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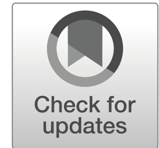

Macrophages skew towards M1 profile through reduced CD163 expression in symptomatic apical periodontitis

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Abstract

Objectives To explore the macrophage profiles in symptomatic and asymptomatic forms of AP through phenotypic and functional analyses.

Material and methods Cross-sectional study. Apical tissue/lesion samples were collected from patients with clinical diagnosis of AAP ($n = 51$) or SAP ($n = 45$) and healthy periodontal ligament (HPL) from healthy patients as controls ($n = 14$), all with indication of tooth extraction. Samples were digested, cells were stained for CD14, M1 (CD64, CD80), and M2 (CD163, CD206) phenotypic surface markers and analyzed by flow cytometry. Functional cytokine profiles L-6, IL-12, TNF-α, IL-23 (M1), IL-10, and TGF-β (M2) were determined by qPCR.

Results Higher macrophage M1/M2 ratio ($CD64^+CD80^+/CD163^+CD206^+$) along with lower CD163 mean fluorescence intensity (MFI) were found in SAP compared to AAP and controls ($p < 0.05$). IL-6, IL-12, TNF-α, IL-23 (M1), and IL-10 mRNA (M2) were upregulated, whereas TGF-β mRNA (M2) was downregulated in apical lesions compared to controls. Specifically, IL-6 and IL-23 (M1) were upregulated in SAP compared with AAP and controls ($p < 0.05$). The data were analyzed with Kruskal-Wallis test.

Conclusions Macrophages exhibited a polarization switch towards M1 in AL. SAP exhibited a reduced M2 differentiation profile based on a reduction of CD163 expression levels in SAP over AAP. Specifically, IL-6 and IL-23 were augmented SAP over AAP, suggesting a role in the severity of apical lesions.

Clinical relevance Deciphering the macrophage polarization and functions in apical periodontitis can contribute to explain AP dynamics, its clinical presentation and systemic impact.

Keywords Apical periodontitis · Innate immunity · Macrophage polarization · Interleukin-6 · Interleukin-23

Introduction

Apical periodontitis (AP) is a prevalent dental infection comprising the pulpal tissue of the root canal system. If left untreated, an osteolytic apical lesion (AL) will be formed and tooth loss will occur over time. [1]. Additionally, emerging epidemiologic and mechanistic evidence sustains a link between AL and low-grade systemic inflammation involving higher risk of cardiovascular diseases (CVD) and diabetes mellitus [2–4].

ALs are clinically heterogeneous and can vary over time between asymptomatic (AAP) and symptomatic (SAP) forms. The nature, extent, and duration of host's immune response to endodontic infection are the ultimate determinants of apical tissue breakdown versus healing processes during AP [5–7]. In fact, a more severe pro-inflammatory response [8] and higher bone resorptive activity, based on tartrate-resistant acid phosphatase (TRAP) levels [9], have been reported in SAP reflecting a more active or progressive form of AP [9–11].

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Though diverse infiltrating leukocyte and periodontal resident cells are involved in the pathogenesis of AP, macrophages are among the most relevant inflammatory cell populations [12, 13]. Resident macrophages are often the first immune cell to encounter pathogens, and naïve monocytes/macrophages will be recruited from the bloodstream to the periapical tissues via chemokine gradients. Macrophage activation is initiated by the ligation of Toll-like receptors (TLRs) with pathogen-associated molecular patterns (PAMPs), such as LPS, released from gram-negative bacteria or endogenous danger-associated molecular patterns (DAMPs), released from necrotic or dying cells. The ligation of TLRs leads to macrophage activation and triggers a range of functions including phagocytosis and production of inflammatory mediators, such as cytokines, reactive oxygen species, and matrix metalloproteinases (MMPs), and activation of humoral and cellular responses, making them essential in regulating inflammation, immune response, bone homeostasis, and tissue remodeling [13–15].

Macrophages show wide plasticity and differentiation dynamics towards two main phenotypic profiles, M1 and M2 [14]. The M1 profile is the result of the classical activation pathway. This will result in the release of pro-inflammatory cytokines, such as IL-6, IL-12, TNF-α, and IL-23, enhancement of antigen presentation and phagocytosis [16], and promotion of Th1 and Th17 patterns of immune responses, which are directly associated with destruction of periodontal tissues [17]. On the other hand, M2 macrophages are the result of the alternative activation pathway and are involved in the modulation of the immune system and repair by secreting anti-inflammatory cytokines, such as IL-10 and TGF-β; however, they are not efficient in antigen presentation nor microbial destruction. In addition, M2 macrophages promote the Th2 and Treg phenotypes, which are periodontally protective [18, 19].

A switch from M2 towards M1 macrophage profile, manifested as an increased ratio of $NOS2^+/CD206^+$ macrophages, has previously been reported in a mouse model of marginal periodontitis [20]. Additionally, cytokines and chemokines associated with the M1 profile, such as TNF-α, IL-1β, IL-6, and IL-8, increased their levels in symptomatic apical lesions [21, 22], but few is known about anti-inflammatory M2 cytokines. Overall, the macrophage immune profiles and their association with the symptomatic forms of AP remain unknown. Deciphering macrophage polarization and functions in different clinical forms of apical periodontitis is central to understand and optimize antibacterial defense, dampen detrimental inflammation, and/or control alveolar bone loss. This study aimed to explore the macrophage profiles in symptomatic and asymptomatic forms of AP through phenotypic analysis of M1 and M2 surface markers and functional profiling of cytokines in ALs. We hypothesized that that the dynamic balance between asymptomatic and symptomatic apical periodontitis associates with a macrophage profile switch from M2 to M1.

