LUCAS MOREIRA MAIA

PAPEL PROTETOR DAS CITOCINAS IL-9/IL-10 EM LESÕES PERRIRADICULARES HUMANAS.

Faculdade de Odontologia

Universidade Federal de Minas Gerais

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Dissertação apresentada ao Colegiado do Programa de Pós-Graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do grau de Mestre em Odontologia – área de concentração em endodontia

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Ata de Defesa

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“A vida é como andar de bicicleta. Para ter equilíbrio, você tem que se manter em movimento...” *Albert Einstein*

Resumo

Este estudo tem como objeitvo Identificar a expressão gênica de um novo grupo de linfócitos T, as células Th9, caracteristicamente responsável por produzirem a IL-9 nos tecidos perirradiculares de indivíduos portadores de infecções endodônticas submetidos à terapia endodôntica de rotina, na presença e ausência de infecção assim como as citocinas TNF-α, IL-1. IL-9, INF-ɣ e IL-10 e das quimiocinas CCL-2/MCP-1 e CCR-6 no fluido intersticial periapical de infecções de canais radiculares humanas. As amostras foram coletadas imediatamente após os procedimentos de limpeza e formatação e 7 dias mais tarde (após redução da carga microbiana intracanal) para caracterizar a expressão destes genes. A reação em cadeia da polimerase em tempo real demonstrou níveis significativamente maiores de marcadores de IL-1, IL-9, INF-ɣ, TNF-α e IL-10 no dia 7 quando comparado com ao dia 0. Por sua vez, as quimiocinas CCL-2/MCP-1 e CCR-6 e a citocina IL-17A não apresentaram diferenças significativas na expressão de mRNA entre os 2 períodos analisados. Ao analisar a variação clínica pós terapia endodôntica sobre a condição imune periapical, este estudo demonstrou que a resposta pró-inflamatória mediada por citocinas e quimiocinas parece ser modulada de forma IL-10/IL-9 dependente.

Palavras-chave: Interleucina. IL-9. TH9. Periodontite apical. Citocina e Quimiocina.

ABSTRACT

Protective role of IL-9 and IL-10 in human periradicular lesions

This study aims to identify the gene expression of a new group of T lymphocytes, Th9 cells, characteristically responsible for producing IL-9 in the periradicular tissues of individuals with endodontic infections submitted to routine endodontic therapy, in the presence and absence of infection as well as the cytokines TNF-α, IL-1. IL-9, INF-γ and IL-10 and CCL-2 / MCP-1 and CCR-6 chemokines in the periapical interstitial fluid of human root canal infections. Samples were collected immediately after cleaning and formatting procedures and 7 days later (after reduction of intracanal microbial load) to characterize the expression of these genes. Real-time polymerase chain reaction demonstrated significantly higher levels of IL-1, IL-9, INF-ɣ, TNF-α and IL-10 markers at day 7 compared to day 0. In turn, the CCL-2 / MCP-1 and CCR-6 chemokines and IL-17A cytokine showed no significant differences in mRNA expression between the 2 periods analyzed. In analyzing the clinical variation after endodontic therapy on periapical immune status, this study demonstrated that the cytokine and chemokine-mediated proinflammatory response appears to be modulated in a IL-10 / IL-9 dependent manner.

Keywords: Interleukin. IL-9. TH9. Apical periodontitis. Cytokine and Chemokine.

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LISTA DE ABREVIATURAS E SIGLAS

SCR: Sistema de Canais Radiculares

IFN- ɣ: Interferon Gamma

IL: Interleucina

TNF- α: Fator de Necrose Tumoral

Th: Célula T Helper

CCL2/MCP: C-C Ligante de Quimiocinas 2/ Monocyte Chemotactic Protein

CCR6: C-C Ligante de Quimiocinas CCl20

IL-10: Interleucina 10

IL-1: Interleucina 1

IL-9: Interleucina 9

IL-17A: Interleucina 17A

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

O complexo dentino-pulpar, é constituído pelo órgão pulpar e paredes dentinárias circundantes, formando uma barreira física contra patógenos oportunistas (Stashenko & Wang 1992; Jontell., *et al* 1998). Em condições normais, os sistemas de canais radiculares (SCR) não apresentam uma microbiota residente. Entretanto, nos dentes cujo suprimento vascular encontra-se comprometido, o ambiente torna-se favorável à contaminação por patógenos oportunistas (Sundqvist, 1992; Stashenko *et al.*, 1998).

As patologias pulpares e perirradiculares iniciam-se no momento em que microorganismos oportunistas violam a estrutura dental através de lesões cariosas, traumas, restaurações mal adaptadas e defeitos estruturais, como trincas e fissuras produzidas pelo estresse mastigatório (Rittling *et al*., 2009).

A destruição tecidual, corresponde a reações imuno-inflamatórias, em uma tentativa de conter as toxinas secretadas pelo metabolismo bacteriano no interior do sistema de canais radiculares (Takahashi *et al*., 1998; Fukada *et al*., 2009; Brito *et al*., 2012).

Sendo assim a infecção dos SCR é necessária e suficiente para que ocorra a instalação e o desenvolvimento de uma lesão perirradicular. Seu desenvolvimento dependerá, contudo, da composição da microbiota infectante, bem como da resposta do próprio hospedeiro (Ribeiro Sobrinho *et al*., 2002; 2005). Sendo caracterizadas pela destruição de tecidos mineralizados que rodeiam o ápice radicular em consequência da resposta imune inflamatória (Nowak EC, *et al.,* 2009)

As células adjacentes ao forame radicular são responsáveis pela liberação de mediadores pró-inflamatórios: quimiocinas e moléculas de adesão que ao alcançar os vasos sanguíneos aumentam sua permeabilidade e são responsáveis por promover e estímular o recrutamento de leucócitos para o sitio agredido (Márton & Kiss, 2014). As quimiocinas ao se ligarem as moléculas da matriz extracelular, promovem um estimulo para que ocorra a migração de células de defesas. Estas são responsáveis pela

destruição de patógenos e por lesar os tecidos adjacentes, além de atrair células de defesa do sistema imune inato e adaptativo (Márton & Kiss, 2014).

