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Effects of combustible cigarettes and electronic nicotine delivery systems on the regenerative properties of mesenchymal stem cells derived from periodontal ligament (PDL-MSCs)

Nikolina Kastratovic[1], Olivera Milosevic-Djordjevic[1,2], Carl Randall Harrell[3], Valentin Djonov[4] and Vladislav Volarevic[1,5]

[1]Center for Harm Reduction of Biological and Chemical Hazards, Department of Genetics, Faculty of Medical Sciences, [2]Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia, [3]Regenerative Processing Plant, LLC Palm Harbor, Florida, United States of America, [4]Institute of Anatomy, University of Bern, Bern, Switzerland and [5]Department of Microbiology and Immunology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

Summary. Introduction. Periodontal ligament-derived mesenchymal stem cells (PDL-MSCs) are promising cells with crucial roles in maintaining and repairing periodontal tissue. However, their regenerative capacity can be influenced by various factors, including cigarette smoke and electronic nicotine delivery system (ENDS) aerosols. Smoking and vaping can impair their regenerative potential, and even though ENDS are perceived as safer tobacco products, there is a lack of evidence to guarantee this assumption.

Material and methods. Changes in the viability and proliferation of PDL-MSCs will be investigated after smoke and aerosol generation and cell exposure. In addition, the effects of smoke and aerosols on the immunomodulatory capacity of PDL-MSCs co-cultured with T lymphocytes will be further determined via the evaluation of cytokine profiles and flow cytometry analysis of T-cell phenotypes.

Results. Combustible cigarettes (CCs) induced more severe impairment in the viability and proliferation of PDL-MSCs compared with ENDS. Also, CCs promoted a proinflammatory immune response that could cause tissue damage to progress. On the other hand, ENDS had the potential to generate an immunosuppressive response that would prevent further cell decay.

Discussion. The regenerative capacity of PDL-MSCs decreased after treatment with both cigarette smoke and ENDS-aerosols. Even though the results demonstrate less severe effects with ENDS, further research is essential to evaluate their safety and impact on the capacity of PDL-MSCs to prevent and restore oral injuries caused by chronic exposure to aerosols.

Key words: Cigarette smoke, Electronic nicotine delivery systems, Aerosol, Periodontal ligament-derived mesenchymal stem cells

Corresponding Author: Prof. Vladislav Volarevic, Center for Harm Reduction of Biological and Chemical Hazards, Department of Genetics, Faculty of Medical Sciences, 69 Svetozar Markovic Street, 34000 Kragujevac, Serbia. e-mail: drvolarevic@yahoo.com
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Introduction

Periodontal ligament-derived mesenchymal stem cells (PDL-MSCs) are a specific type of stem cell sourced from the periodontal ligament, the connective tissue that surrounds and supports the teeth. PDL-MSCs possess unique regenerative and immunomodulatory properties that make them ideal for the repair and regeneration of injured periodontal tissues (Bojic et al., 2014). PDL-MSCs are multipotent and self-renewable cells with the ability to rapidly proliferate and spontaneously differentiate into cells of mesodermal origin (Volarevic et al., 2017). They can differentiate into cementoblasts (responsible for the formation of cementum), osteoblasts (involved in bone formation), and fibroblasts (essential for the production of connective tissue). This huge differentiation potential allows PDL-MSCs to contribute to the regeneration of periodontal tissues. Another important property of PDL-MSCs is their self-renewal capacity. These cells can divide and replicate themselves, ensuring a sustainable number of multipotent cells for successful tissue repair (Bojic et al., 2014).

Periodontal diseases, such as gingivitis and

periodontitis, can lead to the destruction of the periodontal tissues, including the gums, periodontal ligament, and alveolar bone. Alarmins and damage-associated molecular patterns, released from injured periodontal cells, activate endogenous PDL-MSCs and induce their differentiation into cementoblasts, osteoblasts, and fibroblasts, enabling periodontal tissue regeneration. Similarly, exogenous PDL-MSCs may be used for the cell-based therapy of periodontal diseases. Furthermore, PDL-MSCs have also been studied for their potential in the regeneration of other oral tissues, such as dental pulp. PDL-MSCs can differentiate into odontoblasts, which are responsible for the formation of dentin, the hard tissue that makes up the bulk of the tooth. By using PDL-MSCs, it may be possible to regenerate dental pulp and restore the functionality of damaged teeth (Tomokiyo et al., 2018).

Additionally, PDL-MSCs have immunomodulatory properties, meaning they can regulate the detrimental immune response and reduce inflammation. This is beneficial in the context of immune cell-driven inflammatory periodontal diseases as these conditions often involve an exaggerated immune response that leads to tissue destruction (Harrel et al., 2019). By suppressing detrimental immunity in the oral cavity, PDL-MSCs attenuate ongoing inflammation, promote tissue healing, and restore tissue homeostasis. Therefore, PDL-MSCs are considered potential new therapeutic agents in the treatment of oral inflammatory diseases, oral lichen planus, or recurrent aphthous stomatitis.