Materials and methods

Study design

This is a cross-sectional study. The protocol was approved by the Institutional Review Boards from the Faculty of Dentistry, Universidad de Chile (#2016/08) and Central Metropolitan Health Service (# 2017/70). The investigation was carried out according to Helsinki declaration. The protocol was clearly explained to all selected participants. An IRB-approved informed consent was signed by all participants and corresponding forms for their legal guardians in case of underage patients.

Patients and sample collection

Individuals consulting at the Dental Clinic from the Faculties of Dentistry from Universidad Andrés Bello and Universidad de Chile from October 2017 until October 2018 were consecutively enrolled if they had the clinical diagnosis of AAP or SAP with no previous treatment. The inclusion criteria were individuals with at least one tooth diagnosed with apical periodontitis in association with dental caries that did not respond to pulp sensitivity tests and had a radiographic apical radiolucency. Teeth with a painful response to biting and/or percussion were diagnosed as SAP, and those with no clinical symptoms as AAP, according to previously defined criteria [6]. All teeth had clinical indication of tooth extraction. As controls, healthy periodontal ligaments (HPLs) were obtained from healthy teeth extracted for orthodontic purposes. They were defined as teeth with normal periradicular tissues with no abnormal sensitivity to percussion or palpation testing, and showing intact lamina dura and uniform periodontal ligament space surrounding the root in a periapical radiograph, as previously defined [10]. Exclusion criteria were apical abscess, systemic diseases, and/or antibiotic and/or anti-inflammatory treatments in the last 3 months prior to the study.

A total of 110 samples were obtained and further analyzed, 51 AAP lesions, 45 SAP lesions, and 14 HPL controls. Demographic parameters, smoking habit, and clinical data, including the lesion size expressed as the mean diameter, were recorded. Apical lesions or healthy periodontal ligament tissues were obtained from extracted teeth and either stored in RNAlater 100 μl (Qiagen, Valencia, CA, USA) at − 80 °C for total cytoplasmic RNA extraction or immediately placed into transport medium consisting of RPMI 1640 (Corning, New York, NY, USA) supplemented with antibiotics for tissue digestion and flow cytometry analysis.

Antibodies

For immunofluorescence staining, the following mouse monoclonal anti-human antibodies conjugated with their respective fluorochromes were used: CD14 conjugated with Alexa Fluor 700 was used as a pan macrophage marker; CD64 conjugated with Brilliant Violet 510 (BV510) and CD80 conjugated with fluorescein isothyocyanate (FITC) were used to identify M1 macrophage subpopulations; CD163 conjugated with PE/Cy7 and CD206 conjugated with Brilliant Violet 421 (BV421) were used to identify M2 macrophages subpopulations (Biolegend, San Diego, CA, USA).

Enzymatic digestion

To obtain total cell suspension, all sample tissues/lesions were placed into Petri dishes, minced with sterile scalpel, and digested for 90 min with 0.2 U/ml of type IV collagenase (Gibco, Thermo Fisher, Waltham, MA, USA) and 10 U/ml of DNase I (Invitrogen, Thermo Fisher) in 5 ml of RPMI 1640 medium at 37 °C. The obtained cell suspension was filtered through a 0.70 μm cell strainer in order to remove any tissue debris. Then, they were washed with 1 ml of PBS supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher) and incubated with red blood cell lysis buffer for 5 min at 37 °C inactivated with PBS 10% FBS. Finally, cells were counted, and the cell viability was determined by Trypan Blue exclusion (Sigma-Aldrich, St. Louis, MO, USA), usually around 95%.

Flow cytometry

From the obtained samples ($N = 38$ individuals, AAP $n = 16$, SAP $n = 17$, and HPL $n = 5$) at least 2×10^5 cells/well were placed in a V bottom 96-well plate in 20 μl PBS. For complete staining, the cells were incubated with a mixture of all the abovementioned antibodies at appropriate dilutions in PBS 2% FBS, for 30 min at 4 °C in the dark. The cells were also single stained with anti-CD14, CD80, CD64, CD163, or CD206 antibodies conjugated with the respective fluorochromes at the same conditions as controls. After washing with PBS, the cells were fixed with 2% paraformaldehyde (Invitrogen) and analyzed by flow cytometry (LSR Fortessa X-20®; Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ, USA). Flow cytometric data analysis was performed with Flow Jo v10 software (TreeStar, Ashland, OR, USA). Macrophages were first gated according to their forward- and side-scatter profiles, then gated over the $CD14^+$ cells. For the determination of the frequencies of M1 and M2 macrophage subpopulations, the $CD14^+$ cells were gated over $CD64^+CD80^+$ or $CD163^+CD206^+$. The threshold in each case was determined by the comparison with unstained samples. The M1/M2 ratio was calculated as the frequency of M1 double-positive macrophages divided by the frequency of the M2 double-positive macrophages in each sample/individual. Also, the intensity of expression of each specific cell surface marker was expressed as mean fluorescence intensity (MFI).

The calibration of the flow cytometer was tested with Immuno-Check fluorospheres (Coulter-Immunotech) daily, and the instrument amplifier setting was adjusted with Immuno-Brite microbeads (Coulter-Immunotech) monthly.

Isolation of RNA

Samples ($N = 72$ individuals, AAP $n = 35$, SAP $n = 28$, and HPL $n = 9$) were homogenized, and RNA extraction was performed using a commercial kit (Qiagen), following the manufacturer's protocol and recommendations. RNA was quantified (260/280) using a spectrophotometer (Bio-Tek, Winooski, VT). Then, 12.5 ng of RNA from each sample was converted to single-stranded cDNA, using a reverse transcription kit (Thermo Fisher Scientific, Carlsbad, CA).