Os linfócitos T apresentam um papel importante na resposta imunoinflamatória. São compostos pelos subtipos $CD8^+$(citotóxico) e $CD4^+$ (auxiliar/helper). Os linfócitos Th helper são divididos em vários subgrupos que incluem as células: Th1, Th2, Th17 e T regulatórias (T_{reg}) que se diferem mediante ao grupo de citocinas expressados (Aranha *et al.,* 2013). A resposta do tipo Th1 é caracterizada pela produção e expressão das citocinas IL-1, IL-2, IL-12 e interferon gamma (INF-ɣ), resultando na progressão das lesões e destruição óssea perirradicular (Stashenko *et al.*, 1998). Por sua vez, as citocinas IL-4, IL-5 e IL-13, são componentes da resposta Th2. (Márton & Kiss, 2014), relacionando-se com a cicatrização e regeneração dos tecidos perirradiculares (Stashenko *et al.*, 1998; Teixeira-Salum *et al.*, 2010). As células Th17 são caracterizadas pela produção das interleucinas IL-17, IL-1β, fator de necrose tumoral (TNF-α), IL-6, IL-8, IL-23 e metaloproteinases, e estão relacionadas com o desenvolvimento de condições autoimunes e exacerbação do processo inflamatório (Yang, S. *et al*., 2014). Já os linfócitos linfócitos T_{reg} expressam o fator de transcrição forkhead box P3 (Foxp3), atuando na inibição da formação e função dos osteoclastos (Yang, S. *et al*., 2014).

As citocinas Th1 e Th17 são considerados citocinas pró-inflamatórias e as citocinas Th2 e T_{reg} anti-inflamatória. Estas foram previamente investigadas em lesões periapicais (Brito LC *et al*., 2012; Fukada *et al*., 2009). Sendo que as citocinas do tipo Th1 e Th17 foram associadas a destruição óssea e progessão da lesão, e seus antagonistas Th2 e T_{reg} foram descritos por reparar o dano tecidual (Nowak Ec, *et al.,* 2009; Tan C, *et al.,I* 2010; Li H, 2010; Wan YY, 2010).

Recentemente, com a descoberta de novos subconjuntos Th, como as células Th9, que apresentam papeis importantes na modulação da resposta do hospedeiro interagindo com as subpopulações Th mencionadas anteriormente (Jager A *et al.,* 2010).

Aranha *et al* (2013) enfatizam a necessidade de reavaliar o paradigma das atuais citocinas na patogênese das lesões periapicais.

As células Th9 produzem caracteristicamente IL-9, inicialmente designada como citocina Th2 que apresenta propriedades interessantes de pleiotropia cujas múltiplas ações sobre diferentes tipos celulares demonstram sua importante participação em patologias inflamatórias, variando de ações anti-inflamatória a imunossupressora (Nowak Ec *et al.,* 2009; Tan C *et al.,* 2010; Li H, 2010; Wan, 2010). A resposta Th9 é capaz de promover uma mudança em seu fenótipo para Th1 ou Th17, mas um interruptor preferencial para Th2 é descrito reforçando as descrições iniciais das características anti-inflamatórias do subconjunto Th9 (Tan C *et al.,* 2010).

Dados mais recentes mostram que células T produtoras de IL-9 são distintas da linhagem Th2 convencional, sendo Th9 um nome cogitado para tais células. A diferenciação das células Th naive para o fenótipo Th9 é fortemente induzida pelas citocinas IL-4 e TGF-β, as quais são também responsáveis pela sinalização de fatores de transcrição que regulam a síntese de IL-9 (Jabeen R & Kaplan, 2012).

Em um estudo Fawaz *et al* (2007) observou que o sinergismo entre IL-4 e IL-9 estava associado a síntese de IgE nos linfócitos B, também observou que a produção de IL-9 sugeria a participação dessa citocina não apenas no desenvolvimento, mas também na manutenção da doença (asma).

Sendo assim, na presença de IL-4, a célula TCD4+ se diferencia em célula Th2, enquanto na presença de TGF-β e IL-4, esta célula se diferencia em Th9 (Veldhoen M *et al.,* 2008).

Aranha *et al* (2013), avaliou os níveis de expressão das citocinas Th9 em granulomas ativos e inativos, sua cinética de expressão e sua correlação entre outras citocinas para explorar seu potencial na patogênese das lesões periapicais. As lesões foram categorizadas em ativas e inativas, com base no perfil molecular de RANKL / OPG (RANKL>OPG ativas, RANKL<OPG inativas). Os resultados demonstraram a

sobre-expressão de IL-9 em granulomas inativos e uma correlação negativa com TNF-α, IFN-ɣ e IL-17 nas lesões periapicais.

No mesmo estudo Aranha *et al* (2013), utilizou um modelo animal para uma analise sequencial da expressão de citocinas durante os períodos de desenvolvimento das lesões, os resultados demonstraram que a expressão de IL-9 parece contribuir para um nível relativo de estabilidade da lesão. Em resumo, os resultados sugerem que a via Th9 pode contribuir para a estabilidade da lesão periapical humana e experimental, sendo necessários mais estudos para descobrir o mecanismo exato das ações imunossupressoras da IL-9 no ambiente periapical inflamado.

Em um estudo Araujo-Pires *et al* (2014) analisaram simultaneamente a expressão diferencial de vários subconjuntos de células Th em lesões periapicais humanas, e demonstraram que as citocinas são responsáveis pelo estado de atividade e inatividade das lesões. O estudo demonstrou a associação de IFN-ɣ, TNF-α, IL-17 e IL-21 com a atividade das lesões e a associação de IL-4, IL-9, IL-16, IL-22 e FOXp3 com a inatividade das lesões periapicais em humanos.

