ORIGINAL RESEARCH
Immunology

Influence of genetic regulatory effects modified by environmental immune activation on periapical disease

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Abstract: The objective of this study was to compare the periradicular responses in endodontic infections among members of two populations: an urban Brazilian population and a non-mixed indigenous population. Samples were collected immediately and 7 days after the cleaning and shaping procedures (after reducing the intracanal microbial load) in an attempt to characterize the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-9, interferon (IFN)-γ, IL-17, IL-10, and the chemokines CXCR4, CCL2/monocyte chemotactic protein (MCP)-1, and CCR6. The endogenous cytokine and chemokine expression levels were analyzed using real-time PCR. Only the urban population showed a significant increase in TNF-α, CCL2/MCP-1, CXCR4, and CCR6 expression following the cleaning and shaping of the root canal system. The IFN-γ levels were increased at the 2nd collection ($p < 0.05$) in the indigenous population. In turn, a significant increase in IL-10 and IL-17 expression ($p < 0.05$) was observed after the cleaning and shaping procedures (2nd collection) in both populations. No significant differences in the IL-1β, IL-9, and CCL4 expression levels were observed between the 1st and 2nd collections in both populations. The results demonstrate a cytokine and chemokine expression profile that is specific to each analyzed population. However, immune modulation mediated by IL-10 began on the 7th day after the beginning of the endodontic treatment in both populations.

Keywords: Cytokines; Root Canal Therapy; Population Groups; Indigenous Population.

Introduction

The host response against microorganisms present in the root canal system (RCS) involves immunological mechanisms, with some components acting to protect pulp and periapical tissue and other mediators being involved in periapical bone destruction, specifically bone resorption.[1] Our immune system is incredibly complex, with diverse armies of white blood cells and signaling proteins coursing through our veins, ready to mount an attack on potential invaders. Every individual's immune system is slightly different and contains a unique mixture of hundreds of these cells and proteins. However, the main driver of this variation is unclear.[2]

A wide variety of cells are present in human periradicular lesions, including CD4+ and CD8+ T lymphocytes, macrophages, antigen-presenting cells, mast cells, and eosinophils. However, T cells are the most numerous cells in these lesion.[3]

CD4+ T lymphocytes are subdivided into Th1 and Th2 subsets according to the cytokines they produce. Other cells, such as Th17 and regulatory T (Treg) cells, also modulate the periapical immune response. The Th1 response is characterized by the production of interferon (IFN)-γ, interleukin (IL)-2, IL-12, and tumor necrosis factor (TNF), which are involved in the development and progression of periradicular bone destruction.[3] The Th2 response induces the synthesis and activity of the IL-4, IL-5, IL-6, IL-9, and IL-13 cytokines, which are involved in the healing and regeneration of periradicular tissues.[4] The Th17 subtype produces IL-17, which is a proinflammatory cytokine that acts on several cells involved in the innate response and is considered a bridge between the innate and adaptive responses.[5] Treg cells, which produce transforming growth factor (TGF)-β and IL-10, have an inhibitory effect on bone resorption during osteoclast formation and differentiation and regulate the immune response against infection.[3]

Chemokines are proteins that regulate and determine the nature of the immune response and control leukocyte trafficking. They are essential because they act as chemotactic agents and are precursors of osteoclasts. Their respective receptors (CXCR4 and CCR1) are also involved in the bone resorption process and homeostasis.[6] Monocyte chemotactic protein (CCL2/MCP-1) has been detected in periapical granulomas and is associated with the modulation of human periapical lesions. MCP-1/CCL2-CCR2 activation plays a role in the activation and migration of macrophages while limiting polymorphonuclear (PMN) leukocyte infiltration.[7] CCR6 has been shown to be important for B-lineage maturation and antigen-driven B cell differentiation and may regulate the migration and recruitment of dendritic and T cells during inflammatory and immunological responses.[8]

The systemic conditions of individuals affect these periapical immunoinflammatory responses.[6,9] Recently, analyses of genetic polymorphisms have demonstrated their effects on pulpal-periradicular responses.[10,11,12] Additionally, researchers have recently shown that most immune responses are genetic, very personalized, and finely tuned.[13] As a result, we are likely to respond to an infection in a very individualized manner. Moreover, the diversity and activity of leukocytes are controlled by genetic and environmental influences to maintain balanced immune responses.[14] These factors may influence the extent and severity of the periapical inflammatory response.