RNA expression

In order to quantify the expression levels for IL-6, IL-12, TNF-α, IL-23 (M1 macrophages), IL-10, and TGF-β (M2 macrophages) mRNAs, 12.5 ng of cDNA were amplified using their appropriate 5′-3′ forward primer and 5′-3′ reverse primer sets, respectively, as follows: CTCAACACGGGAAACCTCAC and CGTCCACCACTAAGAACG for 18S rRNA, GCCCAGCTATGAACTCCTTCT and GAAGGCAGCAGGCAACAC for IL-6, AGCTTCATGCCTCCCTACTG and CTGCTGAGTCTCCCAGTGGT for IL-23, CAGCCTCTTCTCCTTCCTGAT and GCCAGAGGGCTGATTAGAGA for TNF-α, TGGGGGAGAACCTGAAGAC and CCTTGCTCTTGTTTTCACAGG for IL-10, and CACGTGGAGCTGTACCAGAA and CAGCCGGTTGCTGAGGTA for TGF-β.

All primer sets and reagents (KAPA™SYBR® Fast; KAPA Biosystems, Woburn, MA, USA) were used in a qPCR equipment (StepOnePlus®; Applied Biosystems, Singapore), as follows: 95 °C for 3 min and 40 cycles of 95 °C for 30 s and 60 °C for 30 s. For detection of non-specific product formation and false-positive amplification, a melt curve of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s was performed. As an endogenous control, 18S rRNA expression levels were determined.

Analysis of results

Relative quantification of mRNA was analyzed using the $2^{-\Delta\Delta CT}$ method. The normalization of gene expression was done against the 18S rRNA, and HPL was used as a fold change reference. The Shapiro-Wilk test was used to determine the

distribution of the continuous data. Inferential analyses were performed using Kruskal-Wallis test followed by pairwise comparisons according to the calculated adjusted p value (0.0083) for non-normally distributed continuous data and ordinal variables, and chi square test for categorical variables. The statistical analysis was performed using STATA 12® (StataCorpLP, TX, USA). The figures were performed in Flow Jo v10 software (TreeStar, Ashland, OR, USA) and Graph Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). No formal sample-size calculation was performed due to the exploratory nature of this study.

Results

Demographic parameters and smoking habit of study participants are shown in Table 1. All parameters, including gender, smoking habit, educational level, as well as diameter of the lesions (mm) showed even distributions among groups except for age, which was lower in healthy periodontal ligament (HPL) controls compared to AAP and SAP groups ($p < 0.05$).

A total of 110 samples were obtained, 51 AAP lesions, 45 SAP lesions, and 14 HPL controls. Among them, cell suspensions were obtained after digesting the respective tissues (AAP $n = 16$, SAP $n = 17$, and HPL $n = 5$), cells were stained for CD14, M1 (CD64, CD80), and M2 (CD163, CD206) phenotypic surface markers and analyzed by flow cytometry. Macrophages were gated on the basis of forward scatter (FSC) and side scatter (SSC) (R1), as presented in Fig. 1 a, b, and c.

Over the R1-gated cells, the frequencies of CD14$^+$ cells tended to be higher in both asymptomatic and symptomatic lesions in relation to controls. The (median) frequency in HPL controls was 64.4%, whereas in AAP and SAP were 93.15% and 87.4%, respectively ($p > 0.05$; Fig. 1d). Based on the values of mean fluorescence intensity (MFI), CD14 did not show significant differences between the evaluated conditions (Fig. 1e, f).

To characterize the different macrophage subpopulations, the percentage of double-positive M1 markers CD64$^+$CD80$^+$ and double-positive M2 markers CD163$^+$CD206$^+$ were analyzed over CD14$^+$-positive cells. As shown in Fig. 2, the median frequency of double-positive CD64$^+$CD80$^+$ M1 macrophages progressively increased from healthy to AAP and SAP. The frequency of M1 subpopulation in HPL controls was 8.2%, whereas in AAP and SAP was 27.2% and 32.5%, respectively ($p > 0.05$). A similar tendency was also seen for double positive CD163$^+$CD206$^+$ M2 macrophages. The median frequency of M2 subpopulation was 16.2% in HPL controls, 26.1% in AAP and 35.2% in SAP ($p > 0.05$). The balance among these macrophage subpopulations was calculated as M1/M2 ratio, which was the lowest in HPL controls and the highest in SAP (0.40, 0.81, and 0.83, respectively), showing a significantly higher M1/M2 ratio in SAP compared with HPL controls ($p < 0.05$). In the case of AAP, a borderline significantly higher M1/M2 ratio was observed compared with HPL controls ($p = 0.0098$).

Additionally, as shown in Fig. 3, we analyzed the median frequency of single CD64$^+$, CD80$^+$, or CD206$^+$ cells over CD14$^+$ cells and observed that these populations tended to be higher in AAP and SAP versus HPL controls, but differences were non-significant ($p > 0.05$). In the case of CD64$^+$, HPL controls were 8.5%, AAP 28.7%, and SAP 40.7%. In the case of CD80$^+$, HPL was 29.9%, AAP 60.1%, and SAP 57.8%; for CD206$^+$, HPL was 16.3%, AAP 31.2%, and SAP 35.8%; finally, the median frequency of single CD163$^+$ remained close to 100% (HPL 89.9%, AAP 96.8%, and SAP 95.8%).

As shown in Fig. 4, based on the analysis of MFI, cell surface expression of the M1 markers CD64 and CD80 did not differ between any experimental condition ($p > 0.05$). On the other hand, the expression of the M2 markers, CD163 and CD206, tended to be progressively downregulated from HPL to SAP. Noticeably, CD163 expression was significantly downregulated in SAP compared to AAP and HPL ($p < 0.05$). In summary, SAP showed a lower M2 macrophage polarization over AAP and healthy controls.