Assim torna-se necessário observar a expressão da IL-9 em processos inflamatórios de origem endodôntica, analisando a sua importância na modulação da resposta imune perriradicular em indivíduos portadores de infeções endodônticas.

2 JUSTIFICATIVA

O recente interesse em analisar o potencial biológico da IL-9 deve-se à descoberta de um novo grupo de linfócitos T, as células Th9, que estão diretamente envolvidas em diferentes respostas imunológicas, assim como no processo inflamatório pulpo-perriradicular. Assim, torna-se relevante conhecer o papel desta citocina no contexto da expressão de mediadores sabidamente envolvidos nos processos imune inflamatórios periapicais.

3 OBJETIVOS

Identificar a expressão gênica de uma citocina produzida pelo novo grupo de linfócitos Th9, a IL-9, em conjunto com citocinas e quimiocinas envolvidas na patogênese das lesões perirradiculares, em indivíduos portadores de infecções endodônticas submetidos à terapia endodôntica de rotina, na presença e na ausência de infecção.

3.1 Objetivos específicos:

Caracterizar, por PCR em tempo real, a expressão gênica das citocinas e quimiocinas (TNF-α, IL-1, IL-17A, IL-9, INF-ɣ, IL-10, CCR6 e MCP1) no fluído intersticial periapical de dentes com diagnostico de necrose pulpar.

4 ARTIGO CIENTÍFICO

Protective role of IL-9 and IL-10 in human periradicular lesions

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Abstract

Introduction: Cytokines, chemokines and receptors are essential for the periapical immune response to infection. Recently, new subgroups of T cells have been discovered, triggering the need to evaluate further their participation in the periapical immune response.

Methodology: The objective of this study was to identify the gene expression of the cytokines TNF-α, IL-1, IL-9, INF-δ, IL-17A and IL-10, and the chemokines CCL-2 / MCP-1 and CCR-6 in the periapical interstitial fluid of human root canal infections. 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 these genes. **Results:** Real-time polymerase chain reaction demonstrated significantly higher levels of the IL-9, INF-δ, TNF-α, IL-1, IL-9 and IL-10 markers at day 7 compared to day 0. IL-17A and the chemokines CCL-2 / MCP-1 and CCR-6, however, did not show significant differences in mRNA expression comparing both timepoints. **Conclusions:** The clinical variation of the periapical immune status after endodontic therapy showed that the cytokine and chemokine-mediated pro-inflammatory response appears to be modulated in an IL-10 / IL-9-dependent manner.

Keywords: interleukin, IL-9, TH9, apical periodontitis, cytokine and chemokine

Introduction

The pulp-dentin complex is comprised of the pulp organ and the surrounding dentin walls that form a physical barrier against opportunistic pathogens (Stashenko, 1992; Jontell et al., 1998). Normally, resident microbiota are not present in the root canal systems (RCS); however, when the pulp vascular supply is collapsed, the RCS becomes a fertile environment for opportunistic pathogens to grow (Sundqvist, 1992). Such infections lead to periradicular bone resorption and migration of several inflammatory cells to this site, subsequently promoting immunological surveillance against microbial pathogens (Stashenko et al., 1992; Marton & Kiss, 2014).

The immune response triggered by root canal infections is complex and involves the recruitment of cells, as well as the synthesis of several inflammatory mediators (Fukada et al., 2009; Brito et al., 2012; Marton & Kiss, 2014). Cytokines play an important role in the development of periradicular lesions, modulating the inflammatory immune response (Stashenko, 1992). Conversely, chemokines and their receptors mediate the recruitment of leukocytes to the injured sites, including the periradicular area (Silva et al., 2005). These mediators also take part in the healing process (Rossi & Zlotnik, 2000).

The interactions of the different subtypes of T cells and their mediators, notably Th1, Th2, Th17 and Treg cells, has been extensively investigated in periradicular lesions (Henriques et al., 2011; Brito et al., 2012; Aranha et al. (1999), Araujo-Pires *et al*., 2014; Bambirra *et al*., 2015; de Brito1 *et al*., 2015; Ferreira *et al*., 2015). The progression of the lesion and the bone destruction are consistently associated with Th1 and Th17 responses (Fukada et al., 2009; Brito et al., 2012), while the healing processes are related to the Th2 response (Kawashima & Stashenko, 1999). Both pro- and anti-inflammatory responses are regulated by regulatory T cells (Treg) by the cytokines IL-10 and TGF-b (Fukada et al., 2009; Romagnani, 2006). More recently, new T-cell subgroups have been described, such as Th9 and Th22 (Jager et al., 2010; Mucida & Cheroutre, 2010). This research has elicited the need for further evaluation of their contribution to periapical lesion development.

Th9 cells characteristically produce IL-9, initially designated a Th2 cytokine but also exert pro-inflammatory activities. Its anti- or pro-inflammatory profiles depend on the modulation by Treg and / or Th-17 cells (Nowak et al., 2009; Tan et al., 2010, Li, 2010. IL-9 demonstrates interesting pleiotropic properties with multiple effects on different cell types. This property is evidenced through its participation in inflammatory pathologies, ranging from anti-inflammatory to immunosuppressive actions (Nowak et al., 2009; Li, 2010). The Th9 response is capable of promoting a change in its phenotype, including changing to a Th1 or a Th17 subtype; however, it is described as presenting a preferable tendency towards a Th2 response (Tan et al., 2010).

The aim of this study was to evaluate quantitatively the mRNA expression of the cytokines TNF-α, IL-1, IL-9, INF-δ, IL-17A, and IL-10 and the chemokines CCL-2 / MCP-1 and CCR-6 in samples collected from human periradicular interstitial fluid adjacent to root canal infections. Samples were collected immediately after completion of the procedure and 7 days after the endodontic therapy. Finally, the data obtained at both times of evaluation were statistically compared.