The objective of this study was to compare the periradicular immune responses in two genetically diverse populations: an urban Brazilian population and a non-mixed indigenous population of the Tikuna – Wochtimaücü tribe (Amazonas, Brazil). Clinical samples were collected from the interstitial fluid adjacent to the infected root canal in both groups immediately after instrumentation of the RCS and seven days later. The expression of the cytokines TNF-α, IL-1-β, IL-9, IFN-γ, IL-17, and IL-10 and the chemokines CXCR4, CCL2/MCP-1, and CCR6 was evaluated using real-time PCR. The null hypothesis of this study is that each population carries several minor regulatory variants that have arisen over time in response to environmental factors, which may cause significant quantitative genetic variability among hosts in the regulation of the periapical immune response.

Methodology

Ethical considerations

This study was approved by the ethics committee of UFMG (CAAE: 65529617.0.0000.5149) and was presented to and approved by the xxx leadership. All participants read and signed an informed consent form.

Patients

Clinical samples were collected from 24 individuals with RCS infections. Twelve individuals belonged to the Tikuna tribe (Amazonas, Brazil) and were treated at the Endodontics Clinic of Paulista University, Manaus campus, Amazonas, Brazil. Twelve other individuals

living in the city of Belo Horizonte, Minas Gerais, Brazil, were treated at the Endodontics Clinic of the Federal University of Minas Gerais.

Collection of clinical specimens

Clinical samples were taken from teeth with pulp necrosis and apical periodontitis, which were diagnosed based on clinical and radiographic analyses and pulp sensitivity tests. The teeth did not present acute periapical symptoms at the time of the appointment. The sampling procedures were performed as previously described.[7] Each tooth was isolated, and the root canals were cleaned and shaped using ProTaper universal NiTi files (Dentsply Maillefer, Ballaigues, Switzerland) and 2.5% sodium hypochlorite. Samples were collected immediately after root canal cleaning, when the cytokine expression corresponded to the response to the ongoing infection. After cleaning and drying, three paper points (#20) were introduced into the root canal and passively passed through the root apex (2 mm) into the periapical tissue, where they remained for 1 min.

The paper points were cut 4 mm from the tip, placed into microcentrifuge tubes, and stored at -70°C. This procedure was used to extract RNA from the periapical interstitial fluid. No endodontic dressing was inserted into the root canals. The coronal access cavities of the teeth were restored using eugenol-based cement. Seven days later (day 7), the teeth were opened, and the periapical interstitial fluid was resampled to characterize cytokine/chemokine expression in the teeth with reduced root canal bacterial loads, as demonstrated elsewhere.[15] Single- and multiple-root teeth were included in this study. In teeth with multiple canals, the first (day 0) and second (day 7) samples were collected from the same canal. At this time point, no teeth exhibited clinical signs or symptoms, and the root canals were filled using the lateral compaction technique.

Sample preparation

Total RNA was extracted from each sample using TRIzol reagent (Gibco/BRL Laboratories, Grand Island, NY, USA), as described elsewhere [7-9], and then stored at -70°C.

Real-time PCR

Complementary DNA was synthesized using 1 μg of RNA and reverse-transcribed, as described previously.[16] The primer sequences were designed using Primer Express software (Applied Biosystems, Foster City, USA) based on nucleotide sequences available in the GenBank database. Real-time PCR assays were performed using Primer Express software (Applied Biosystems). The primer sequences used for the quantitative PCR analysis of TNF-α, IL-1-β, IL-9, IFN-γ, IL-17, IL-10, CXCR4, CCL2/MCP-1, and CCR6 mRNA expression are provided in Table 1. PCR was performed under the following standard conditions: a holding stage at 95°C (10 min); a cycling stage of 40 cycles at 95°C (15 s), followed by 60°C (1 min); and a melting curve stage at 95°C (15 s), 60°C (1 min), and 95 °C (15 s). A SYBR Green detection system (Applied Biosystems) was used to visualize primer amplification. Following amplification, a melting curve analysis was performed to determine the specificity of the amplified products. The melting curve was obtained from 60°C to 95°C, and continuous fluorescence measurements were recorded for every 1% increase in temperature. PCR products with melting temperatures that diverged from those established for standard DNA were considered false positives; for these cases, a null fluorescence value was assigned. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization and was assayed with each set of reactions. All samples were assayed in duplicate. Each reaction was performed using a volume of 25 μL containing 1 μg of cDNA. Sequence Detection System (SDS) Software version 2.4.1 (Applied Biosystems) was used to analyze the data after amplification. The results were obtained as threshold cycle (Ct) values, and the expression levels were calculated using the comparative $2^{-\Delta\Delta CT}$ method.[16] The results were calculated as the mean value of duplicate assays for each patient, and the mRNA expression level for each specific primer in all samples was defined as the ratio to the GAPDH expression level (Table).

