononuclear phagocytic cells ($p < 0.05$).

The results related to the production of three immunoregulatory cytokines in the cultures of PL-ICs were presented in Figure 3. The levels of IL-10 and TGF-β were significantly higher in the PL-ICs cultures of larger-size lesions ($p < 0.01$ and $p < 0.05$, respectively). In contrast, the level of IL-27 was higher in the cultures of small-size lesions ($p < 0.05$).

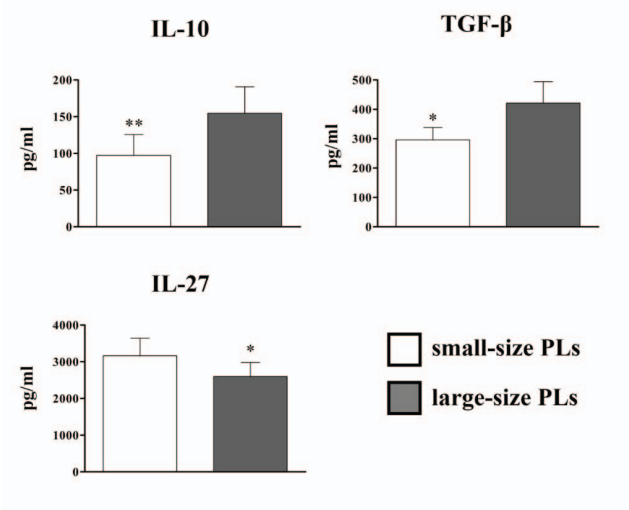

Fig. 3 – The levels of interleukin (IL)-10, IL-27 and transforming growth factor (TGF)-β in culture supernatants of asymptomatic periapical lesions (PL)-inflammatory cells (PL-ICs). Values are given as mean ± standard deviation for n = 12 (PL-ICs from the small-size lesions) or n = 18 (PL-ICs from the large-size lesions).
*** = $p < 0.05$; ** = $p < 0.01$, compared to the values of small-size (PLs).**

In order to explain whether these differences are associated with the cell subset composition in PLs, phenotypical analysis of PL-ICs was performed on the cytospin preparations by using two immunocytochemistry methods. Table 2 and Figures 4 and 5 show that the proportion of B cells/plasma cells ($CD19/CD38^+$ cells), the subset of T cells ($CD8^+$) and $CD14^+$ cells were higher in the large-size lesions ($p < 0.005$; $p < 0.05$; $p < 0.05$, respectively). In contrast, the proportion of total T cells ($CD3^+$ cells) was higher in the small-size lesions ($p < 0.05$). No significant differences were observed in the proportion of $CD4^+$ T cells and $HLA\text{-}DR^+$ cells.

When the levels of cytokines in each group of lesions were correlated with the cellular composition of these lesions, no statistically significant correlation was found (data not shown).

Table 1

Total cellularity and cellular composition of periapical lesions (PLs)

Cell type	Small-size PLs (n = 12)	Large-size PLs (n = 18)
	mean ± SD	mean ± SD
Total number of cells ($\times 10^6$)	1.1 ± 0.8	2.3 ± 1.0**
Lymphocytes (%)	50.2 ± 7.2	48.0 ± 10.2
Plasma cells (%)	14.8 ± 6.6	17.1 ± 4.9
N.granulocytes (%)	16.4 ± 6.9	12.1 ± 7.5
Macrophages (%)	12.2 ± 3.7	17.9 ± 6.8*
Mast cells (%)	4.1 ± 1.6	2.9 ± 2.2
Other cells (%)	2.3 ± 1.9	2.0 ± 1.8

SD – standard deviation; *$p < 0.05$; **$p < 0.01$, compared to the values of small-size PLs.

Table 2

Phenotypic characteristics of inflammatory cells isolated from periapical lesions (PLs)

Markers	Small-size PLs (n = 12)	Large-size PLs (n = 18)
	mean ± SD	mean ± SD
CD3	38.2 ± 6.9	32.4 ± 7.1*
CD4	19.2 ± 5.2	22.3 ± 6.5
CD8	6.8 ± 4.6	12.2 ± 5.8*
CD19/38	20.4 ± 6.9	30.8 ± 5.5***
CD14	13.8 ± 4.7	21.7 ± 8.6*
HLA-DR	15.1 ± 3.6	20.9 ± 8.8

SD – standard deviation; *$p < 0.05$; ***$p < 0.005$, compared to the values of small-size PLs.