PDL-MSCs within the oral cavity are continuously exposed to various hazards and, therefore, their regenerative and immunomodulatory properties could be influenced by various factors (Harrel et al., 2021). Many experimental studies have shown that smoke derived from combustible cigarettes (CCs) had harmful effects on PDL-MSCs, negatively impacting their therapeutic potential. Cigarette smoke contains numerous toxic compounds and free radicals that can induce oxidative stress in PDL-MSCs. CC-derived smoke impairs the viability and capacity of PDL-MSCs to differentiate into cementoblasts, osteoblasts, and fibroblasts. Therefore, continuous exposure to CC-sourced smoke represents a known risk factor for the development and progression of periodontal diseases, particularly gingivitis and periodontitis.

Electronic nicotine delivery systems (ENDS), including e-cigarettes, vaping devices, and heated tobacco products (HTPs), have emerged as an alternative to CCs (Harrel et al., 2022). They are designed to deliver nicotine, the most desirable tobacco-related compound, or other substances extracted from tobacco to users without the combustion of tobacco, thereby reducing exposure to the harmful chemicals associated with traditional smoking. One of the main arguments in favor of ENDS is their potential for harm reduction. Unlike CCs, ENDS do not produce tar or many of the harmful chemicals generated by the combustion of tobacco. While not completely risk-free, the overall toxicant levels in ENDS-derived aerosols are significantly lower compared with cigarette smoke. However, the molecular mechanisms responsible for the effects of ENDS-derived aerosols on oral activity and the therapeutic potential of PDL-MSCs are completely unknown as yet.

Therefore, in this study, we compared the viability, proliferation, and immunomodulatory properties of air, CC, and ENDS-exposed PDL-MSCs, and analyzed differences in phenotype and function of T cells isolated from healthy volunteers co-cultured with treated PDL-MSCs. This study aimed to pave the way for a better understanding of the biological effects of ENDS-derived aerosols on the viability, proliferation, and immunomodulatory properties of PDL-MSCs.

Materials and methods

Cell passaging

Human PDL-MSCs cell lines were kindly provided by OMJ (Jakovljević et al., 2022) and propagated according to instructions. The cells were maintained in a humidified atmosphere with 5% CO_2 in air at 37°C, with Dulbecco′s Modified Eagle′s Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA) standard culture medium changing every 2-4 days, until 70-80% confluence. Cells in passage 3 were used for experiments (Zhu and Liang, 2015).

Smoke and aerosol generation and cell exposure

The Borgwaldt LM1 smoking and LM4E Vaping Machines (Hamburg, Germany) were used for cell culture exposure. Cells were exposed to CC-sourced smoke using 1R6F-certified reference cigarettes (University of Kentucky) with 1,896 mg nicotine per cigarette under a Health Canada Intense (puff volume, duration, and frequency of 55 mL, 2 s, and 30 s (55/2/30), with bell shape and filter holes closed by tape) smoking regime for a total of nine puffs. Vaping puffs were set to reach the same nicotine concentration as cigarette smoking (12 puffs from one IQOS Heatstick were generated every 15 minutes following the Health Canada Intensive puffing regime of a 55-mL puff every 30 seconds, for a total of 5 Heatsticks). An IQOS 3 DUO device was used with Heets “Sienna selection” (Red) with a nicotine content of 0.5 mg/Heatstick.

The aqueous aerosol extracts (AqE) from cigarettes and IQOS were generated by bubbling through 20 ml of AqE capture media (DMEM, Sigma-Aldrich, Saint Louis, MO, USA). AqE 100% stocks were diluted with appropriate volumes of media to produce a range of AqE concentrations for cell exposure (25, 50, 75, and 100%). One day after seeding PDL-MSCs for all the assays, standard medium was replaced with AqE capture medium and incubated for at least 24h. AqE capture medium was filtered through a 0.2 μm syringe filter before treatment (Caruso et al., 2023).

Evaluation of cell viability and proliferation

Viability and Cytotoxicity Assays – MTS and Neutral Red Uptake (NRU) tests were used to assess the viability of PDL-MSCs exposed to CCs (PDL-MSCs^{CCs}) and ENDS (PDL-MSCs^{ENDS}). PDL-MSCs cultured in standard DMEM, only exposed to fresh clean air, were used as control cells (PDL-MSCs^{AIR}).

PDL-MSCs were seeded at a cell density of 1×10^4 cells/100 µL in each well of 96-well multiplates for both assays. The next day medium was removed, cells were washed with 100 µL prewarmed DPBS (Sigma-Aldrich, Saint Louis, MO, USA), and then treated with different AqE concentrations from cigarettes and IQOS. Each treatment concentration was investigated in triplicate; AqEs were serially diluted in DMEM to concentrations of 25, 50, 75, and 100%.

For the NRU assay, treated cells were incubated for 24h at 37°C in a 5% CO_2 atmosphere. NRU solution (NRU, Sigma Aldrich, Saint Louis, MO, USA) was prepared in DMEM+HEPES buffer one day before the assay and placed in the incubator overnight. The following day, NRU solution was filtered through a 0.2 µm syringe filter before use and added after removing supernatants (150 µL/well). Following the 3-h incubation, the NRU solution was removed, and 330 µl of NRU destain solution (50% ethanol, 49% distilled water, 1% glacial acetic acid) was added to elute the incorporated solution from the cells. Plates were shaken for 10 min, and optical density was read at 540 nm using a reference filter of 630 nm (Agilent BioTek Epoch Microplate Spectrophotometer, Santa Clara, CA, USA) (Caruso et al., 2021).