Methodology

Patient selection

Ten patients (n = 10) between 20 and 52 years of age were referred to the School of Dentistry of the Federal University of Minas Gerais (UFMG), in Belo Horizonte, Brazil to receive endodontic treatment after diagnosis of pulp necrosis, regardless of whether they presented with radiographically detectable periradicular lesions. The collection of clinical specimens was performed at the Clinic of Endodontics of the School of Dentistry of the Federal University of Minas Gerais (FO-UFMG). The exclusion criteria included the use of systemic antimicrobial and anti-inflammatory medication in the three months prior to collection and patients who presented any systemic disorders. This study was approved by the Research Ethics Committee of the Federal University of Minas Gerais (CAAE: 56942016.2.0000.5149).

Clinical specimen sample collection

After the initial clinical procedures, the selected tooth had its clinical crown fully isolated using a rubber dam. This isolation was followed by a thorough asepsis following the method proposed by Möller (1966), which includes using 10% hydrogen peroxide for five minutes, 5% iodine dye for five minutes followed by a 5% sodium thiosulfate for another five minutes. The cleaning and formatting of the root canal system was performed using ProTaper Universal Nickel-Titanium files (Dentsply Maillefer, Ballaigues, Switzerland). Root canals were rinsed using 5.25% sodium hypochlorite. After the chemical and mechanical preparation of the RCS, as described by Brito et al. (2010), three # 20 absorbent paper tips were inserted into the wider canal of the tooth, exceeding 1 mm above the root foramen, as to stay in direct contact with the periradicular tissues for two minutes. No intracanal medication was placed into the canal between sessions. The coronary access of the dental element was closed with zinc oxide/eugenol paste. After 7 days, coronary accesses were reopened, and the periapical interstitial fluid was collected again to characterize the angiogenic factors and cytokines present after the reduction of the microbial load. In teeth with multiple canals, the first and the second collection (seven days after the first one) were performed in the same canal. At that moment, teeth that did not present any clinical signs or symptoms underwent obturation using the lateral condensation technique. The final four millimetres of the paper tips used to collect the samples were cut, inserted into an Eppendorf and immediately frozen at -80 °C.

Sample preparation

Total RNA was extracted from each sample with the TRIzol reagent (GIBCO / BRL Laboratories, Grand Island, NY), according to the previous description by Bambirra et al., (2015). RNA was then stored at -70 °C.

Real-time polymerase chain reaction

Complementary DNA was synthesized using 1 mg of RNA through the reverse transcription reaction as described by Barbosa Silva et al., (2007). Polymerase chain reaction (PCR) was performed according to standard conditions: a holding stage at 95 °C (10 minutes), a cycling stage with 40 cycles at 95 °C (15 seconds), and 60 °C (1 minute) and a melting curve stage at 95 °C (15 seconds), 60 °C (1 minute) and 95 °C (15 seconds). The primer sequences used for quantification of the RT-PCR of the cytokines TNF-α, IL-1, IL-9, INF-δ, IL-10, and IL-17A and the chemokines CCL-2 / MCP-1 and CCR-6 are shown in Table 1. Human primer sequences were designed using PRIMEREXPRESS software (Applied Biosystems, Foster City, CA) based on the nucleotide sequences available in the GenBank database. The real-time PCR reactions were performed with the Step One Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR-Green detection system (Applied Biosystems, Foster City, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used to normalize the mRNA expression levels. All samples were run in duplicate in a reaction volume of 10 µl with 1 µg of the cDNA. Sequence Detection Software, version v 2.4.1 (Applied Biosystems) was used to do the data analysis after amplification. The results were obtained as threshold cycle values (Ct), which represents how many times each fluorescence signal passes through a fixed threshold. Expression levels were calculated by the ΔΔCt method. The Ct values are the mean of two independent measurements, and the mRNA expression levels of the samples are the ratio between the expression of the gene of interest and the GAPDH gene.

TABELA 1. Sequência de primers

Gene	Sequência *(5'-3')*	Mt* (^{o}C)	bp*
GAPDH	FW 5'-GCA CCA CCA ACT GCT TAG CA-3' RV 5'-TGG CAG TGA TGG CAT GGA GGA-3'	65	96
TNF-α	FW 5'-TTC TGG CTC AAA AAG AGA ATT G- 3' RV 5'-TGG TGG TCT TGT TGC TTA AGG- 3'	54	76
IL-10	FW 5'-GGT TGC CAA GCC TTG TCT GA-3' RV 5'-TCC CCC AGG GAG TTC ACA T- 3'	62	107
IFN-g	RV 5'-GAA CTG TCG CCA GCA GCT AAA-3' FW 5'-TGC AGG CAG GAC AAC CAT TA- 3'	80	95
IL-10	RV 5'-GGT TGC CAA GCC TTG TCT GA- 3' FW 5'-TCC CCC AGG GAG TTC ACA T- 3'	81	107
IL-1	RV 5'-TGG CAG AAA GGG AAC AGA A- 3' FW 5 -ACA ACA GGA AAG TCC AGG CTA- 3'	73	59
IL-9	FW 5'-CAT CAG TGT CTC TCC GTC CCA ACT G-3' RV 5'- GAT TTC TGT GTG GCA TTG GTA G-3'	62.9	47.8
CCL2	FW 5'-CGG AGT TTG GGT TTG CTT GT-3' RV 5'-AAG ACC ATT GTG GCC AAG GA- 3'	80	93
CCR6	FW 5'- CCA TTC TGG GCA GTG AGT CA-3' RV 5'- AGA AGC ATC CCG CAG TTA-3'	60.5	55
ILB17A	FW5' -BCAA TGA CCT GGA ATT ACC CAAB 3' RV 5' -BTGA AGG CAT GTG AAA TCG AGAB 3'	70	52

FW (*forward primer*), RV (*reverse primer*).