Table. Primer sequences.

MARKERS	5′- 3′ Sequence	Mt (°C)
GAPDH	5′-GCA CCA CCA ACT GCT TAG CA- 3′ 5′ -GGC ATG GAC TGT GGT CAT GAG – 3′	80
IL-1β	5′ -TGG CAG AAA GGG AAC AGA A- 3′ 5′ -ACA ACA GGA AAG TCC AGG CTA- 3′	73
IFN-γ	5′ -GAA CTG TCG CCA GCA GCT AAA- 3′ 5′ -TGC AGG CAG GAC AAC CAT TA- 3′	80
TNF-α	5′ -TTC TGG CTC AAA AAG AGA ATT G- 3′ 5′ -TGG TGG TCT TGT TGC TTA AGG- 3′	76
IL-9	5′-CAT CAG TGT CTC TCC GTC CCA ACT GATG-3′ 5′-GAT TTC TGT GTG GCA TTG GTC AG-3	62.9
IL-10	5′ -GGT TGC CAA GCC TTG TCT GA- 3′ 5′ -TCC CCC AGG GAG TTC ACA T- 3′	81
IL-17	5′ -CAA TGA CCT GGA ATT ACC CAA- 3′ 5′ -TGA AGG CAT GTG AAA TCG AGA- 3′	70
CXCR4	5′ -TGT TGG CTG AAA AGG TGG TC- 3′ 5′ -AAA GAT GTC GGG AAT AGT C- 3′	80
CCL2/MCP-1	5′ -AAG ACC ATT GTG GCC AAG GA- 3′ 5′ -CGG AGT TTG GGT TTG CTT GT- 3′	80
CCR6	5′-CCA TTC TGG GCA GTG AGT CA-3′ 5′ -AGCAGCATCCCGCAGTTAA-3	60.5
CCL4	5′ -TCT CCT CAT GCT AGT AGC TGC CTT- 3′ 5′ -GCT TCC TCG CAG TGT AAG AAA AG- 3′	78

Statistical analysis

SPSS for Windows (version 15.0; SPSS, Chicago, USA) was used to perform the data analysis. The data were subjected to the Shapiro–Wilk test to characterize normality. Because the samples did not present a normal distribution, the Wilcoxon test was used to determine significant differences between groups ($p < 0.05$).

Results

The mRNA expression levels were determined by real-time PCR and quantified by comparison with the internal control gene GAPDH. The assessment revealed significant increases in the expression of TNF-α, MCP-1, CXCR4 and CCR6 in teeth with reduced bacterial loads (second collection) compared to the initial levels (first collection) only in the urban individuals (Figure 1b, Figure 2a, c, d). Conversely, the mRNA level of the IFN-γ gene was increased on day 7 ($p < 0.05$) (Figure 2b) in the indigenous population. The expression levels of IL-17A and the Treg mediator IL-10 were increased in both populations on day 7 ($p < 0.05$) (Figure 1c, d).

Significant differences were not observed in the IL-1β, IL-9, or CCL4 mRNA expression levels between the first and second collections in either population (Figure 1a, Figure 3a, b).