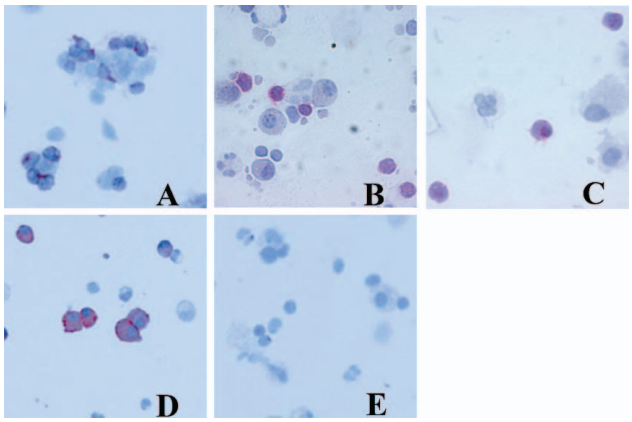

Fig. 4 – Representative images of cytospins stained with monoclonal antibodies (mAbs) to the lymphocyte cell subsets by an immunoalkaline phosphatase method: A) CD3; B) CD4; C) CD8; D) CD19/CD38; E) Negative control (Magnifications: × 600).

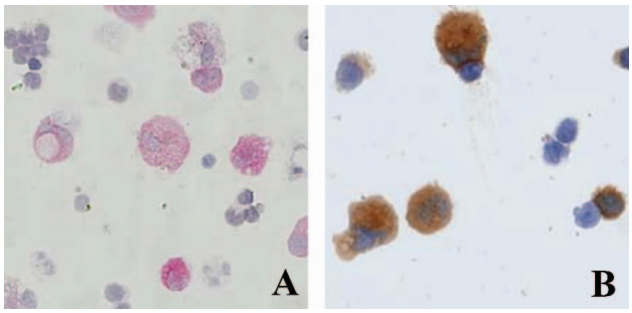

Fig. 5 – Representative images of cytospins stained with mAbs to CD14 (A) and HLA-DR (B).
A) immunoalkaline phosphatase method;
B) immunoperoxidase method [Magnifications: × 600].

Discussion

This is the first study investigating the production of immunoregulatory cytokines (IL-10, IL-27 and TGF-β) in clinically asymptomatic PLs, which were analyzed according to the size of PLs. IL-10 and TGF-β were implicated in the suppressive mechanisms mediated by different PL cells, including $CD4^+CD25^+Foxp3^+$ Tregs [19]. In contrast, IL-27 was identified as both pro-inflammatory and immunosuppressive cytokine in PLs [15]. We decided to study the clinically asymptomatic lesions based on a large number of data showing that immunosupressive mechanisms are more operative in this type of lesions [3, 4, 6, 20]. In addition, the large-size lesions are usulally an advanced stage of the PL development, characterized as large granulomas or cysts. In contrast, the small-size lesions usually represent an early stage of PL induction [6, 20]. The division into these two groups of PLs was arbitrary and based on the size of our PL collection. To avoid possible errors in the PL measurement [21] we excluded from the study those PLs whose greatest diameter was higher than 3.5 mm and PLs whose smallest diameter was less than 4.5 mm.

Both types of PLs had similar cellular content, composed predominantly of mononuclear infiltrating cells, which is a typical hallmark of asymptomatic lesions, whereas the proportion of granulocytes which are characteristic for symptomatic lesions (acute phase of PL development or PL exacerbation) was lower [3, 17, 20]. Our results showed that ICs from the large-size PLs produced higher levels of IL-10 and TGF-β than ICs from the small-size PLs. This finding correlates with a higher proportion of mononuclear phagocytic cells, which are an important source of these immunosuppressive cytokines [22]. Except macrophages, IL-10 is produced by dendritic cells, different subsets of T and B cells and various innate immune cells [23]. TGF-β is additionaly secreted by different stromal cells [24] and both cytokines are produced by Tregs [25]. In our previous paper [19], we showed positive correlations between the frequency of Tregs and the levels of IL-10 and TGF-β in culture supernatants of mononuclear cells (MNC) isolated from PLs. It is interesting that all Tregs, isolated from PLs, expressed IL-10, but only a half of them were TGF-β-positive [19]. Based on all these data, it can be supposed that the proportion of Tregs in the large-size PLs is higher than that in the small-size lesions, but this hypothesis, which has not been tested so far, needs to be proved in further experiments.

