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*Original article*

# Mesenchymal Stem Cells from Periapical Lesions Upregulate the Production of Immunoregulatory Cytokines by Inflammatory Cells in Culture

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## SUMMARY

**The pathophysiology of periapical lesions (PLs) is under control of pro-inflammatory and anti-inflammatory (mainly immunoregulatory) cytokines. We have recently established mesenchymal stem cells (MSCs) from PLs and showed their suppressive effects on the production of pro-inflammatory cytokines from PLs inflammatory cells (ICs). In this work we studied the production of interleukin (IL)-10, IL-27 and transforming growth factor (TGF)-β, by PL-ICs in direct or indirect contacts with PL-MSCs. PL-ICs, which were isolated from four different asymptomatic PLs, predominantly composed of lymphocytes, followed by neutrophil granulocytes, macrophages and plasma cells. PL-MSCs, expressing typical MSC markers, were co-cultivated with PL-ICs at 1:10 ratio, either in direct contact or in a transwell-system, for 24 hours. The levels of cytokines in cell-culture supernatants were tested by ELISA. The results showed that PL-MSCs up-regulated the production of all three immunoregulatory cytokines by PL-ICs. PL-MSCs stimulated the production of IL-10 and IL-27 via soluble factors, whereas the up-regulation of TGF-β required direct cell-to-cell contacts. In conclusion, our results showed for the first time the involvement of PL-MSCs in restriction of inflammation in PLs by up-regulation of immunoregulatory cytokines.**

*Key words:* **periapical lesions, mesenchymal stem cells, inflammatory cells, immunoregulatory cytokines, culture**

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## INTRODUCTION

Demineralization of solid dental tissue (enamel, dentin and dental root cement) and exposure of dental pulp to microorganisms and their products has as a consequence of dental caries and triggering the local inflammatory response. As a result of descending bacterial invasion and inflammation, necrosis of dental pulp arises followed by spreading the inflammatory process to periapical dental tissues and generation of periapical lesions (PLs) (1). Chronic PLs stand for granulomatous inflammation, where the process of exacerbation (symptomatic PLs) and their restriction (asymptomatic PLs) are present (2). It has been shown that the symptomatic PLs are characterized by the up-regulation of adhesive molecules, chemotactic factors (3), T helper (Th)1 and Th17 cytokines as well as bone resorptive cytokines such as interleukin (IL)-1, IL-6, and tumour necrosis factor (TNF)-$\alpha$ (2, 4). On the other hand, the generation of asymptomatic PLs is followed by the Th2 immune response, which promotes PL chronicity with restriction of inflammation (5). The predominant cytokines in chronic PLs are IL-10 and transforming growth factor (TGF)-$\beta$, which are involved in healing and regression of the inflammation within PLs (2). IL-27 seems to be involved in both pro- and anti-inflammatory effects in PLs (6), but the evidence about factors contributing to the secretion of anti-inflammatory mediators in PLs is largely unknown (2).

Considering the great capacity for tissue regeneration, and their immunosuppressive properties, mesenchymal stem cells (MSCs) could serve as an excellent tool for the therapy of chronic inflammation (7). MSCs are characterised by a high self-renewal capacity and the multi-lineage differentiation potential (8). Up to now, several types of MSCs have been isolated from dental tissue such as dental pulp, exfoliated deciduous teeth, periodontal ligament, apical papilla, dental follicle and gingiva (9). The inflamed dental tissue could also be a source of MSCs, but only a few papers have described the presence of MSCs at the site of an inflamed periodontal ligament or dental papilla (10, 11). We have recently isolated MSCs from PLs and found their significant anti-proliferative and immunomodulatory effects on lymphocytes in culture, and the ability to suppress the production of proinflammatory cytokines by inflammatory cells (ICs) isolated from PLs (PL-ICs) (12). In this work we studied the effect of PL-MSCs on the production of immunoregulatory cytokines by PL-ICs in vitro, using either direct or indirect co-culture assays.