Discussion

Subsets of pathogens and human hosts have been geographically compartmentalized over long periods of time. This study aimed to determine whether changes in human society brought about by technological advances, such as the ease of travel

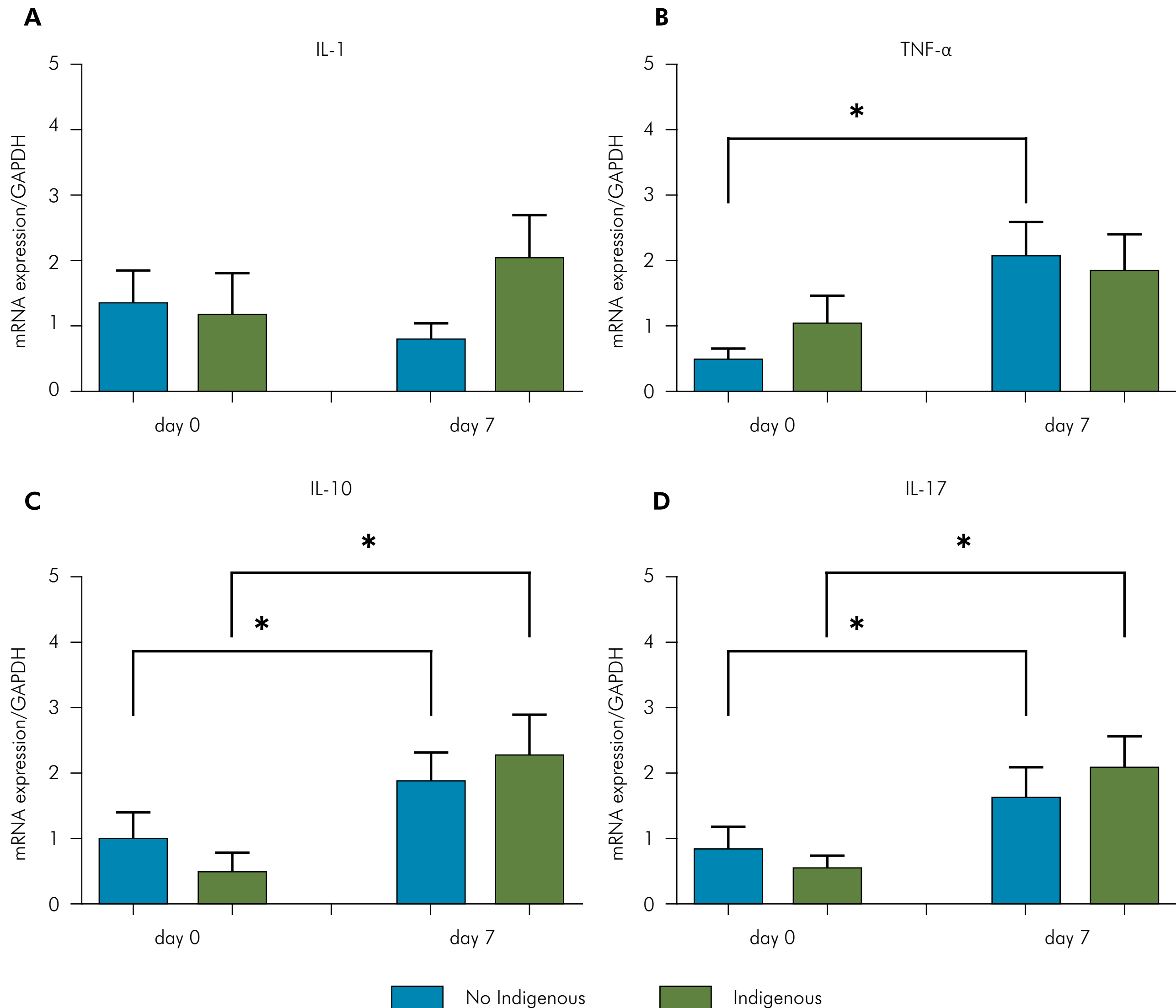

Figure 1 (a-d). Expression of TNF-α, IL-1, IL-17 and IL-10 genes in the periradicular tissues of indigenous and urban individuals with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with an internal control (GAPDH). Bars represent the mean values of samples recovered from teeth that did or did not receive endodontic treatment; lines represent the standard error of the mean. $^*p < 0.05$ by Wilcoxon or Mann–Whitney test.

and wide availability of antibiotics, have led to an increased danger of pathogenic infections and human genetic variation that interfere with the periapical immune response.