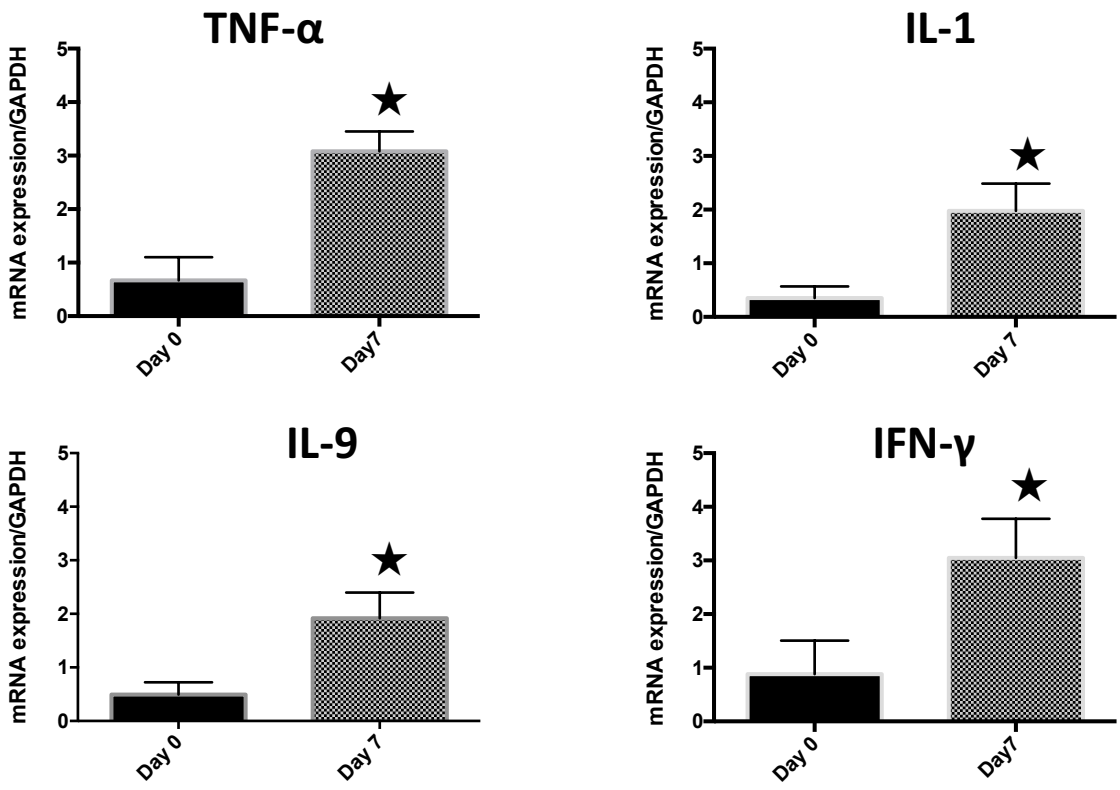
*Mt: melting temperature; bp: base pairs of amplicon size.

Statistical analysis

All data were analysed using SPSS (SPSS Inc., version 15.0, Chicago, IL, USA). Data were then subjected to the Shapiro-Wilk test to verify normality. The Wilcoxon test was used to determine significant differences ($p<0.05$) for data that did not display a normal distribution pattern.

Results

The mRNA expression was determined by RT-PCR and quantified by comparing to the GAPDH internal control gene. The analysis revealed a significant increase in mRNA levels of TNF-α, IL-1, IL-9, INF-δ and IL-10 on day 7 compared to day 0 ($p<0.05$) (Fig. 1). IL-17A and the chemokines CCL-2 / MCP-1 and CCR-6 did not show significant differences in mRNA expression between the two periods analysed ($p>0.05$) (Fig. 2).



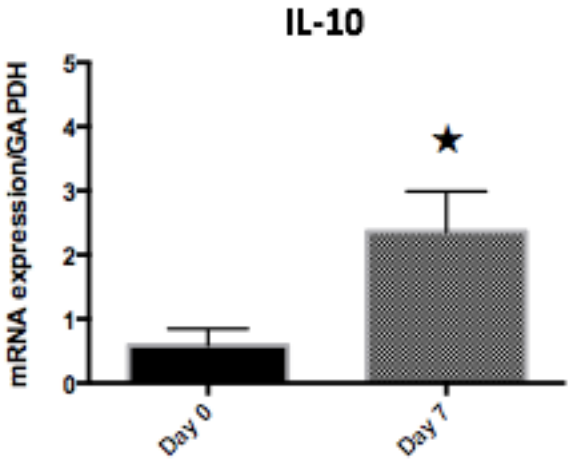
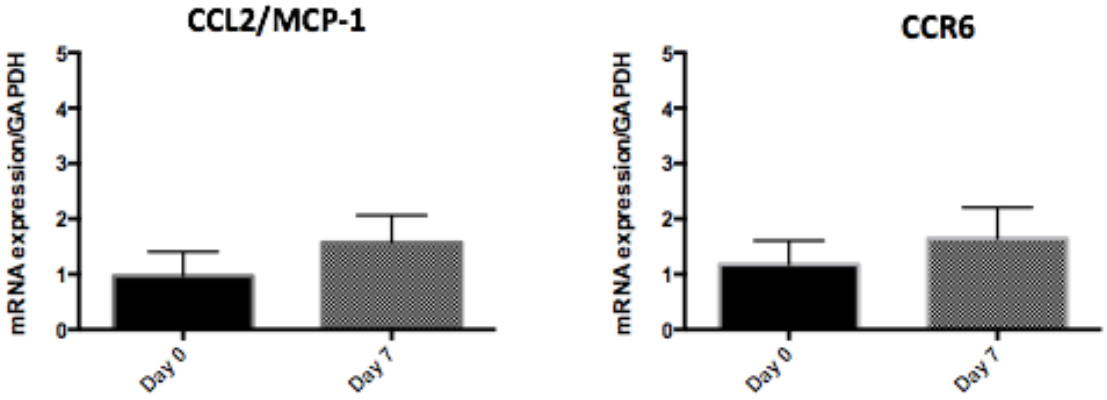


Figure 1. TNF-α, IL-1, IL-9, INF-δ, IL-10 and IL-17A mRNA expression in the interstitial fluid of the infected periapical region of human teeth. Expression levels were determined by RT- PCR and quantified by comparing to the internal control gene (GAPDH). The bars represent significant sample values of 10 patients. The lines represent the standard error of the mean. * represents $p<0.05$ by the Wilcoxon test.