## MATERIAL AND METHODS

### Isolation and cultivation of cells

PLs (total n = 5), used for the isolation of PL-MSCs (n = 1) and PL-inflammatory cells (PL-ICs) (n = 4), were collected from patients who signed Consent Forms at the Clinic for Maxillofacial and Oral Surgery, Military Medical Academy (MMA), Belgrade. The donors (ranging from 22 to 54 yrs) were without systemic diseases and had radiographic evidence of PLs. PLs were extracted at the time of teeth extraction. PLs were excised by the curettage of firmly attached periodontal tissue from dental radices with a scalpel. All PLs were asymptomatic. The study has been approved by the Ethics Committee of the MMA.

The detailed protocol for the isolation of MSCs has been described in our previous paper (12). Briefly, PLs tissues were digested in a Dulbecco's modified Eagle's medium (DMEM; Sigma, Munich, Germany) solution with a type I collagenase (1mg/mL; Sigma) and DNAase (25 mg/mL; Sigma) for 1 hr in an incubator. The cells were cultured in the standard medium composed of DMEM-low glucose (Sigma), 10% foetal calf serum (FCS; Sigma) and antibiotics [penicillin/ streptomycin/ gentamicin (Galenika, Belgrade, Serbia), 1% each]. Passaging was performed by using 0.2% trypsin (Sigma)/0.02% NaEDTA phosphate-buffered saline (PBS) solution. The fourth passage of one MSC line was used in the experiments.

PL-ICs were isolated from the PLs tissues by the collagenase/DNAase digestion (12). PL-ICs were then washed by centrifugation and counted. Cytospins were prepared from the cells by a cytocentrifuge (Shandon, CytoSpin 4, ThermoFisher SCIENTIFIC, Waltham, MA USA), stained by May Grünwald-Giemsa (MGG) and analyzed by a light microscope.

### Phenotypic characterization of PL-MSCs

The flow cytometry was used for characterization of a clone after the fourth passage, as previously described (12, 13), using the following monoclonal antibodies (mAbs) conjugated with fluorescein isothyocyanate (FITC): anti-CD14, anti-CD45, anti-CD29, anti-CD105, anti-CD44, anti-CD19 (Immunotools, Friesoythe, Germany), anti-CD146, anti-CD46, anti-CD166, anti-CD90, anti-CD106, mouse IgG1a negative control (Serotec). Anti-human

leukocyte antigen (HLA)-DR- phycoerithryne (PE), and mouse IgG1a negative control–PE were also from Serotec. The indirect labelling was performed using anti-STRO-1 (Millipore/Chemicon) mAb followed by secondary anti-mouse IgG1-FITC mAb. After staining, the cells were analyzed with a flow cytometer (Partec, CyFlow® Cube 6, Germany). The isotype control was carried out for each experiment.

## Co-culture assays

The effect of PL-MSCs on the production of cytokines by allogenic PL-ICs was evaluated in the co-culture and Transwell system experiments. In the co-culture experiments, PL-ICs (1 x $10^5$ cells/well) were cultivated with PL-MSCs (1 x $10^4$/well), using 96-well plates, in the presence of phorbol myristate acetate (PMA; 20 ng/mL; Sigma) and $Ca^{2+}$ ionophore (A23187, 1 μM; Sigma) for 24 hours. Separate cultures of PL-ICs and PL-MSCs treated identically served as controls. The levels of cytokines produced in the PL-MSCs/PL-ICs co-cultures were compared with the sum of cytokines produced from separate PL-ICs and PL-MSCs cultures. The experiments, each carried out in duplicate, were established with four different PL-ICs and one PL-MSC line.

In the Transwell system experiments, PL-MSCs (5 x $10^4$ cells/insert) were seeded onto the upper chamber of a transwell insert (0.4 μm pore size) (Falcon) in 24 well plates, whereas PL-ICs (5 x $10^5$ cells/ml) were in the lower chamber. Control PL-ICs were cultivated in 24-well plates without inserts. After 24 hours of co-culture, the inserts with PL-MSCs were removed. PL-ICs from all cultures were washed and stimulated with PMA/$Ca^{+2}$ ionophore for an additional 24 hours, followed by collection and analysis of the supernatants.