IL-10 and TGF-β have the potent anti-inflammatory properties that plays a central role in limiting the host immune response to pathogens, thereby preventing the tissue damage, including osteolysis in PLs [3, 7, 20]. Dysregulation of IL-10 is associated with the enhanced immunopathological processes in response to infection and increased risk for the development of autoimmune diseases [23]. It is interesting that the systemic administration of IL-10 for autoimmune therapy was shown to be paradoxically proinflammatory, whereas the localized IL-10 delivery proved to be therapeutic [26]. IL-10

mediates its immunosuppressive activity by the heterodimeric IL-10 receptors (IL-10R1, IL-10R2), which is expressed at varying degrees in many cell types, especially in monocytes and macrophages. The ligation of receptor is followed by the activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, and subsequent transcription of various immunomodulatory genes, which, in turn, inhibit the production of pro-inflammatory mediators, stimulate the release of anti-inflammatory molecules such as interleukin-1 receptor antagonist (IL-1RA), soluble tumor necrosis factor-α (TNF-α) receptor, and interleukin IL-27 and down-regulate antigen presentation and phagocytosis [23, 26]. Additionally, through the release or via physical interactions with T cells, IL-10 can directly or indirectly enhance the Treg function [26], but it is also able to activate a certain type of immune cells, including the B cells, and to stimulate their proliferation. Such finding and association of IL-10 with the Th2 immune response are in agreement with a higher percentage of $CD19^+/CD38^+$ B cells/plasma cells and with previous findings that the advanced stage of PL development is associated with the predominance of humoral immune response [3, 20, 23].

TGF-β participates in several important pathophysiological processes and plays a bidirectional activity in the immune regulation. Its dual role in modulating the macrophage function is rapidly gaining recognition. TGF-β functions as a macrophage suppressing agent and as a monocyte activator [24]. It also exerts the inhibitory and stimulatory effects on the bone marrow cells, stimulates the chemotactic activity of osteoblasts and supports the differentiation of mesenchymal stem cells into osteoblasts and chondroblasts [24, 27]. In our previous paper, we showed that the production of TGF-β by PL-ICs is upregulated by the mesenchymal stem cells isolated from PLs [28]. TGF-β also stimulates the production of extracellular matrix and collagen type I, the molecules which are of key importance for the promotion of tissue healing [29]. It is known that healing is a characteristic of advanced stage of the PL development [20], a finding which is in correlation with our present results.

IL-27 is a relatively new cytokine member of the IL-6/IL-12 family, exerting both pro-inflammatory and anti-inflammatory properties. It consists of the Epstein-Barr virus-induced gene 3 (EBI3) and p28 subunits and acts through the IL-27 receptor complex formed by WSX-1 and gp130 subunits. DCs and activated macrophages are its main source [30]. In the PLs endothelial cells [15] as well as the mast cells [31] are also $IL\text{-}27^+$. It is well documented that IL-27 plays an important role in the initial immune response by stimulating the production of Th1 cytokines, which are important for the development of granulomatous diseases (tuberculosis, sarcoidosis and Crohn's disease) [32]. However, IL-27 has general inhibitory effects on the activity of Th1, Th2, Th17 and regulatory T cells [30]. The inhibitory effect of IL-27 on osteoclasts [33] is in accordance with this hypothesis, suggesting the role of this cytokine in the restriction of unwanted immune responses and bone destruction in PLs. In our previous experiment, we showed that the production of IL-27 by PL-MNC, especially in symptomatic PLs, was a significantly higher compared to its level in the cultures of peripheral blood MNC and correlated with the frequency of $CD14^+$ and $CD3^+$ cells. Exogenous IL-27 stimulated Th1 and downregulated the Th17 cytokine production by PL-MNC from symptomatic PLs, but the downregulated Th1 and Th2 responses in asymptomatic PLs suggested its complex biological functions in PLs [15].