To evaluate the functional cytokine profiles associated to macrophage plasticity in ALs, cytokine mRNA levels in AAP ($n = 35$), SAP ($n = 28$), and HPL ($n = 9$) groups were determined by qPCR as presented in Fig. 5. For M1 cytokines, the

Table 1 Demographic parameters and smoking habit of study individuals

Parameters	HPL ($n = 15$)	AAP ($n = 51$)	SAP ($n = 45$)
Age (years, mean ± SD)	22.2 ± 6.2*	41.9 ± 13.4	36.4 ± 12.5
Females (n, %)	10 (76.9%)	26 (50.1%)	20 (44.44%)
Smokers (n, %)	3 (23.1%)	18 (35.3%)	23 (51.11%)
Educational level (median)	Full high school	Full high school	Full high school
Diameter of the lesion (mm, years, median, IQR)	–	3.83 (3.43)	2.85 (1.55)

Gender and smokers were evaluated by Pearson's chi-squared test, age, education level and average diameter of the lesion was evaluated by Kruskal-Wallis test

AAP asymptomatic apical periodontitis, *SAP* symptomatic apical periodontitis, *HPL* healthy periodontal ligament

*Overall $p < 0.05$

ǂ Data not reported in one individual

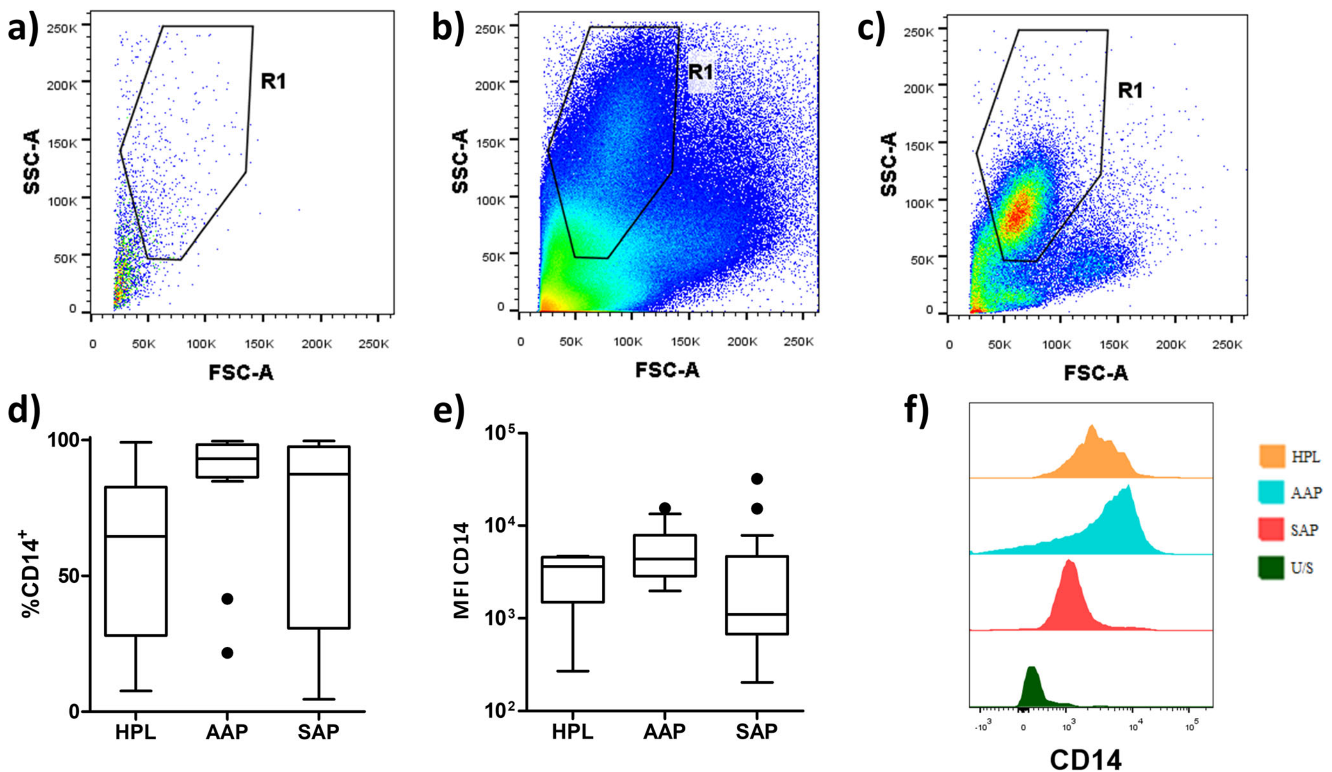

Fig. 1 Identification of monocytes/macrophages in endodontic apical lesions and controls. Macrophages were gated on the basis of forward scatter (FSC) and side scatter (SSC) (R1 region). **a** Healthy periodontal ligament (HPL). **b** Asymptomatic apical periodontitis (AAP). **c** Symptomatic apical periodontitis (SAP). **d** $CD14^+$ cell percentage over the R1 gated cells. **e** CD14 mean fluorescence intensity (MFI) over the R1-gated cells. **f** Representative histograms of CD14 MFI. US unstained. Data were analyzed by Kruskal-Wallis test

expression levels of IL-6 were the highest in SAP, followed by AAP, and the lowest in HPL, with significant differences among all groups ($p < 0.05$). The expression levels of IL-12 and TNF-α were higher in SAP and AAP compared to HPL controls ($p < 0.05$); IL-23 was significantly higher in SAP compared to AAP and HPL ($p < 0.05$). In the case of M2 cytokines, IL-10 mRNA levels were higher in SAP and AAP compared to HPL, whereas TGF-β expression levels were the lowest in SAP compared to AAP and HPL controls ($p < 0.05$). In summary, compared with HPL, AAP and SAP showed a pro-inflammatory M1 cytokine balance, whereas IL-6 and IL-23 were significantly upregulated compared to AAP.