For the MTS assay, cells were incubated under standard conditions (at 37°C in a 5% CO_2 atmosphere) for 72h. After incubation, the supernatants were removed and 20 µL of MTS solution (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) was added to each well, and cells were incubated for an additional 3h in a humidified atmosphere of 5% CO_2 in air. The plates were shaken for 10 min, and absorbance was measured at 490 nm (Agilent BioTek Epoch Microplate Spectrophotometer, Santa Clara, CA, USA) (Wang et al., 2021).

Evaluation of the cytokine profile of PDL-MSCs

Commercially available ELISA sets (R&D Systems, Minneapolis, MN, USA) were used to measure the concentration of immunosuppressive IL-10 and transforming growth factor-β1 (TGF- β1), as well as inflammatory cytokines (TNF-α, IFN-γ) in the supernatants of PDL-MSCs^{CCs}, PDL-MSCs^{ENDS}, and PDL-MSCs^{AIR} 72 hours after exposure to AqEs from CCs and ENDS at different concentrations. The assays were performed according to the manufacturer's protocol. Absorbance was measured at a wavelength of 450 nm, and the concentration of biomolecules was calculated from a standard curve. All measurements were performed in triplicate (Harrel et al., 2021).

Isolation of T cells from human peripheral blood

Blood samples were obtained from two healthy volunteers at the Center for Research of Harm Reduction of Biological and Chemical Hazard, the Faculty of Medical Sciences of the University of Kragujevac. Two samples per patient were obtained with participant consent as well as institutional ethical approval. Volunteers were aged above 18 and below 55 years, with a normal physical examination, and exclusion criteria were that subjects had been previously diagnosed with any disease or medical condition.

Peripheral blood mononuclear cells (pbMNCs) were isolated using Histopaque-1077 (Sigma-Aldrich, Saint Louis, MO, USA) density gradient centrifugation (Volarevic et al., 2012). T cells were separated from pbMNCS by using the Pan human T cell isolation kit (cat. No. 130-096-535, Miltenyi Biotec) and magnetic separation. T cells from each volunteer were maintained separately in standard Dulbecco′s Modified Eagle′s Medium (DMEM).

Co-culture of CC and ENDS-exposed PDL-MSCs with T lymphocytes

The co-culture of MSCs with T cells represents a great way to study the immunomodulatory effects of MSCs on adaptive immune cells (El-Sherbiny et al., 2018). T lymphocytes and PDL-MSCs were seeded in Transwell® 24-well plates at a suitable density (1:10) in complete medium. Lymphocytes were seeded in the apical compartment of each well (inserts), while PDL-MSCs were seeded in the basal compartments of each well (under the inserts), thus the two types of cells were physically separated. Cells were seeded 48 hours before exposure to AqEs from CCs and ENDS at different concentrations. T cells were collected and analyzed 72 hours after the treatment by flow cytometry (Laing et al., 2019).

Intracellular staining and flow cytometry analysis of T cells

To detect the cell surface expression of a variety of molecules, isolated T cells were analyzed using standard staining methods. Cells were washed and stained with fluorochrome-conjugated anti-human monoclonal antibodies (CD3, CD4, CD8) for 30 min in the dark at 4°C. After incubation, cells were washed twice with DPBS (Sigma-Aldrich, Saint Louis, MO, USA) and pelleted by centrifugation (250 g, 5 minutes). Cells were then incubated in a BD fixation/permeabilization solution (BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit; Cat. No. 554714) for 20 min at 4°C. After washing cells twice in the BD Perm/Wash™ buffer and centrifugation, intracellular staining for IFN-γ, TGFβ,

IL-17, and IL-10 was performed. All the anti-human monoclonal antibodies used were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), or allophycocyanin (APC). Cells were incubated at 4°C for 30 minutes in the dark. Upon incubation, cells were washed twice with BD Perm/Wash™ buffer and resuspended in a staining buffer before flow cytometry analysis. Flow cytometry analysis was conducted on a BD Biosciences FACSCalibur and analyzed by the application of the flowing software analysis program (Turku Bioscience Centre).

Statistical analyses

The statistical analysis was conducted using SPSS 21.0 software for Windows. The study used one-way ANOVA to determine differences between the mean values within groups. In cases where the ANOVA showed a significant difference, further examination was performed using two-tailed Student's t-tests with Tukey's correction for multiple comparisons. All data were expressed as the mean ± standard error of the mean (SEM). SEM was used instead of standard deviation because we investigated every sample through measurements in triplicate. Thus, SEM is an option with a wider range and more suitable for this study. All reported p-values were two-sided, and $p<0.05$ was considered statistically significant.

Results

CC-derived smoke and ENDS-derived aerosols impair the viability and proliferation of PDL-MSCs in a dose-dependent manner

Results obtained by NRU (Fig. 1) and MTS assay (Fig. 2) showed that exposure to CC-derived smoke and ENDS-sourced aerosols negatively affected the viability and proliferation of PDL-MSCs. While air exposure enabled normal growth and proliferation, the total number of viable and proliferating PDL-MSCs significantly decreased after exposure to the different concentrations of CC-derived smoke and ENDS-derived aerosols (Figs. 1, 2).