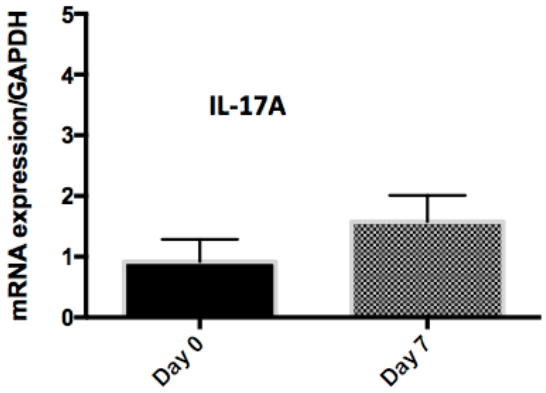


Figure 2. CCL-2 / MCP-1, CCR-6 and IL-17A mRNA expression in the interstitial fluid of the infected periapical areas of human teeth. Expression levels were determined by RT-PCR and quantified by comparing to the internal control gene (GAPDH). The bars represent significant sample values of 10 patients. The lines represent the standard error of the mean. * represents $p<0.05$ by the Wilcoxon test.

Discussion

The presence of microorganisms and their by-products inside the root canal system activates the host immune response in an attempt to contain the infection and prevent its dissemination via the periapical foramen (COTTI et al., 2014). Cytokines play an important role in the pathogenesis of periradicular lesions. Although the cytokines belonging to the Th1, Th2, Th17, and Treg subgroups have been well evaluated at these sites (Henrique et al., 2011; de Brito et al., 2012, by Brito1 et al., 2015), few studies have investigated the role of Th9 cells in periradicular lesions (Araujo-Pires et al., 2014; Aranha et al., 2013).

In this study, cytokine expression was evaluated under two distinct microbial conditions inside the RCSs. Specimens were first collected immediately after the cleaning of the RCS, where cytokine expression corresponded to the response to the ongoing infection. After 7 days, samples were collected in the presence of a reduced intracanal microbial load due to the previous cleaning procedures (Brito et al., 2012, Bambirra et al., 2015). All evaluated mediators, TNF-α, IL-1, IL-9, IFN-γ, IL-17A and IL-

10, as well as the chemokines CCL2 and CCR6, had detectable expression levels in the interstitial fluid, but their expression varied depending on the time of their collection. These data enabled the comparison between active lesion conditions, considering their microbial stimulus, and the conditions at the beginning of the healing process. This step provides an appraisal of the clinical variations that succeed endodontic therapy and provides a robust analysis of the periradicular immune status under these two conditions.

A significant increase in the pro-inflammatory profile of the cytokines TNF-α, IL-1 and IFN-γ was observed 7 days after cleaning and shaping of the RCS was performed. These results are dramatically contrary to those of other studies that used similar methodologies (Brito et al., 2012; Bambirra et al., 2015). Nonetheless, similar results to this study were obtained by Tavares et al., (2012), who suggested that reinfection of the RCS is due to the lack of a physical barrier in those teeth that did not receive intracanal medication or where a restoration between treatment sessions failed. TNF-α and IL-1 are cytokines classically described as pro-inflammatory and osteoclastogenic, and are involved in the progression of periradicular lesions (Sastahenko et al., 1992).

A type 1 immune response, characterized by increased production of IFN-γ, TNF-α and IL-1β, as observed in this study, is associated with bone loss, remodelling and the progression of periradicular lesion (Takeichi et al., 1996). On the other hand, cytokine-mediated mechanisms derived from Treg cells, including production of IL-10 and TGF-b, are responsible for restricting the inflammatory immune response (Kawashima & Stashenko, 1999; Bazzoni et al., 2010). In this study, the greater expression of type 1 cytokines correlates with an increased expression of IL-10 after the reduction of the intracanal microbial load after 7 days. Taken together, these results suggest that the imune modulation promoted by regulatory cytokines is likely to progress, as was observed 7 days after the cleansing procedures in our study, due to cross-regulation in which type 1 cytokines are inhibited by type 2 cytokines, and vice-versa (Alayan et al., 2007). These effects have been shown to be due to IL-10 and TGF-b, since CD4 + CD25 + Foxp3 + cells (Treg) express these cytokines at high levels in the periradicular lesions (Colic et al., 2009). In contrast to that observed in HIV + patients, an increase in

the expression of IFN-γ, TNF-α, IL-1 and IL-17A was demonstrated at day 7 reducing the bacterial load but without correlation to an increase in the expression of regulatory cytokines (Brito et al., 2015). The authors suggested that the pro-inflammatory profile persists in the periradicular lesions of these HIV + individuals with no initiation of immune response modulation, which is not in keeping with what was observed in this study.