## Cytokines detection

Concentrations of IL-10, IL-27 and TGF-β1 (referred as TGF-β) from the culture supernatants were determined using the commercial ELISA kits (R&D Systems, DuoSet) by calculating the unknown concentrations of the cytokines from the standard curves.

## Statistical analysis

Kruskal-Wallis or Mann-Whitney tests were used to evaluate the differences between the experimental and corresponding control samples. Values at $p < 0.05$ or less were considered to be statistically significant.

# RESULTS

We have previously described in details morphological, phenotypical and differentiation characteristics of PL-MSCs isolated from three different donors (12). In this work we used one of the lines to study its immunoregulatory properties.

Flow cytometry data showed that almost all cells of the line expressed CD105, CD166, CD44, CD29 and CD90 (Table 1). About half of the cells expressed CD46 and CD146, whereas a relatively low percentage of the cells (9.3%) expressed STRO-1 molecule. The expression of HLA-DR, CD45, CD19 and CD14 were not detected (data not shown).

***Table 1. Phenotypic characteristics of PL-MSC***

| Markers | Expression (%) |
|---|---|
| CD90 | 99.9 |
| CD44 | 99.5 |
| CD29 | 98.8 |
| CD166 | 91.8 |
| CD105 | 98.3 |
| CD46 | 57.0 |
| CD146 | 50.4 |
| STRO-1 | 9.3 |

***Note:*** *PL-MSCs from passage 4 were harvested and prepared for the flow cytometry analysis, as described. The cells were gated according to the cell-specific forward scatter/side scatter parameters (12) and the percentage of positive cells for the indicated molecules was determined based on the isotype control. Results are given as % of positive cells.*

The effect of PL-MSCs on the production of immunoregulatory cytokines by PL-ICs was studied by using co-culture experiments. The analysis of cytospins, prepared from PL-ICs, showed the predominance of lymphocytes, followed by

neutrophil granulocytes, macrophages and plasma cells (Table 2). PL-ICs from individual donors and PL-MSCs were cultivated either alone or together for 24 hours in the presence of PMA/Ca2+ ionophore, an additional stimulus for the cytokine production.

***Table 2. Composition of inflammatory cells isolated from periapical lesion (PL-ICs)***

| Cell types | (%) ± SD |
|---|---|
| Neutrophil granulocytes | 29.4 ± 6.2 |
| Macrophages | 20.5 ± 4.7 |
| Lymphocytes | 38.1 ± 10.1 |
| Plasma cells | 10.2 ± 2.9 |
| Other cells | 1.8 ± 1.0 |

*Note: PL-ICs were isolated from four different asymptomatic PL, as described. Cytospins were prepared and analyzed by light microscopy. The results are given as mean % ± SD (n = 4)*

Figure 1 shows that PL-ICs produced detectable levels of IL-10, IL-27 and TGF-β, whereas the supernatants of PL-MSCs cultures contained low levels of IL-27, and two times higher quantity of TGF-β levels, compared to PL-ICs cultures. IL-10 was present in PL-MSCs cultures at the baseline level. The levels of IL-10 and TGF-β in PL-ICs/PL-MSCs co-cultures were significantly higher, compared to the sum of cytokines from individual PL-ICs and PL-MSCs cultures. However, no significant differences were found regarding the levels of IL-27.