Apical periodontitis is a condition characterized by inflammation or destruction of the periradicular tissues and is considered a defensive reaction of the organism against microorganisms and their byproducts. This inflammatory response involves the recruitment and activation of macrophages, plasma cells, mast cells, eosinophils, PMN cells, and T ($CD4^+$ and $CD8^+$) and B lymphocytes, as well as the expression of mediators, such as cytokines, chemokines, arachidonic acid and its metabolites, and neuropeptides.[9,17] The diversity and activity of leukocytes are controlled by genetic and environmental influences to maintain a balanced immune response.[14] However, the relative contribution of environmental factors compared with genetic factors to variations in immune traits is unknown. Here, we analyzed the

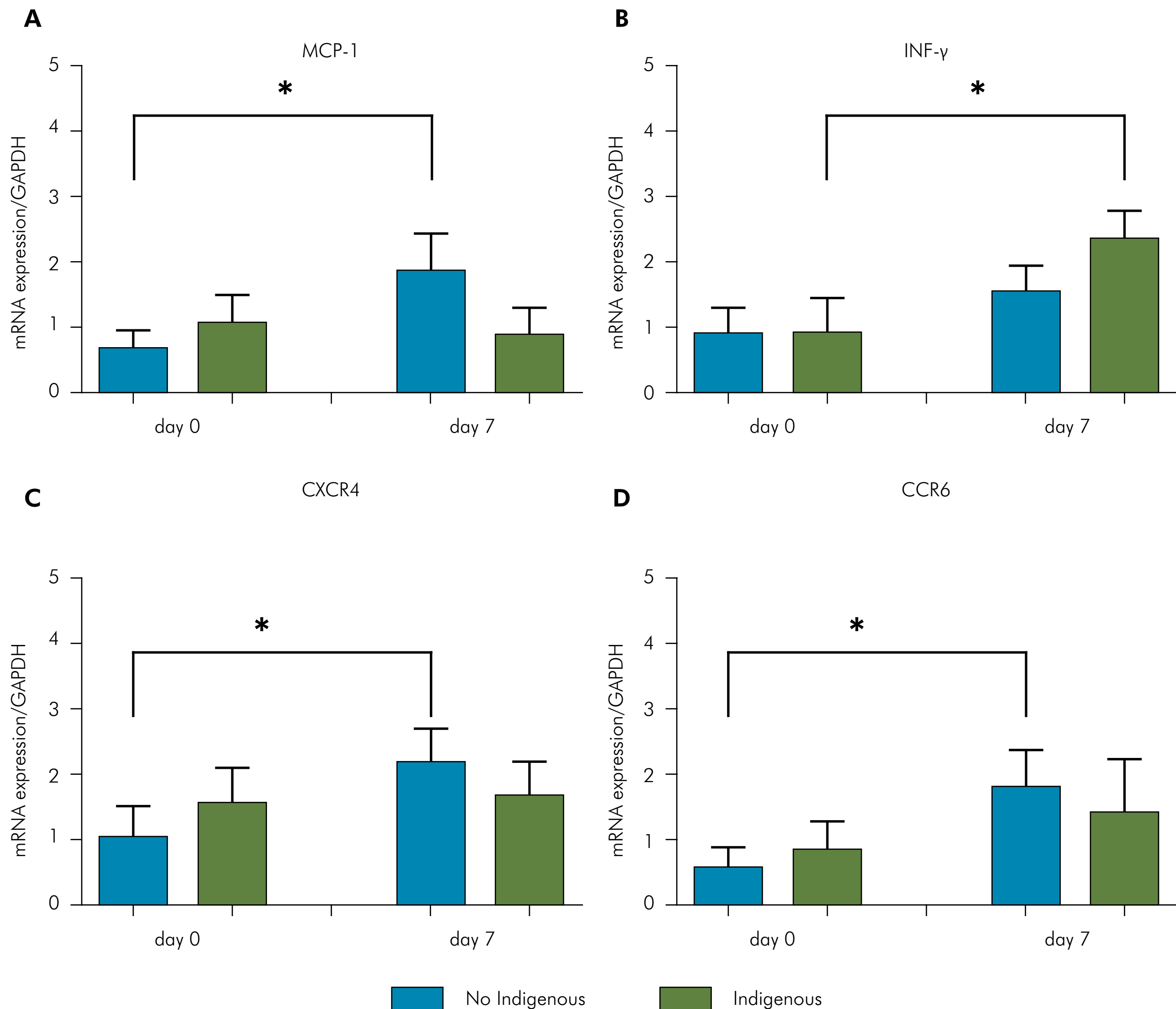

Figure 2 (a-d). Expression of MCP-1, INF-γ, CXCR4 and CCR6 genes in the periradicular tissues of indigenous and urban individuals with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with an internal control (GAPDH). Bars represent the mean values of samples recovered from teeth that did or did not receive endodontic treatment; lines represent the standard error of the mean. *$p < 0.05$ by Wilcoxon or Mann–Whitney test.

periradicular immune responses in two genetically and environmentally diverse populations located more than 3,000 km apart: an urban Brazilian population and a non-mixed indigenous population of the Tikuna – Wochtimaücü tribe (Amazonas, Brazil).