We showed in this study for the first time that the small-size asymptomatic PLs produce the higher levels of IL-27 than the large-size PLs. These results suggest that the immunoregulatory mechanisms are differently regulated during the progression of PL development, having in mind the opposite production of IL-10 and TGF-β. Our results also suggest that, during initiation of the PL development, IL-27 may have a more important down-modulatory role in suppressing the production of Th1 and Th2 cytokines, than the other two cytokines may have. However, under the certain conditions, IL-27 can stimulate the Th1 response, which is mediated by the activated $CD4^+$ T-cells, recently migrated into the periapical tissue. This finding was already observed in our previous paper, in which we showed that exogenous IL-27 stimulated the production of IFN-gama by PL-MNC, especially during the exacerbation phase of the PL development [15].

Conclusion

The results of this study, performed on clinically asymptomatic PLs, showed that the small-size lesions differ from the large-size lesions in terms of IL-10, IL-27 and TGF-β production. ICs from the small-size PLs produce significantly much higher quantity of IL-27 than the large-size PLs. It was the opposite with IL-10 and TGF-β. Cumulatively, it can be hypothesized that these immunoregulatory cytokines may play different roles in the suppression of unwanted immune/inflammatory responses in asymptomatic PLs, depending on the extension of the pathological process.

Acknowledgement

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Declaration of interest

Authors declare no conflict of interest.