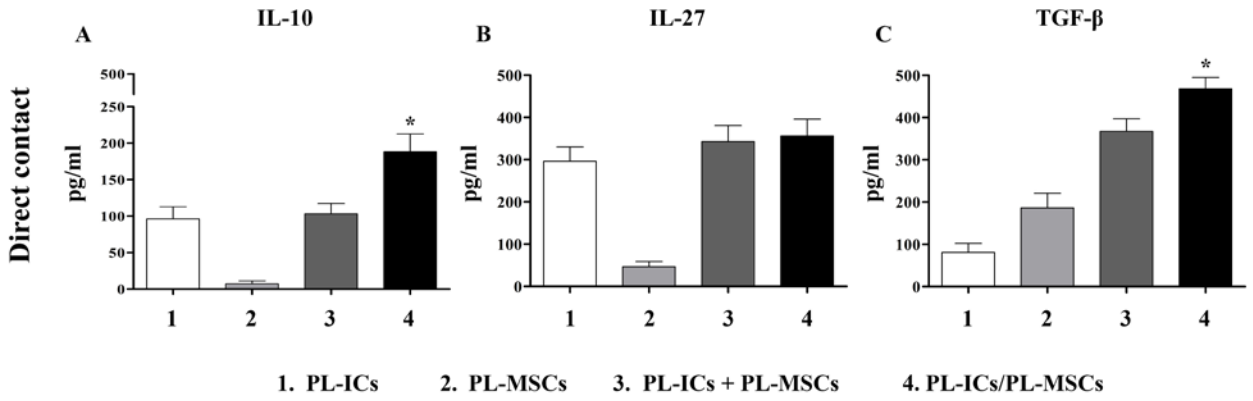


***Figure 1. The effect of PL-MSC on the production of IL-10, IL-27 and TGF-β by PL-ICs using direct co-culture assay. PL-MSCs were co-cultivated with 4 different allogenic PL-ICs for 24 hours in direct co-culture system using 1/10 PL-MSCs/PL-ICs cell ratio, as described. The production of cytokines was stimulated with PMA (20 ng/mL) and Ca2+ ionophore (1 μM). PL-ICs and PL-MSCs cultivated separately and treated the same way served as controls. The levels of cytokines produced in the PL-MSCs/PL-ICs co-cultures were compared with the sum of produced cytokines in the separate control cultures. Values are given as mean ± SD (n=4), * p<0.05 compared to the sum (PL-ICs + PL-MSCs) (Mann-Whitney's tests).***

To evaluate whether a direct cell-to-cell contact was necessary for the observed effect, we performed the Transwell system experiments (Fig. 2). The results showed that PL-ICs pre-cultivated with PL-MSCs without direct contact produced a significantly higher quantity of IL-10, but also IL-27, compared to the control PL-ICs. However, the production of TGF-β was not modulated significantly.

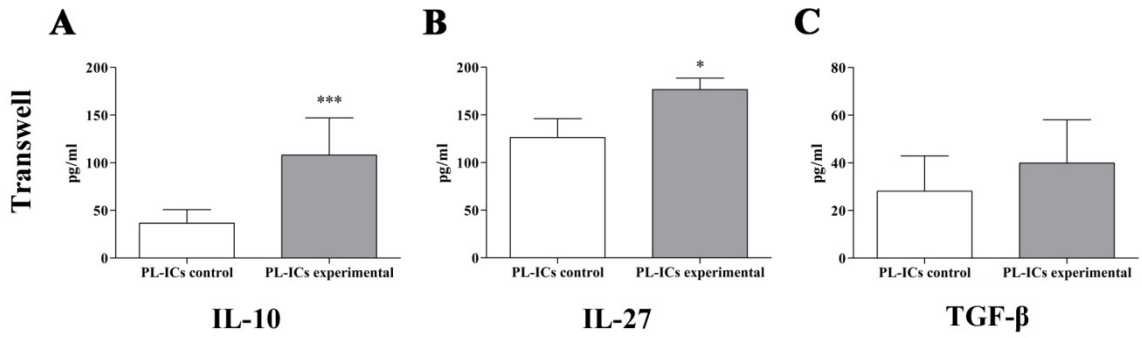


*Figure 2. The effect of PL-MSCs on the production of IL-10, IL-27 and TGF-β by PL-ICs using transwell system. Transwell system experiments were performed as described in Materials and Methods. After collection of supernatants and removal of PL-MSCs, PL-ICs (experimental and control cultures) were washed and treated with PMA/Ca2+ ionophore for an additional 24h to measure cytokines' production. PL-ICs cultivated in the same way, but without PL-MSCs, served as controls. The results are shown as mean ± SD (n=4), * p<0.05; *** p<0.001 compared to control PL-ICs culture (Mann-Whitney's tests).*

## DISCUSSION

In our previous study (12), we isolated PL-MSCs from three donors and showed that all these lines possessed typical MSCs properties, including a fast proliferation rate, clonogenic ability, multy-linage (osteogenic, chondrogenic and adipogenic) differentiation potential in vitro and, of substantial significance, the anti-proliferative and anti-inflammatory properties.