CC favored the generation of inflammation, while ENDS promoted the generation of an immunosuppressive phenotype in PDL-MSCs

PDL-MSCs treated with CC-sourced smoke expressed a pro-inflammatory phenotype. An increased

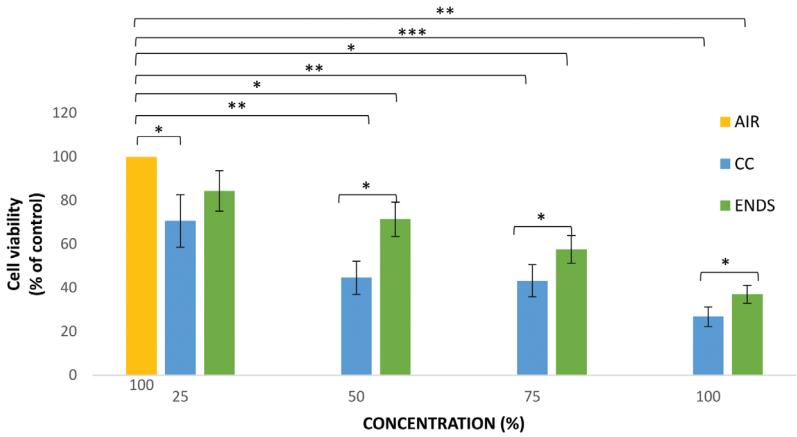

Fig. 1. The viability of PDL-MSCs was significantly impaired after treatment with CC and ENDS at various concentrations compared with PDL-MSCs exposed to air only. The NRU (Neutral red uptake) cytotoxicity assay showed significantly lower viability values in CC-exposed compared with ENDS- and Air-exposed cells. Values are presented as percentage of control (untreated cells); n=2 samples per group; *p<0.05, **p<0.01, ***p<0.001.

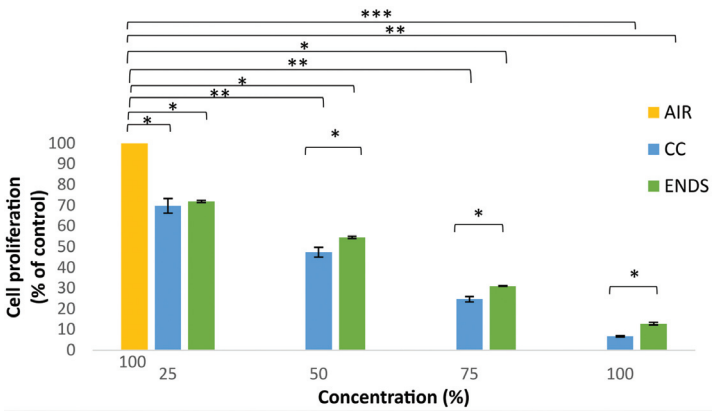

Fig. 2. The proliferative capacity of PDL-MSCs was significantly impaired after treatment with CC at various concentrations compared with PDL-MSCs treated with ENDS. MTS assay for cell viability and proliferation showed significantly lower values in CC-exposed compared with ENDS- and Air-exposed cells. Values are presented as percentage of control (untreated cells); n=2 samples per group; *p<0.05, **p<0.01, ***p<0.001.

concentration of inflammatory cytokines (IFN-γ, TNF-α) was observed in the supernatants of CC-treated cells compared with air-exposed and ENDS-exposed PDL-MSCs (Fig. 3A,B). Both IFN-γ and TNF-α are known to be inflammatory cytokines involved in the Th1 cell-mediated immune response, therefore, increased concentrations indicate the generation of an inflammatory phenotype in CC-exposed PDL-MSCs. Importantly, IFNγ and TNF-α supernatant concentrations were lower in ENDS-treated than CC-treated PDL-MSCs.

On the contrary, exposure to ENDS-derived aerosols significantly enhanced the secretion of TGF-β and IL-10 in PDL-MSCs. Importantly, concentrations of anti-inflammatory TGF-β1 and IL-10 were higher in ENDS-treated compared with air-exposed and CC-exposed PDL-MSCs (Fig. 3C,D).

PDL-MSCs^{CC} favored the generation of inflammation, while PDL-MSCs^{ENDS} favored the generation of an immunosuppressive phenotype in T cells

Intracellular staining and flow cytometry analysis of T cells co-cultured with CC-exposed PDL-MSCs (PDL-MSCs^{CC}) showed a pro-inflammatory phenotype (Fig. 4A-D), while T-cells co-cultured with ENDS-exposed PDL-MSCs (PDL-MSCs^{ENDS}) had an immuno-suppressive phenotype (Fig. 4E,F). The total number of inflammatory IFN-γ and IL-17-producing CD3+CD4+ (Fig. 4A,B) and CD3+CD8+ T cells (Fig. 4C,D) increased when T cells grew in the presence of PDL-MSCs^{CC}, which were previously exposed to increasing concentrations of CC-derived smoke. In contrast, the total number of immunosuppressive IL-10 and TGF-β-producing CD3+CD4+ (Fig. 4E,F) increased when T cells grew in the presence of PDL-MSCs^{ENDS}, which were previously exposed to increasing concentrations of ENDS-derived aerosols. Importantly, a significantly higher number of IFN-γ and IL-17-producing CD3+CD4+ and CD3+CD8+ T cells and a lower number of TGFβ and IL-10-producing CD4+ cells were observed when T cells were co-cultured with PDL-MSCs^{CC} rather than PDL-MSCs^{ENDS}, confirming opposing effects for CC and ENDS on the immuno-regulatory properties of PDL-MSCs.