Discussion

AP is a prevalent dental infection that manifests as a localized bone resorptive lesion with well-defined signs of chronic inflammation [7, 23]. Its clinical variability and the progression of the associated AL are expected to depend ultimately on the host's immune response to endodontic infection [5–7]. Based on pro-inflammatory cytokine levels, bone resorptive activity, and larger-sized lesions [21, 22, 24, 25], SAP is proposed to represent a more progressive form of the disease compared to AAP that manifests with clinical symptoms [9, 11]. Macrophages play important roles in the progression of inflammatory diseases, but their role and phenotypic profiles remain elusive in AP forms. In this study, we explored and confirmed a predominant M1 macrophage polarization profile over M2 in AL, based on $CD64^+CD80^+/CD163^+CD206^+$ macrophage phenotypes, which was also reflected in the overall pro-inflammatory cytokine profile. Specifically, SAP exhibited a weaker M2 differentiation profile, based on a reduction of CD163 expression levels, along with overexpression of the M1 cytokines, IL-6 and IL-23 when compared to AAP.

In the present study, we characterized M1 and M2 macrophage subpopulations based on the expression of their cell surface markers. Since in vivo M1 and M2 macrophage profiles represent the extremes of a continuum of intermediate cells, human studies have been hampered by the lack of validated phenotypic markers. Recent studies have systematically validated the surface markers CD64 and CD80 for M1, as well as CD163 and CD206 for M2, suggesting their combined use with a pan-macrophage marker, such as CD14 [19, 26, 27]. A tendency to a higher percentage of $CD14^+$ cells was identified in SAP and AAP compared with HPL controls, though non-statistically significant. Still, these results point to an increased population of tissue macrophages in AL as a result of endodontic infection and consequent

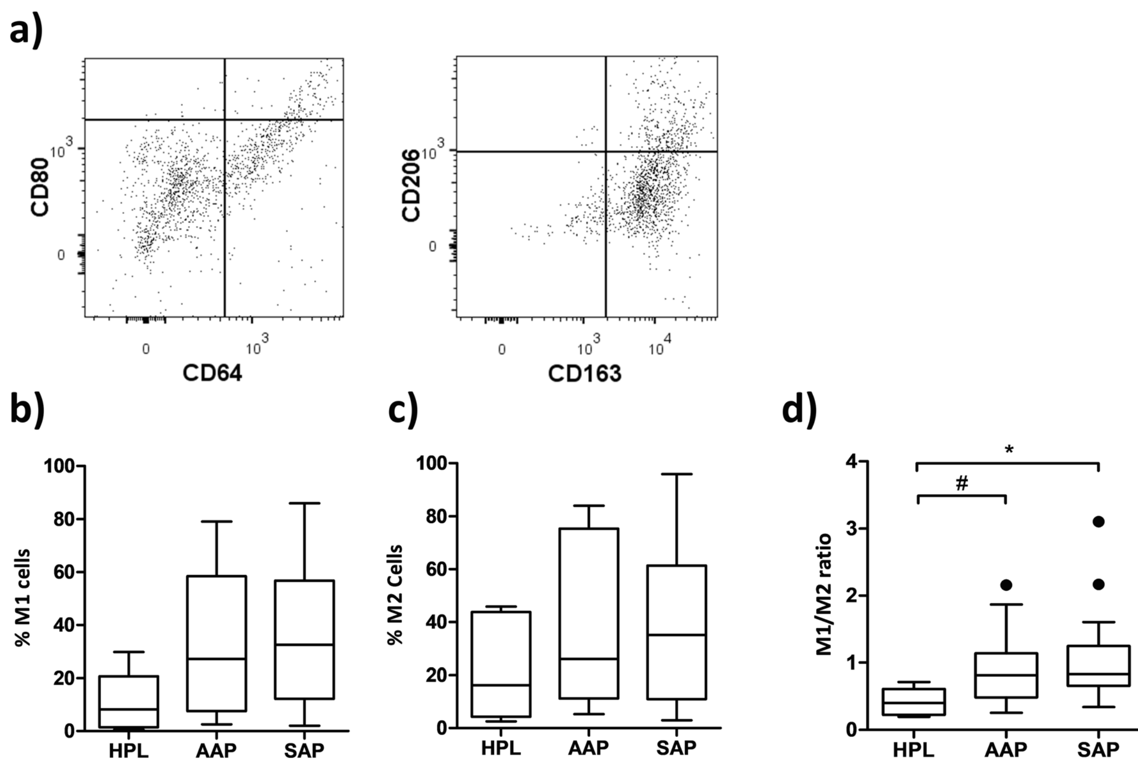

Fig. 2 Percentages of M1 and M2 macrophage populations in endodontic apical lesions and controls. Cells were stained with anti-CD14, anti-CD64, anti-CD80, anti-CD163, and anti-CD206 antibodies as are described in the "Materials and methods" section. **a** Representative flow cytometry dot plots of macrophages for the determination of M1 and M2 macrophages. **b** Percentage of CD64$^+$ CD80$^+$ for M1 macrophages. **c** CD163$^+$ CD206$^+$ for M2 macrophages over total population of CD14$^+$ cells. **d** M1/M2 ratio from HPL, APP, and SAP. HPL healthy periodontal ligament, AAP asymptomatic apical periodontitis, SAP symptomatic apical periodontitis. Data were analyzed by Kruskal-Wallis test, $*p < 0.05$, $^{\#}p = 0.0098$ for adjusted p value = 0.0083