The phenotype of PL-MSCs, including the line used in this study, characterized by high experession of CD105, CD166, CD44, CD29 and CD90, is also in accordance with other findings on MSCs isolated from inflamed periodontal tissue (10). However, some phenotypical specificities, such as higher expression of CD44 by PL-MSC, compared with MSCs from other dental tissues, could be due to the inflammatory origin of these cells (14). We found that STRO-1, a marker for MSCs (15), showed a lower membrane expression. However, this marker including CD46, was localized predominately intracellularly, as demonstrated by immunochystochemistry (12).

Except for their substantial differentiation capacity and low immunogenicity, it was demonstrated that different MSCs, including our PL-MSCs lines, have profound anti-proliferative and anti-inflammatory effects (9, 12, 13). Since the production of proinflammatory cytokines is controlled by the immunoregulatory cytokines, the principal aim of this study was to check the involvement of PL-MSCs in these processes. We used a model of PL-MSCs co-culture with infiltrating PL cells. These cells, as shown in this study and our previous paper (16), are composed of lymphocytes and B-cell effectors (plasma cells) together with innate immune cells (neutrophil granulocytes and macrophages). Therefore, this model reflects better the function of PL-MSCs in vivo compared to the models using peripheral blood mononuclear cells.

We showed for the first time the ability of PL-MSCs to augment the secretion of IL-10, IL-27 and TGF-β immunoregulatory cytokines, but the mechanisms involved seem to be different. By using direct cell-to-cell co-culture assay and indirect approach (transwell system), which prevents direct cell-to-cell contacts, we demonstrated that the up-regulation of IL-10 and IL-27 are most probably mediated by soluble factors. However, the regulation of TGF-β secretion seems to require direct cellular contacts.

IL-10 is one of the key immunoregulatory cytokines with potent anti-inflammatory and immunosuppressive properties. It inhibits the activity of Th1, Th2 and Th17 cells, NK cells and the production of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, IL-12 and TNF-α, from macrophages, neutrophils and dendritic cells (DCs). The main source of IL-10 is T regulatory cells (Tregs), but other cells, such as Th-cell subsets (predominantly Th2), some CD8+ T-cells, macrophages, DCs and B-cells produce this cytokine as well. Although the knowledge of IL-10 regulation is limited, it is known that the regulatory mechanisms differ between different cell types. Up to now, p38 is one of the best

proven signalling pathway involved in the up-regulation of genes responsible for IL-10 transcription (17).

At the moment we do not know how PL-MSCs up-regulate IL-10 secretion by PL-ICs, since the complex pathways between different pro-inflammatory and anti-inflammatory cytokines exist in PL and other chronic inflammatory diseases (16). The best examined pathway includes the balance between Th17 cells and Tregs (18). Because PL-MSCs down-regulate IL-17 production by human T cells (Colic et al., unpublished results), it can be postulated that such a mechanism could be responsible for the counterbalanced action of Th17 cells and Tregs in our model. Of course, many other soluble factors, produced by both PL-MSCs and PL-ICs might be involved too, by a direct or indirect manner.

TGF-β, together with IL-10, plays a substantial role in the regulation of inflammation and immune responses. This cytokine is produced by different subsets of leukocytes, macrophages, fibroblasts, osteoclasts and, most importantly by Tregs. Although TGF-β stimulates initially the inflammatory responses by attracting lymphocytes and monocytes to the inflammatory site, it suppresses the proliferation of these cells as well as the production of different pro-inflammatory cytokines (19). TGF-β is also expressed in PLs (16, 20), irrespective of whether PLs are asymptomatic or symptomatic. It can be postulated that this cytokine is important for down-regulation of inflammatory and immune responses within PLs and for the repair of periapical bone loss. PL-MSCs, like other dental MSCs, also produce TGF-β (12, 13). In our co-culture model, the number of PL-MSCs was significantly lower than the number of PL-ICs (1:10, respectively), therefore the contribution of TGF-β produced by PL-MSCs in the co-culture, could be considered as less important. We found that PL-MSCs up-regulated the secretion of TGF-β from PL-ICs and that direct cell-to-cell contacts are needed for such a mechanism.