CCs and ENDS have different effects on the survival of PDL-MSCs and T cells

CC-derived smoke and ENDS-derived aerosols induced a lower number of viable cells compared with the control. Additionally, co-culture of PDL-MSCs with T cells showed that CC can increase the number of activated T cells and modulate their phenotype (Fig. 5).

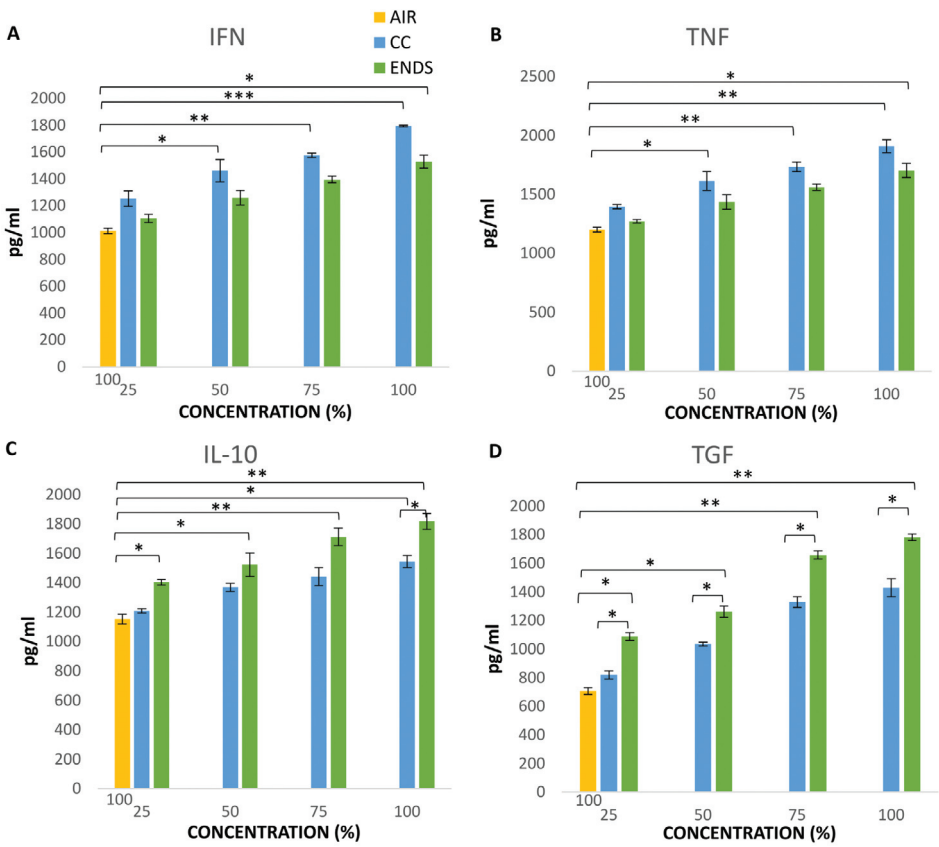

Fig. 3. Concentrations of pro-inflammatory cytokines (IFNγ, TNFα) were higher in the supernatants of CC-exposed than ENDS-exposed cells **(A, B)**. However, concentrations of anti-inflammatory cytokines (TGFβ, IL-10) were lower in the supernatants of CC-exposed than ENDS-exposed cells **(C, D)**. Values are presented as Mean ± SEM; *n*=6 supernatants per group; ***p*<0.05, ***p*<0.01, ****p*<0.001.

Discussion

Long-term and frequent use of CCs increases the risk of developing periodontal diseases (Warnakulasuriya et al., 2010). CC-derived smoke contains several thousands of toxic substances that alter the composition of the oral microbiome and enhance the production of inflammatory cytokines in immune cells, crucially contributing to the destruction of periodontal tissue (Warnakulasuriya et al., 2010). PDL-MSCs play a crucial role in regenerating injured periodontal tissue through their unique properties of self-renewal and differentiation. Upon injury, PDL-MSCs migrate to the damaged area, where they differentiate into osteoblasts, odontoblasts, cementoblasts, chondrocytes, and fibroblasts and secrete growth factors, enabling repair and regeneration of injured periodontal tissue (Greenberg et al., 2017; Harrel et al., 2022). Additionally, PDL-MSCs produce immunosuppressive cytokines (TGF-β and IL-10) and attenuate detrimental inflammatory responses, creating a more favorable environment for the restoration of periodontium and ultimately enhancing overall oral health (Kamel et al., 2020).