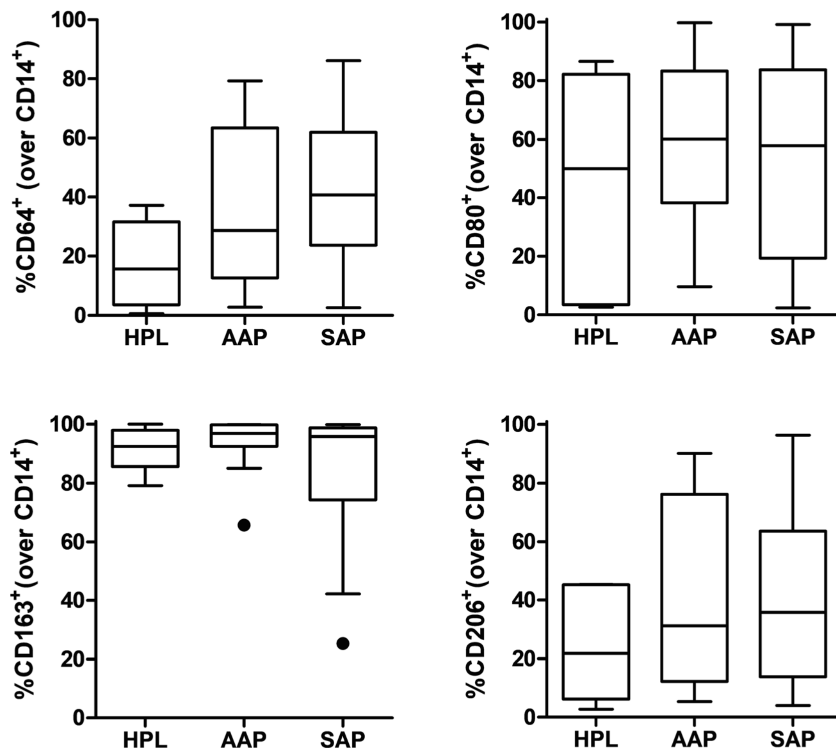

Fig. 3 Percentage of single stain for M1 and M2 markers in endodontic apical lesions and controls. Percentage of CD64$^+$, CD80$^+$, CD163$^+$, or CD206$^+$ over the total population of CD14$^+$ cells in HPL, APP, and SAP. HPL healthy periodontal ligament, AAP asymptomatic apical periodontitis, SAP symptomatic apical periodontitis. Data were analyzed by Kruskal-Wallis test, $*p < 0.05$

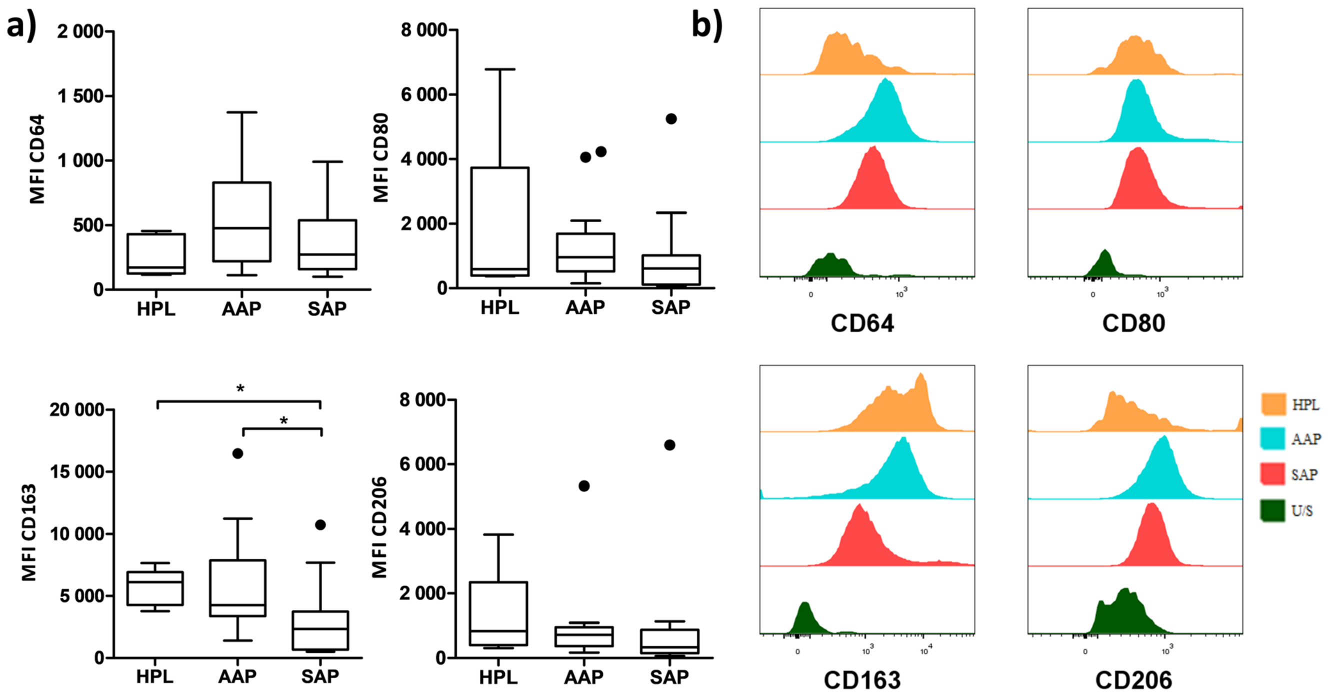

Fig. 4 Expression of M1 and M2 markers form apical lesions. **a** Mean fluorescence intensity (MFI) of CD64, CD80, CD163, and CD206 over $CD14^+$ cells endodontic apical lesions and controls. **b** Representative histogram of $CD14^+$ cells for M1 and M2 macrophage markers. HPL healthy periodontal ligament, AAP asymptomatic apical periodontitis, SAP symptomatic apical periodontitis, US unstained. Data were analyzed by Kruskal-Wallis test, $*p < 0.05$

chemoattractant signals. Recent evidence sustains that adult tissue-resident macrophages of embryonic origin are involved in tissue remodeling, as occurs during periodontal tissue homeostasis; whereas monocyte-derived cells can complement tissue compartments to primarily assist in host defense [28]. Accordingly, it is likely