This finding is opposite compared to the regulation of IL-10 and IL-27 by the influence of secretory products. Although we did not investigate the molecular pathways involved in these processes, it can be postulated that interplay between PL-MSCs, antigen-presenting cells (APC) and Tregs in the co-cultures is of particular importance. This hypothesis is based on the knowledge that for the development of Tregs, which are significant producers of TGF-β, a direct contacts between T cells, APC and probably other cells, is necessary (17).

IL-27 is a new member of the IL-12 family, consisting of the Epstein-Barr virus-induced gene 3 and p28 subunits. Like two other members of the IL-12 family (IL-12 and IL-23), IL-27 is mainly produced by the activated macrophages and DCs (21). Our results demonstrated additional expression of IL-27 in endothelial cells within PLs and significant levels of this cytokine was detected in culture supernatants of PL-ICs (6). IL-27 exerts both pro- and anti-inflammatory properties (21). We showed its stimulatory activity on Th1 and down-modulatory activity on Th17 cytokine production by PL-ICs from symptomatic PLs. However, IL-27 down-regulate Th1 and Th2 responses in asymptomatic PLs (6). Therefore, it can be postulated that augmented production of IL-27 by PL-ICs in the co-culture with PL-MSCs might be immunomodulatory, since all PL-ICs in this study were isolated from the asymptomatic PLs. Such an effect was observed using an indirect cell-culture model where soluble factors from PL-MSCs are involved. It is not known why direct contact between PLMSCs and PL-ICs inhibited the effect of soluble mediators and this phenomenon deserves further investigations.

## CONCLUSION

Our results support the hypothesis that PL-MSCs, by up-regulating the secretion of IL-10, IL-27 and TGF-β, are another cell type in PLs responsible for controlling the excessive inflammatory and immune responses and for promoting healing and bone reparation. These characteristics could make PL-MSCs useful in the treatment of periodontal diseases or other chronic inflammatory processes followed by tissue destruction.

## Acknowlegments

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# Mezenhimske matične ćelije iz periapeksnih lezija stimulišu produkciju imunoregulacijskih citokina od strane inflamacijskih ćelija u kulturi

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## SAŽETAK

**Patofiziologija periapikalnih lezija (PLs) je pod kontrolom proinflamacijskih i antiinflamacijskih (uglavnom imunoregulacijskih) citokina. U našem ranijem radu uspostavili smo ćelijsku liniju mezenhimalnih matičnih ćelija (MSCs) iz PLs i pokazali njihov supresivni efekat na produkciju proinflamacijskih citokina od strane inflamacijskih ćelija iz periapekalnih lezija (PL-ICs). U ovom radu smo proučavali produkciju IL-10, IL-27 i transformišućeg faktora rasta (TGF)-β, od strane PL-ICs u direktnom ili indirektnom kontaktu sa PL-MSCs. Izolovane PL-ICs iz četiri različite asimptomatske PLs pretežno čine limfociti, zatim granulociti, makrofagi i plazma ćelije. PL-MSCs, za koje je pokazano da eksprimiraju tipične MSCs markere, kultivisane su sa PL-ICs u odnosu 1:10 u direktnoj ko-kulturi ili korišćenjem umetaka koji onemogućavaju direktan međućelijski kontakt u toku 24 časa. Nivo citokina u supernatantima ćelijskih kultura određivan je pomoću ELISA metode. Rezultati pokazuju da PL-MSCs povećavaju produkciju sva tri imunoregulatorna citokina od strane PL-ICs. PL-MSCs stimulišu produkciju IL-10 i IL-27 preko solubilnih faktora, dok je za povećanje TGF-β neophodan direktan ćelijski kontakt. U zaključku, naši rezultati pokazuju po prvi put da PL-MSCs imaju ulogu u ograničavanju inflamacije PLs povećanjem produkcije imunoregulacijskih citokina.**

*Ključne reči:* **periapikalne lezije, mezenhimalne matične ćelije, ćelije zapaljenja, imunoregulatorni citokini, ćelijska kultura**