Herein, we were the first to demonstrate that exposure to CC-derived smoke significantly impaired the viability and proliferative abilities of PDL-MSCs and lessened their regenerative properties. CC-derived smoke contains 1, 3-butadiene, acrylonitrile, and ethylene oxide, which increase the frequency of large deletions in the hypoxanthine-guanine-phosphoribosyltransferase (*HPRT*) gene and impair DNA synthesis, negatively affecting the cell cycle progression of rapidly proliferating cells (Liu et

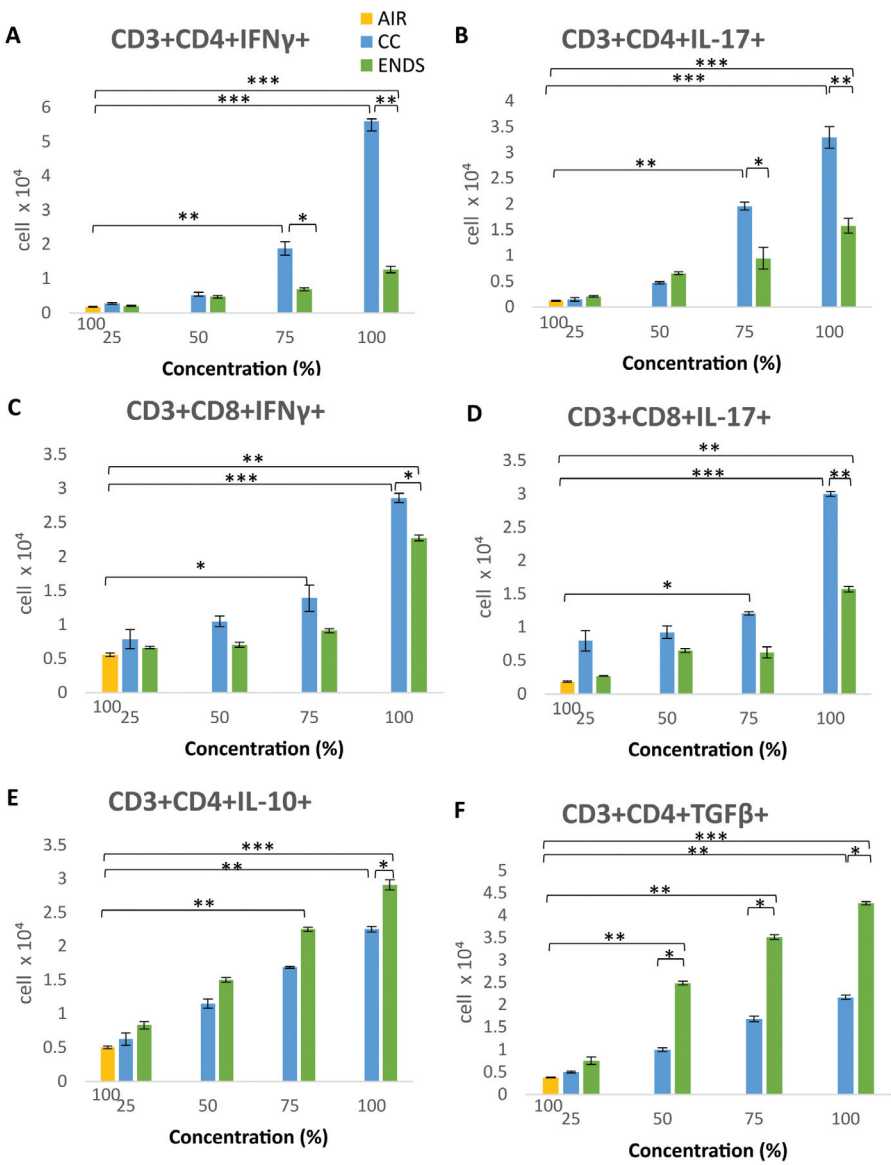

Fig. 4. A lower number of proinflammatory (IFN-γ+, IL-17+) CD4+ and CD8+ lymphocytes and a higher number of immunosuppressive (IL-10 and TGF-β-producing) CD4+ T cells are observed when T cells were co-cultured with ENDS-exposed PDL-MSCs (PDL-MSCs^{ENDS}) compared with CC-exposed PDL-MSCs (PDL-MSCs^{CC}). Values are presented as Mean ± SEM; n=2 samples per group; *p<0.05, **p<0.01, ***p<0.001.

al., 2008; Pero et al., 1981). In line with these findings, these harmful constituents likely were those mainly responsible for the detrimental effects of CC-derived smoke on the viability and proliferation of PDL-MSCs.

PDL-MSCs exposed to ENDS-derived aerosols also had reduced viability compared with untreated, air-exposed PDL-MSCs. Importantly, the detrimental effects of ENDS-derived aerosols on the viability and proliferative capacity of PDL-MSCs were less severe than those caused by CC-derived smoke. Since concentrations of 1,3-butadiene, acrylonitrile, and ethylene oxide in ENDS-derived aerosols were 99.3-99.9% lower than in CC-sourced smoke (Uguna and Snape, 2022), it is highly likely that the difference in the concentrations of these harmful compounds between ENDS-sourced aerosols and CC-derived smoke was mainly responsible for the distinct effects of CCs and ENDS on the viability and proliferation of PDL-MSCs.