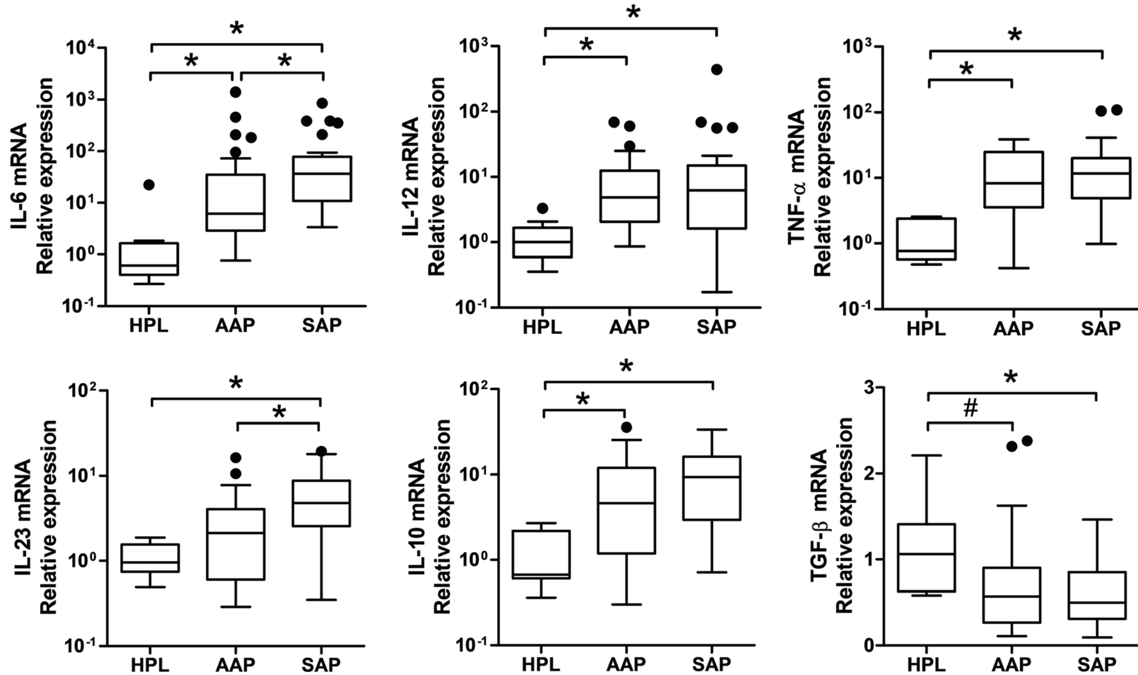

Fig. 5 Cytokine expression in endodontic apical lesions and controls. Pro-inflammatory cytokines (IL-6, IL-12, TNF-α, and IL-23) and anti-inflammatory cytokines (IL-10 and TGF-β) relative gene expression was normalized relative to 18S rRNA and expressed as the difference between $2^{-\Delta\Delta CT}$. HPL healthy periodontal ligament, AAP asymptomatic apical periodontitis, SAP symptomatic apical periodontitis. Data were analyzed by Kruskal-Wallis test, $*p < 0.05$, $^{\#}p = 0.0085$ for adjusted p value = 0.0083

that macrophage enrichment in apical lesions might be explained by the recruitment and differentiation of peripheral blood monocytes.

CD64 is a well-characterized M1 marker, which binds IgG with high affinity [29] and triggers functional pro-inflammatory responses [30], whereas CD80 is a co-stimulator for T cell activation during antigen presentation, expressed in macrophages, dendritic cells, and activated B lymphocytes. If combined with CD14, CD80 is also a useful and specific M1 marker [26, 27]. On the other hand, CD163 and CD206 are well-characterized M2 markers. CD163 is a scavenger receptor for the hemoglobin-haptoglobin complex that is exclusively expressed on monocytes/macrophages and CD206 is a C-type lectin receptor, both involved in the phagocytic capacity of macrophages to scavenge debris and apoptotic cells and therefore performing reparative functions [31].

The analysis of the double positive expression of CD64 and CD80 M1, and CD163 and CD206 M2 mean percentage cells in SAP, AAP, and HPL controls revealed a tendency for both subpopulations to increase from healthy HPL to AAP and SAP, though non-significant. Instead, the balance among these macrophage subpopulations expressed as M1/M2 ratio was the highest in SAP, followed by AAP and HPL controls (0.83, 0.81, and 0.40, respectively), showing a statistically significant difference for SAP compared to controls ($p < 0.05$), and borderline significance between AAP and controls. Despite no similar studies are available, previous reports based on histologic techniques and semiquantitative approaches showed the highest M1 to M2 macrophage degree in inflammatory radicular cysts, followed by apical granulomas, and were the lowest in dentigerous cysts, which are non-inflammatory in nature. Also, macrophage counts were lower in the later lesions [32]. Similarly, higher M1 macrophage population and M1/M2 ratio in gingival tissue from patients with marginal periodontitis compared with gingivitis and healthy individuals were reported through immunofluorescence. Interestingly, the gingivitis group showed increased M2 phenotype and functionally lower IL-6 levels versus healthy and periodontitis tissues [33]. Our findings further highlight the role of macrophages plasticity and differentiation dynamics in AL, showing a phenotype balance towards M1 over M2. Bacterial components, such as Gram-negative's lipopolysaccharide (LPS), diffuse to the periradicular tissues inducing apical inflammation. Also, endodontic bacterial DNA has frequently been identified in AL, supporting bacterial translocation beyond the tooth structures [34]. During AP, endodontic pathobionts and their motifs would switch macrophages to the functional M1 phenotype at the time of TLR ligation displaying increased antigen presentation and microbial killing capabilities, by modifying their profiles of cytokines and chemokine production, NO metabolism, phagocytosis, and transcriptional profiles [16]. A more striking imbalance towards M1 polarization over M2 might mediate the onset of SAP from previously existing AAP. In fact, the later might represent a more balanced stage between pathogen killing functions and immune modulation/healing [16, 19, 35].