Still focused on the main drivers of periradicular lesion inactivity, attributed to increased levels of IL-4, FOXp3, IL-10, IL-9 and IL-22, this study evaluated the expression of IL-9 in periradicular lesions after intracanal microbial load reduction. Th9 cells have been shown to have an interesting plasticity, either acting synergistically with Th2 cells in some inflammatory processes, or by performing immunosuppressive actions via the production of IL-10 (Tan et al., 2010; Xing J et al., 2011). Th9 cells are also capable of changing their phenotype to Th1 or Th17 in the presence of IL-17 (Tan et al., 2010). In this study, an increase of IL-9 mRNA expression was observed at day 7 after the reduction of intracanal microbial load. This correlates with an increase in IL-10 expression, with no change in IL-17A expression. Such a result suggests that this immune modulation is IL-9 / IL-10-dependent, reinforcing the preference of this cytokine for a type 2 profile, as demonstrated in different studies (Jager et al., 2010; Tan et al., 2010; Araujo-Pires et al., 2014). Corroborating this result, it has recently been demonstrated in an animal model that the high expression of IL-9 contributes to the stability of the periapical lesions (Aranha et al., 2013). If analysed in conjunction, these results reinforce that despite the increased expression of type 1 cytokines 7 days after reducing the microbial load, immunomodulation starts and will probably lead to the onset of subsequent healing processes.

Finally, this study evaluated the expression of the chemokines CCL2 / MCP-1 and CCR6 at the two timepoints described. Chemokines have an important role in directing T-cell movement to initiate immune responses in the face of different pathogens, as well as in the recruitment of effector cells to sites of inflammation (Silva et al., 2005). Despite the detectable levels of CCL2 / MCP-1 and CCR6 in the samples collected, no significant differences were observed between the two evaluation times ($p>0.05$). Similar results were previously demonstrated for CCL2 / MCP-1 after the reduction of the

microbial load in the RCS (Brito et al., 2012; Ferreira et al., 2015). In contrast, diverging results were observed in lesions that were refractory to endodontic treatment (Henriques et al., 2011) and in HIV + patients (Brito et al., 2015), where increased expression of CCL-2 was observed. This chemokine influences the recruitment of inflammatory cells, but also has effects on effector T cell differentiation, where it leads to a decreased production of IL-12 by activated macrophages (Chensue et al., 1996).

In sites where inflammation is present, osteoblasts express the chemokine binding receptor CCR-6 to the cytokine CCL-20 (Lisignoli et al., 2009). This constitutes an excellent marker for bone resorption activity. Literature has already demonstrated an increase in the expression of this receptor when in the presence of TNF-α and IL-1β in rheumatoid arthritis (Lisignoli et al., 1999, Kaneko et al., 2001; Santos et al., 2003). In this study, despite the increased levels of TNF-α and IL-1β after the reduction of the intracanal microbial load at day 7, no change in CCR-6 expression was observed at both timepoints. This result reinforces and demonstrates that IL-9 / IL-10-dependent immune modulation is promoted, suggesting that after 7 days, the lesion is already starting its inactivity phase.

To date, there has been a single study focused on the role of CCR-6 in periradicular lesions. In this study, it was demonstrated that an increased expression of $CD3^+$ $CCR6^+$ Th17 cells in knockout mice, for a member of the IL-1(ST2) receptor family, who presented lesions greater than their respective wild controls (Velickovic et al., 2015).

In summary, this study analysed the clinical variations of the periradicular immune status following endodontic therapy. We demonstrated that the cytokine and chemokine-mediated pro-inflammatory response appears to be modulated in an IL-9 / IL-10-dependent manner. Further studies are warranted to reveal the exact mechanism of immunosuppression in the inflamed periradicular environment.

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CONCLUSÃO

Ao analisar a variação clínica pós terapia endodôntica, sobre a condição imune periapical, observamos que a resposta pró-inflamatória mediada por citocinas e quimiocinas parece ser modulada de forma IL-10/IL-9 dependente. Embora obtivemos estes resultados, estudo futuros devem dissecar o exato mecanismo de imunossupressão mediado por tal achado no ambiente periapical inflamado.

TABELA 1. Sequência de primers

Gene	Sequência *(5'-3')*	Mt* (^{o}C)	bp*
GAPDH	FW 5'-GCA CCA CCA ACT GCT TAG CA-3'	65	96
	RV 5'-TGG CAG TGA TGG CAT GGA GGA-3'		
TNF-α	FW 5'-TTC TGG CTC AAA AAG AGA ATT G- 3'	54	76
	RV 5'-TGG TGG TCT TGT TGC TTA AGG- 3'		
IL-10	FW 5'-GGT TGC CAA GCC TTG TCT GA-3'	62	107
	RV 5'-TCC CCC AGG GAG TTC ACA T- 3'		
IFN-g	RV 5'-GAA CTG TCG CCA GCA GCT AAA- 3'	80	95
IL-10	FW 5'-TGC AGG CAG GAC AAC CAT TA- 3'	81	107
	RV 5'-GGT TGC CAA GCC TTG TCT GA- 3'		
IL-1	FW 5'-TCC CCC AGG GAG TTC ACA T- 3'	73	59
	RV 5'-TGG CAG AAA GGG AAC AGA A- 3'		
IL-9	FW 5 -ACA ACA GGA AAG TCC AGG CTA- 3'	62.9	47.8
	FW 5'-CAT CAG TGT CTC TCC GTC CCA ACT G-3'		
	RV 5'- GAT TTC TGT GTG GCA TTG GTA G-3'		
CCL2	FW 5'-CGG AGT TTG GGT TTG CTT GT- 3'	80	93
	RV 5'-AAG ACC ATT GTG GCC AAG GA- 3'		
CCR6	FW 5'- CCA TTC TGG GCA GTG AGT CA-3'	60.5	55
	RV 5'- AGA AGC ATC CCG CAG TTA-3'		

FW (*forward primer*), RV (*reverse primer*).

*Mt: melting temperature; bp: base pairs of amplicon size.