REFERENCES

1. *Warnsinck J, Shemesh H.* The dynamics of the periapical lesion. Endod Pract Today 2017; 11(3): 167–71.
2. *Graves DT, Oates T, Garlet GP.* Review of osteoimmunology and the host response in endodontic and periodontal lesions. J Oral Microbiol 2011; 3(1): 5304.
3. *Colić M, Gazivoda D, Vucević D, Vasilijić S, Rudolf R, Lukić A.*Proinflammatory and immunoregulatory mechanisms in periapical lesions. Mol Immunol 2009; 47(1): 101–13.
4. *Cavalla F, Araujo-Pires AC, Biguetti CC, Garlet GP.* Cytokine networks regulating inflammation and immune defense in the oral cavity. Curr Oral Health Rep 2014; 1(2): 104–13.
5. *Lukic A, Vasilijic S, Majstorovic I, Vucevic D, Mojsilovic S, Gazivoda D,* et al. Characterization of antigen-presenting cells in human apical periodontitis lesions by flow cytometry and immunocytochemistry. Int Endod J 2006; 39(8): 626–36.
6. *Garlet GP.* Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. J Dent Res 2010; 89(12): 1349–63.
7. *Čolić M, Vasilijić S, Gazivoda D, Vučević D, Marjanović M, Lukić A.* Interleukin-17 plays a role in exacerbation of inflammation within chronic periapical lesions. Eur J Oral Sci 2007; 115(4): 315–20.
8. *Takayanagi H.* New developments in osteoimmunology. Nat Rev Rheumatol 2012; 8(11): 684–9.
9. *Belkaid Y, Tarbell K.* Regulatory T cells in the control of host-microorganism interactions. Annu Rev Immunol 2009; 27(1): 551–89.
10. *Garlet GP, Cardoso CR, Mariano FS, Claudino M, de Assis GF, Campanelli AP,* et al. Regulatory T cells attenuate experimental periodontitis progression in mice. J Clin Periodontol 2009; 37(7): 591–600.
11. *Glowacki AJ, Yoshizawa S, Jhunjhunwala S, Vieira AE, Garlet GP, Sfeir C,* et al. Prevention of inflammation-mediated bone loss in murine and canine periodontal disease via recruitment of regulatory lymphocytes. Proc Natl Acad Sci USA 2013; 110(46): 18525–30.
12. *Fukada SY, Silva TA, Garlet GP, Rosa AL, da Silva JS, Cunha FQ.* Factors involved in the T helper type 1 and type 2 cell commitment and osteoclast regulation in inflammatory apical diseases. Oral Microbiol Immunol 2009; 24(1): 25–31.
13. *Marçal JR, Samuel RO, Fernandes D, de Araujo MS, Napimoga MH, Pereira SA*, et al. T-Helper Cell Type 17/Regulatory T-Cell Immunoregulatory Balance in Human Radicular Cysts and Periapical Granulomas. J Endod 2010; 36(6): 995–9.
14. *Menezes R, Garlet TP, Letra A, Bramante CM, Campanelli AP, Figueira RD*, et al. Differential patterns of receptor activator of nuclear factor kappa b ligand/osteoprotegerin expression in human periapical granulomas: possible association with progressive or stable nature of the lesions. J Endod 2008; 34(8): 932–8.
15. *Colić M, Gazivoda D, Majstorović I, Dragicević A, Vasilijić S, Rudolf R,* et al. Immunomodulatory Activity of IL-27 in Human Periapical Lesions. J Dent Res 2009; 88(12): 1142–7.
16. *Menezes R, Garlet TP, Trombone AP, Repeke CE, Letra A, Granjeiro JM,* et al. The potential role of suppressors of cytokine signaling in the attenuation of inflammatory reaction and alveolar bone loss associated with apical periodontitis. J Endod 2008; 34(12): 1480–4.
17. *Čolić M, Lukić A, Vučević D, Milosavljević P, Majstorović I, Marjanović M,* et al. Correlation between phenotypic characteristics of mononuclear cells isolated from human periapical lesions and their in vitro production of Th1 and Th2 cytokines. Arch Oral Biol 2006; 51(12): 1120–30.
18. *Collins DP.* Cytokine and cytokine receptor expression as a biological indicator of immune activation: important considerations in the development of in vitro model systems. J Immunol Methods 2000; 243(1–2): 125–45.
19. *Colić M, Gazivoda D, Vucević D, Majstorović I, Vasilijić S, Rudolf R,* et al. Regulatory T-cells in periapical lesions. J Dent Res 2009; 88(11): 997–1002.
20. *Márton IJ, Kiss C.* Overlapping protective and destructive regulatory pathways in apical periodontitis. J Endod 2014; 40(2): 155–63.
21. *Kruse C, Spin-Neto R, Wenzel A, Kirkevang L.* Cone beam computed tomography and periapical lesions: a systematic review analysing studies on diagnostic efficacy by a hierarchical model. Int Endod J 2014; 48(9): 815–28.
22. *Vannella KM, Wynn TA.* Mechanisms of organ injury and repair by macrophages. Annu Rev Physiol 2017; 79(1): 593–617.
23. *Iyer SS, Cheng G.* Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol 2012; 32(1): 23–63.
24. *Travis MA, Sheppard D.* TGF-β activation and function in immunity. Annu Rev Immunol 2014; 32(1): 51–82.
25. *Tanaka A, Sakaguchi S.* Regulatory T cells in cancer immunotherapy. Cell Res 2017; 27(1): 109–18.
26. *Rojas JM, Avia M, Martín V, Sevilla N.* IL-10: a multifunctional cytokine in viral infections. J Immunol Res 2017; 2017: 6104054.
27. *Yoshimura A, Wakabayashi Y, Mori T.* Cellular and molecular basis for the regulation of inflammation by TGF-. J Biochem 2010; 147(6): 781–92.
28. *Marković M, Tomić S, Djokić J, Čolić M.* Mesenchymal stem cells from periapical lesions upregulate the production of immunoregulatory cytokines by inflammatory cells in culture. Acta Fac Med Naiss 2015; 32(3): 171–9.
29. *Pakyari M, Farrokhi A, Maharlooei MK, Ghahary A.* Critical role of transforming growth factor beta in different phases of wound healing. Adv Wound Care (New Rochelle) 2013; 2(5): 215–24.
30. *Yoshimura T, Takeda A, Hamano S, Miyazaki Y, Kinjyo I, Ishibashi T,* et al. Two-sided roles of il-27: induction of th1 differentiation on naive cd4+ t cells versus suppression of proinflammatory cytokine production including il-23-induced il-17 on activated cd4+ t cells partially through stat3-dependent mechanism. J Immunol 2006; 177(8): 5377–85.
31. *Li J, Huang SG.* Immunomodulatory activity of interleukin-27 in human chronic periapical diseases. Am J Transpl Res 2017; 9(3): 1460–70.
32. *Kastelein RA, Hunter CA, Cua DJ.* Discovery and biology of il-23 and il-27: related but functionally distinct regulators of inflammation. Annu Rev Immunol 2007; 25(1): 221–42.
33. *Kamiya S, Nakamura C, Fukawa T, Ono K, Ohwaki T, Yoshimoto T,* et al. Effects of IL-23 and IL-27 on osteoblasts and osteoclasts: inhibitory effects on osteoclast differentiation. J Bone Miner Metab 2007; 25(5): 277–85.

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