Additionally, our results demonstrated that CC-derived smoke and ENDS-sourced aerosols significantly altered the secretory profile of PDL-MSCs, affecting their immunomodulatory properties. It is well known that, in infected and inflamed periodontal and gingival tissues, PDL-MSCs, through the activity of PDL-MSC-derived inflammatory and immunosuppressive cytokines, aggravate or attenuate immune cell-driven inflammation (De la Rosa-Ruiz et al., 2019). During the initial phase of the anti-microbial immune response,

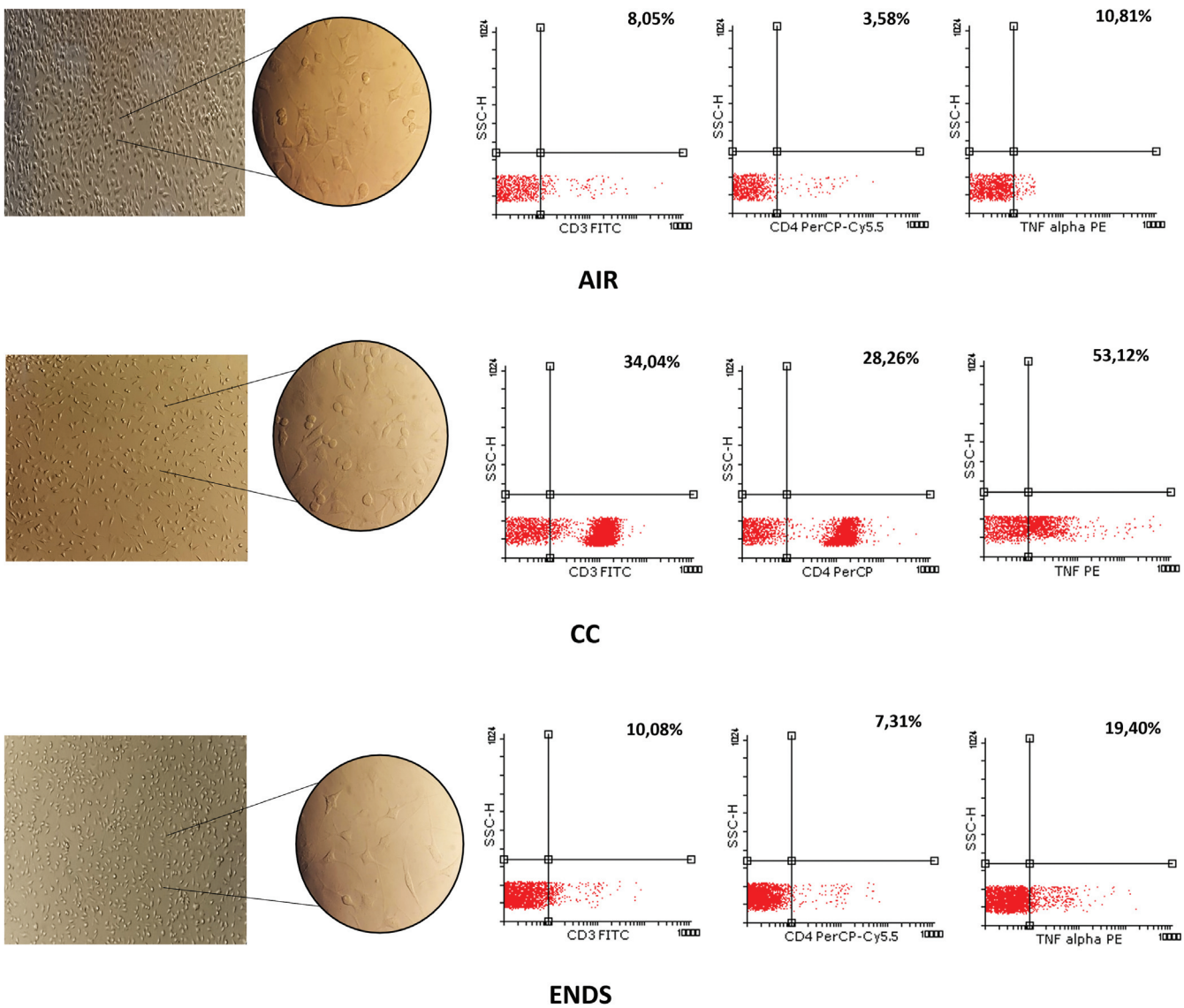

Fig. 5. The lower number of PDL-MSCs and their lower potential to change the phenotype of T lymphocytes (CD3+CD4+TNF-α-producing) were observed after treatment with CC compared with ENDS. The left panels show AIR/CC/ENDS-exposed PDL-MSCs photographed with a digital camera mounted on a light microscope (Olympus BX51, Japan), with a magnification of 100x (square) and 10x (circle). The right panels show representative flow cytometry dot plots of CD3+CD4+TNF-α-producing T cells after co-culture with AIR/CC/ENDS-exposed PDL-MSCs.