Analysis of median cell frequencies (percentages) for single cell markers hinted a similar tendency as seen for double-positive macrophages increasing progressively from healthy controls to AAP and SAP, except for CD163$^+$ cells, which remained high and close to 100% of cells in the three conditions. Of interest, CD163 differentiated from the other cell surface markers evaluated in the fact that it was expressed in most macrophages independently of the apical condition; instead, we observed differences in CD163 intensity levels. Our results demonstrated that CD163 MFI was progressively and significantly downregulated from HPL controls, in which were the highest, followed by AAP and were the lowest in SAP. In line with this, CD163-expressing macrophages have been detected in sites of inflammation such as chronically inflamed arthritis joints, atherosclerotic plaques, and the vicinity of tumor cells (tumor associated macrophages) [36]. Altogether, these findings suggest that during endodontic infection, apical lesion macrophages might express CD64$^+$CD80$^+$ surface phenotype, whereas transition towards SAP from AAP associates with further skewing towards M1 due to a drop in the density of the M2 markers in the cell surface, especially of CD163. During the symptomatic phase of AP, exposure of M2 macrophages to M1 signals, such as higher concentration of LPS or Th1-type of cytokines, might further reprogram them towards M1 phenotype, along with declined M2 scavenger functions. CD163 is a specific marker of anti-inflammatory monocytes/macrophages, which is up regulated during macrophage colony stimulating factor (M-CSF)-dependent macrophage differentiation of human blood monocytes [37]. In tissue macrophages, CD163 transduces signals upon binding of its ligands that lead to release of anti-inflammatory mediators, such as IL-10 and IL-1RA, and inhibition of pro-inflammatory cytokines. In fact, exogenous pro-inflammatory molecules, such as LPS, can decrease the expression of CD163 in SAP and conversely, the expression levels of CD163 on tissue macrophages increase during the resolution of acute inflammatory response or during the wound healing phase, as might occur during AAP [38]. These findings further support that SAP associates with an exacerbated immune response.

Functionally, macrophages can initiate and modulate T cell responses, control tissue homeostasis, and interact with stem cells [32]. In line with a predominant M1 macrophage polarization profile, we also found a significant upregulation of the hallmark M1 cytokines IL-12, IL-23 and TNF-α in SAP and AAP versus controls. Strikingly, IL-6 was up regulated in SAP, followed by AAP and controls respectively, with significant differences among the three conditions, whereas IL-23 was higher in SAP versus AAP and controls. The expression levels IL-10 showed a similar behavior as most M1 cytokines, but TGF-β was significantly lower in SAP over HPL. These results further confirm that macrophages functionally skew

towards a predominant M1 over M2 phenotype especially in SAP, at the expense of higher pro-inflammatory and lower anti-inflammatory cytokine profiles.

SAP has previously been characterized by elevated expression of MMP-1, MMP-2, MMP-8, MMP-13 over AAP [10] and elevated bone resorptive activity compared with AAP and controls based on tartrate-resistant acid phosphatase (TRAP) levels [9]. The perpetuation of inflammation by macrophages leads to the recruitment of the adaptive immune response. M1 macrophage activation results in the secretion of pro-inflammatory cytokines and promotion of Th1 and Th17 patterns of immune responses [31], which are directly associated with periodontal breakdown [17]. Specifically, increments of IL-23 and IL-6 are involved in Th17 differentiation and osteoclastogenesis, by producing receptor-activator of nuclear factor kappa-B ligand (RANKL), in association with the initiation and progression of periodontitis [39, 40]. In contrast, M2 macrophages induce Th2 and Treg responses [31] and are periodontally protective. In line with this, the induction of M2 macrophage phenotype reduced the alveolar bone loss in experimental periodontitis model [18].

IL-6 is also a key cytokine for the crosstalk between the local and systemic inflammatory responses by inducing C-reactive protein (CRP) synthesis in the liver and mononuclear cells. Indeed, our group recently demonstrated CPR synthesis also in healthy periodontal tissues and apical lesions [4, 41], which might contribute to explain their association with systemic inflammation and cardiovascular risk [4, 42–44]. In support of this, an experimental murine model of marginal periodontitis reported an enhancement of M1 to M2 macrophage phenotype markers and respective cytokine profiles in gingival tissue along with systemic inflammation based on higher IL-6 to IL-10 levels in serum compared to healthy controls [20]. These results suggest that predominant M1 macrophage profile in AL might be associated with symptomatic lesions, exacerbated alveolar bone resorption and also higher propensity to systemic inflammation and related non-communicable diseases.

Overall, the present study analyzes for the first time M1 and M2 macrophage profiles in AP through flow cytometry. A strength was the inclusion of a panel of surface markers for M1 and M2 along with a pan macrophage marker [19, 26, 27]. A methodological drawback as previously described was the significant age difference among healthy individuals and patients with AL which is inherent to the inclusion of HPL as controls, though no differences were found between AAP and SAP [9, 12]. It has also been described that smoking influences the progression of marginal periodontitis [45], although no evidence supports this fact in apical periodontitis [46] and smoking habit was evenly distributed among study groups, as well as the other demographic factors. Finally, even so, a smaller number of control samples were analyzed due to methodological limitations, including the low quantities of obtainable cells and mRNA; they showed considerably less variability and provided enough statistical power to detect significant differences.

In summary, in this study, we demonstrated that macrophages exhibited an increased M1/M2 ratio in AL over HPL, based on $CD64^{+}CD80^{+}/CD163^{+}CD206^{+}$ macrophage phenotypes and the overall pro-inflammatory cytokine profile; SAP showed reduced CD163 expression and elevated IL-6 and IL-23, supporting a relevant role in the severity and/or progression of apical lesions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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