The progression of apical periodontitis and consequent bone resorption are attributed to the Th1 response, which induces osteoclast differentiation and activation. Th1 cells produce a proinflammatory response that involves the production of IFN-γ, TNF-α, and IL-1β.[18] TNF-α and IL-1β are proinflammatory and osteoclastogenic cytokines that are involved in the progression of periradicular lesions.[19] In this study, IL-1β expression remained at baseline levels at both time points in the two evaluated populations, in agreement with previous results demonstrating that the levels of this cytokine were not significantly different between diseased teeth and healthy control teeth in urban individuals.[20,21] Conversely, TNF-α expression was significantly increased only in

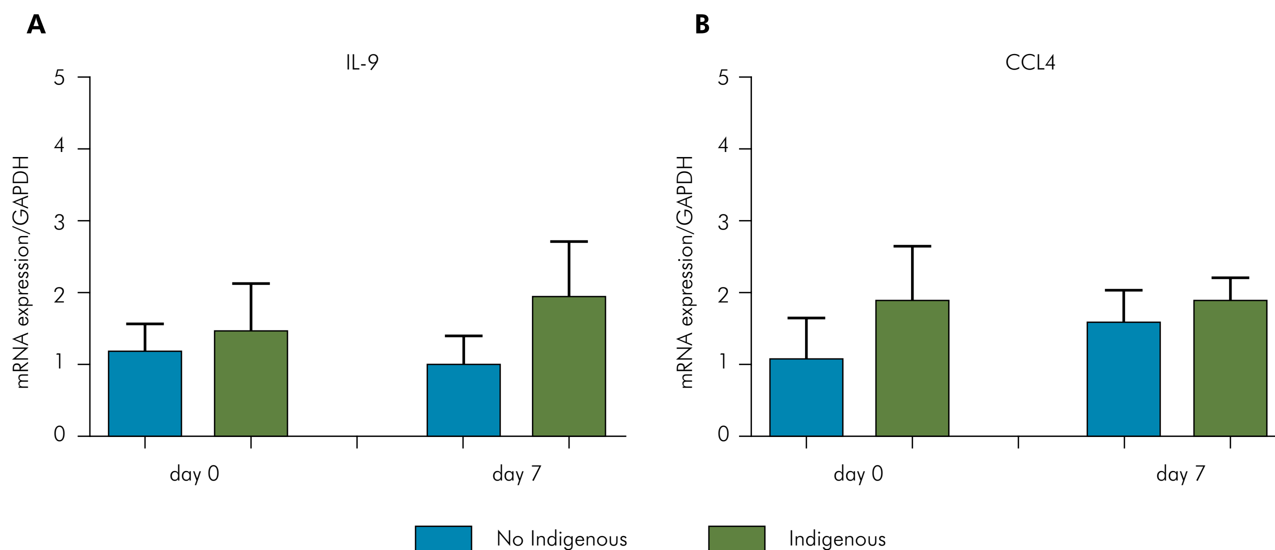

Figure 3 (a, b). Expression of IL-9 and CCL4 genes in the periradicular tissues of indigenous and urban individuals with root canal infections. Levels of expression were determined by real-time PCR and quantified by comparison with an internal control (GAPDH). Bars represent the mean values of samples recovered from teeth that did or did not receive endodontic treatment; lines represent the standard error of the mean. $^{*}p < 0.05$ by Wilcoxon or Mann–Whitney test.

the urban population 7 days after RCS cleaning and shaping. For both populations, the increased expression of mediators of the immune response may have advantages and disadvantages. On the positive side, a more intense immune response, such as that indicated by the increased TNF-α mRNA expression level in the urban population, may clear infections more rapidly. On the negative side, immune effectors can often be harsh and cause collateral damage to host tissues, e.g., increased periapical bone loss.