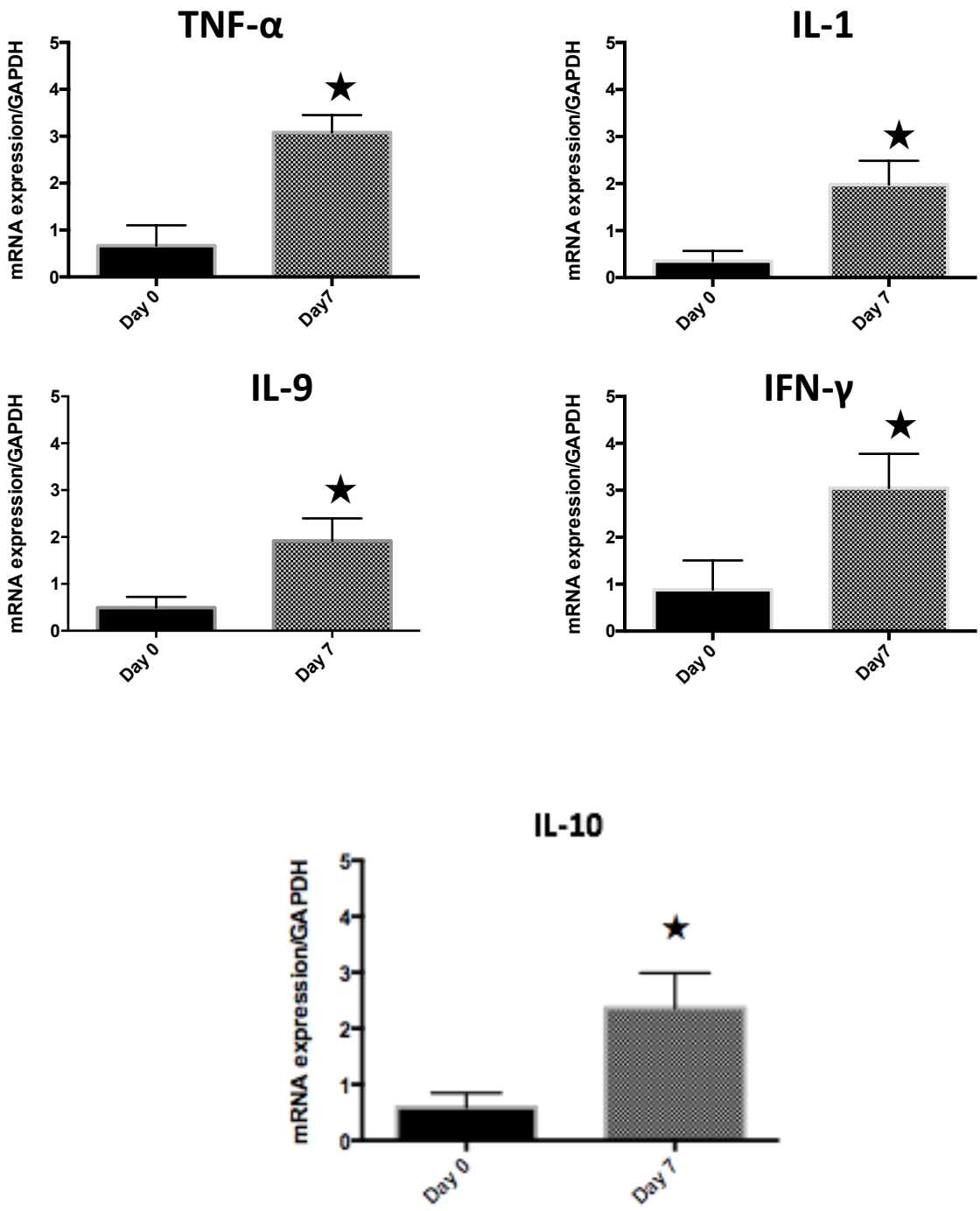

Figura 1. Expressão de mRNA de TNF-α, IL-1. IL-9, INF-δ e IL-10 no fluido intersticial do periapice de dentes humanos com infecções. Os níveis de expressão foram determinados por PCR em tempo real e quantificados em comparação com o controle interno (GAPDH). As barras representam valores amostrais significativos de 10 pacientes; As linhas representam o erro padrão da média. * P <0,05 pelo teste de Wilcoxon.

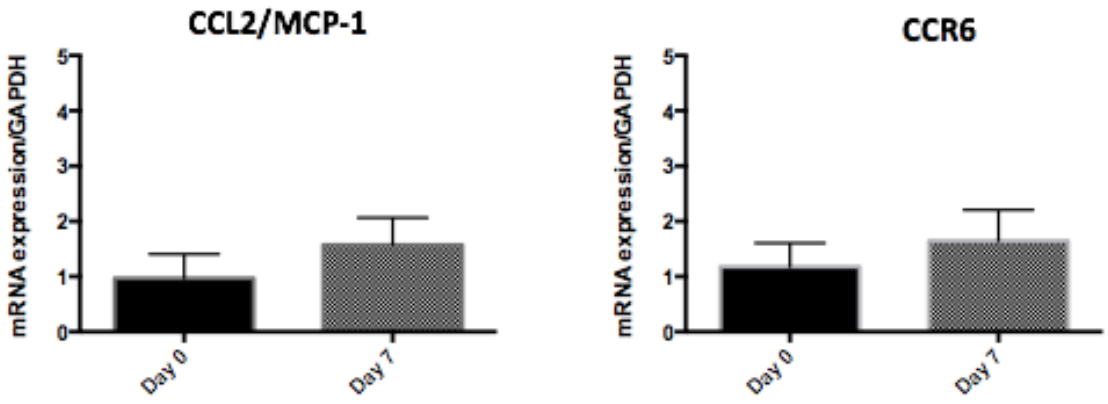

Figura 2. Expressão de mRNA de CCL-2/MCP-1 e CCR-6 no fluido intersticial do periapice de dentes humanos com infecções. Os níveis de expressão foram determinados por PCR em tempo real e quantificados em comparação com o controle interno (GAPDH). As barras representam valores amostrais significativos de 10 pacientes; As linhas representam o erro padrão da média. * P <0,05 pelo teste de Wilcoxon.