PDL-MSCs assume a pro-inflammatory phenotype and produce large amounts of inflammatory cytokines (TNF-α, IFN-γ). These enhance the phagocytic activity of neutrophils and macrophages, induce increased expansion of CD4+, CD8+Th1, and Th17 lymphocytes in regional lymph nodes, and generate a potent T-cell-driven anti-microbial immune response resulting in the efficient elimination of invading pathogens (Volarevic et al., 2017; Trubiani et al., 2019). On the contrary, during the resolution phase of the inflammatory response, upon the removal of microbial pathogens, PDL-MSCs obtain an immunosuppressive phenotype and secrete anti-inflammatory cytokines (IL-10, TGF-β), which induce expansion of tolerogenic dendritic cells, CD4+FoxP3+T regulatory cells, and alternatively activated macrophages that attenuate on-going inflammation, restore tissue homeostasis, and enhance tissue repair and regeneration (Volarevic et al., 2017; Trubiani et al., 2019).

It is well known that CC-derived tar and carbon monoxide (CO) induce oxidative stress, enhance the release of reactive oxygen species (ROS), and activate mitogen-activated protein kinase (MAPKs)-signaling cascades in CC-exposed immune cells, resulting in enhanced production of inflammatory cytokines (Kastratovic et al., 2024). In line with these findings, we assume that CC-dependent generation of inflammatory PDL-MSCs relied on CO and tar-based activation of the MAPK pathway, which led to the increased synthesis of TNF-α and IFN-γ in PDL-MSCs. These inflammatory cytokines promote the expression of major histo-compatibility complex (MHC) class II molecules on PDL-MSC and enhance their capacity for the generation of Th1 and Th17 cells, which play a crucially important pathogenic role in the progression of periodontitis (Trubiani et al., 2019; Chen et al., 2016). CD4+ and CD8+Th1 lymphocytes produce IFN-γ, which activates macrophages and promotes macrophage-driven inflammatory response, leading to tissue destruction in the periodontal environment (Chen et al., 2016). This response can exacerbate bone resorption, further aggravating periodontal tissue damage. On the other hand, Th17 lymphocytes secrete IL-17, a cytokine that enhances the recruitment of neutrophils and stimulates the production of additional inflammatory mediators, ultimately intensifying the inflammatory cascade (Chen et al., 2016). The presence of IL-17 is closely associated with increased osteoclast activity, further contributing to alveolar bone loss (Chen et al., 2016). In line with these findings, it is highly expected that CC-dependent expansion of inflammatory PDL-MSCs, Th1, and Th17 lymphocytes resulted in the aggravation of Th1 and Th17 cell-driven destruction of periodontal tissue, which was frequently observed in CC users.

Interestingly, PDL-MSCs exposed to ENDS-sourced aerosols obtained an immunosuppressive phenotype and massively produced anti-inflammatory cytokines (IL-10 and TGF-β). By inhibiting the activation of the transcription factor NF-kβ in T cells, PDL-MSC-derived IL-10 and TGF-β suppress the production of inflammatory cytokines (TNF-α, IFN-γ, IL-17) and enhance the synthesis of anti-inflammatory factors (Volarevic et al., 2017). Accordingly, ENDS-dependent expansion of immunosuppressive PDL-MSCs was accompanied by an increased number of IL-10 and TGF-β-producing CD4+ and CD8+T cells. These immuno-suppressive T lymphocytes have the potential to suppress periodontitis by impairing the production of inflammatory cytokines in effector T cells and by inhibiting Th1 and Th17 cell-driven inflammation in the oral cavity (Parachuru et al., 2018).

We assume that the different effects of CCs and ENDS on the cytokine profile of PDL-MSCs and PDL-MSC: T-cell interactions were consequences of different tobacco processing (Kastratovic et al., 2024). ENDS utilizes a heating process that occurs at lower temperatures than CC-related combustion (Znyk et al., 2021; Kim et al., 2022). Consequently, the aerosols produced by ENDS contain compounds different from CC-sourced smoke (Znyk et al., 2021; Kim et al., 2022). ENDS does not generate tar, and the concentration of CO is 99% lower in ENDS-derived aerosols than in CC-derived smoke (Zhang et al. 2023). Thus, it is likely that the more pronounced changes in the phenotype and function of PDL-MSCs are primarily due to the harmful effects of CO and tar-containing oxidants, which are present at high levels in CC-sourced smoke and reduced or absent in ENDS-derived aerosols (Znyk et al., 2021; Kim et al., 2022; Zhang et al., 2023).

In summary, our results presented here showed that CC exposure induced more severe impairment of the regenerative and immunomodulatory potential of PDL-MSCs compared with ENDS exposure. However, even though the ENDS-induced detrimental effects were less severe than those caused by CC-derived smoke, it should be emphasized that ENDS should not be seen as a harmless product since ENDS-exposed PDL-MSCs also had impaired viability and proliferation. Finally, it is important to acknowledge that these conclusions were made according to results obtained *in vitro*. Therefore, future animal studies should be conducted to examine the effects of CCs and ENDS on PDL-MSCs phenotypes and function *in vivo*, paving the way for a better understanding of CC and ENDS-induced biological effects in periodontal tissue.

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Conflicts of Interest. The authors declare no conflicts of interest.

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