IFN-γ is the main activator of macrophages, which subsequently produce cytokines and other mediators that play significant roles in the development of periradicular diseases.[22,23] Here, only the indigenous population showed a significant increase in IFN-γ expression on the seventh day. Conversely, a decrease in IFN-γ mRNA expression was demonstrated in the urban population after the root canal cleaning procedures.[6,9] Moreover, epigenetic events may be relevant to IFN-γ modulation in dental pulp.[10] The difference in the cytokine expression levels between the populations demonstrates that it is crucial to understand both the variability of immune responses at the population level and how this variability relates to disease susceptibility.[24]

IL-17A is expressed by members of the Th17 subtype and is believed to regulate osteoclastogenesis.[25] IL-17 expression and maintenance of the inflammatory response may play important roles in the elimination of microorganisms.[26] Here, the levels of this cytokine were significantly increased after the cleaning and shaping procedures (second collection) in both populations, which is important for the exacerbation of inflammation and the clearance of microbial periapical contamination.[3,27]

Treg cells produce TGF-β and IL-10, which are important immunoregulatory cytokines that have an inhibitory effect on bone resorption; these cytokines are also involved in regulating the immune response against infection.[3,28] In this study, both populations presented high IL-10 mRNA expression levels after the cleaning and shaping procedures, which suggested that immune regulation at this time point started to decrease the proinflammatory response that was initiated earlier. Although the results of this study reinforce the idea that the immune system responds to periapical infection in a very individualized manner,[13] periapical immune regulation occurred in a similar manner in both populations after the cleaning and shaping procedures.

Th9 cells have been shown to have interesting plasticity and either act synergistically with Th2 cells in some inflammatory processes or perform immunosuppressive actions via IL-10 production.[29] In this study, no significant differences were observed in the IL-9 mRNA expression levels in either population after the cleaning and shaping procedures. This finding correlates with the increase in IL-10 expression that paralleled the increase in IL-17 expression at the second collection, which may interfere with the differentiation of the Th9 phenotype to the Th2 or Th1/Th17 subtype.

Chemokines are proteins that regulate and determine the nature of immune responses and control immune cell trafficking by directing the movements of cells required for the initiation of T cell-mediated immune responses; these actions are thought to be important for recruiting appropriate effector cells to sites of inflammation, including the differential recruitment of Th1 and Th2 cells.[30] Here, significant differences in mRNA expression of the CCL2/MCP-1 chemokine and the CXCR4 and CCR6 chemokine receptors were observed between the two analyzed time points only for the urban population. These chemokines and receptors, along with CCL4, were expressed at basal levels at both evaluation time points in the indigenous population. For these mediators, we suggest that the immune system responds in an individualized manner to root canal infections, depending on the genetic and environmental conditions. CCL2/MCP-1 not only influences inflammatory cell recruitment but also affects effector T cell differentiation because it leads to decreased IL-12 production by macrophages,[31] which suppresses Th1 responses. In the urban population, its expression was high at the second collection, which paralleled the increase in the expression levels of the proinflammatory cytokines TNF-α and IL-17. No significant difference in CCL2 expression was observed after cleaning procedures elsewhere,[6,9] but an opposite result was demonstrated by others.[32] CXCR4 is the receptor of the anti-inflammatory immune modulator ubiquitin;[33] in this study, CXCR4 was significantly increased in the urban population after the cleaning procedures, in parallel with IL-10, which is congruent with the results of a previous study.[9] Moreover, CXCR4 and CCR6 act as co-receptors for HIV entry.[3] CCR6 is preferentially expressed by immature dendritic cells and memory T cells.[8] In the urban population, CCR6 mRNA expression was increased after the cleaning procedures, which is in agreement with findings showing that this mediator is essential for the recruitment of both proinflammatory IL-17-producing helper T cells (Th17) and Treg cells to sites of inflammation.[35]

The null hypothesis was accepted; minor regulatory variants that have arisen over time in response to environmental factors caused significant quantitative genetic variability among hosts in the regulation of the periapical immune response. Because the functional plasticity and redundancy of the immune system complicate the experimental study of each of its components, the results of this study contribute to the overall understanding of the specific immune responses that predominate in the periapical area in each individualized urban and indigenous population after instituting clinical endodontic therapy. However, to date, the genetic mechanisms and environmental factors that regulate the homeostasis of cell numbers and phenotypes in the peripheral immune system, especially in periapical lesions, remain poorly understood.

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