UNIVERSIDADE DE SÃO PAULO

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The role of Th17 and regulatory T cells (TREGs) in immunomodulation of experimental periapical lesions

Papel das células Th17 e T reguladoras (TREGs) na imunomodulação de lesões periapicais experimentais

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Tese constituída por artigos apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Biologia Oral.

Orientador: Prof. Dr. Gustavo Pompermaier Garlet

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Dedicatória

Dedico este trabalho especialmente à minha família, verdadeira razão do meu viver, base sólida sobre a qual edifico todos os meus sonhos...

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ABSTRACT

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The role of Th17 and Regulatory T cells (TREGs) in immunomodulation of experimental periapical lesions

The pathogenesis of periapical lesions is determined by the balance between host proinflammatory immune response and counteracting anti-inflammatory and reparative responses. In this context, different subtypes of lymphocytes and their products have been implicated in periapical lesion pathogenesis, such as regulatory T cells (Tregs) and Th17. While Tregs has been demonstrated as potential immunoregulatory agents, Th17 has been correlated with greater severity of disease. In this study, we investigated (in a cause-and-effect manner) the involvement of Tregs and Th17, besides the impact of different therapies in the progression of experimental periapical lesions. With this aim, periapical lesions were induced (pulp exposure and bacterial inoculation) in C57Bl/6 (wild-type), IL-17KO and CCR4KO mice and treated with anti–glucocorticoid-induced TNF receptor family regulated gene (anti-GITR) to inhibit Treg function or alternatively with CCL22-releasing, poly lactic-glycolic acid particles to induce site-specific migration of Tregs. Furthermore, WT mice were treated with anti-RANKL using continuous or intermittent protocols, and with anti-TNF therapy as a control. After treatment, lesions were analyzed for Treg or Th17 influx and phenotype, overall periapical bone loss, and inflammatory/immunologic and wound healing marker expression (RealTimePCRarray, ELISA). Treg inhibition by anti-GITR or CCR4 depletion results in a significant increase in periapical lesion severity, associated with upregulation of proinflammatory, Th1, Th17, and tissue destruction markers in parallel with decreased Treg and healing marker expression. The local release of CCL22 in the root canal system resulted in the promotion of Treg migration in a CCR4-dependent manner, leading to the arrest of periapical lesion progression, associated with down regulation of proinflammatory and tissue destruction markers in parallel with increased Treg and healing marker expression. Anti-RANKL treatment arrested lesion development, but prompted a continuous inflammatory response characterized by unremitting elevated expression of proinflammatory cytokines and tissue destructive mediators, and decreased expression of Tregs and wound healing

markers levels. This treatment triggered lesion development relapse and was associated with high TCD4 effector/suppressor cells ratio and active lesions gene expression signature. Anti-TNF treatment limits lesions progression and does not drives lesions relapse upon cessation, being associated with attenuated host response. Finally, the absence of IL-17 results in a significant decrease in periapical lesions severity, associated with upregulation of healing markers and anti-inflammatory cytokines, in parallel with decreased expression of tissue destruction markers and proinflammatory cytokines. Indeed, histomorphometric analysis showed lower concentration of osteoclasts, neutrophils and mononuclear cells in periapical lesions without IL-17. Therefore, we concluded that regulatory T cells are essential in the control of apical periodontitis, while Th17 cells accentuate the lesions severity. Compared with other clinical strategies, such as anti-RANKL therapy, which perpetuates the host inflammatory response prompting lesion relapse, chemoattraction of Treg as well as inhibition of Th17 may be promising strategies for the clinical management of periapical lesions.

Key Words: Periapical Granuloma. Regulatory T cells. Th17 Cells. Wound healing. RANKL.

RESUMO

RESUMO

Papel das células Th17 e T reguladoras (TREGs) na imunomodulação de lesões periapicais experimentais

A patogênese das lesões periapicais é determinada pelo equilíbrio entre a resposta imune pró-inflamatória do hospedeiro e a resposta anti-inflamatória/reparo. Diferentes subtipos de linfócitos e seus produtos têm sido implicados na patogênese dessas lesões, tais como as células T reguladoras (Tregs) e Th17. Enquanto as Tregs parecem ser potenciais agentes imuno-reguladores, Th17 parece estar associada à maior severidade da doença. Neste estudo, foram investigados o envolvimento de Tregs e Th17, além do impacto de diferentes terapias na progressão de lesões periapicais experimentais. Para isso, lesões periapicais foram induzidas (exposição pulpar e inoculação bacteriana) em camundongos C57BL/6, IL-17KO e CCR4KO tratados com anticorpos anti-GITR (inibe a função de Treg) ou com CCL22p, partículas de ácido poliláctico-glicólico (induz a migração de Tregs). Além disso, os camundongos WT foram tratados com anti-RANKL utilizando protocolos contínuos ou intermitentes, ou com anti-TNF como controle. Posteriormente, analisou-se o fluxo e fenótipo de Tregs e Th17, perda óssea periapical e expressão de citocinas inflamatórias/imunológicas e marcadores de reparo (RealTimePCRarray, ELISA). A inibição de Tregs (depleção de CCR4 ou terapia anti-GITR), aumentou significativamente a severidade da lesão, associada com o aumento da expressão de citocinas pró-inflamatórias, Th1, Th17 e mediadores de destruição tecidual em paralelo com a diminuição dos marcadores de Treg e de reparo. A liberação local de CCL22 no canal radicular resultou na migração de Treg, dependente de CCR4, levando à modulação da lesão periapical, associada com a diminuição da expressão de marcadores pró-inflamatórios e de destruição tecidual em paralelo com o aumento dos marcadores de Tregs e de reparo. O tratamento anti-RANKL impediu o desenvolvimento da lesão, mas provocou uma resposta inflamatória contínua caracterizada pela elevada expressão de citocinas pró-inflamatórias e mediadores de destruição tecidual e diminuição da expressão dos marcadores de Tregs e de reparo. Este tratamento levou à recidiva

da lesão e foi associado com o aumento da razão células TCD4 efetoras/supressoras e com o perfil de expressão gênica de lesões ativas. O tratamento anti-TNF limitou a progressão das lesões e não promoveu sua recidiva após o término da terapia, estando associado à resposta do hospedeiro atenuada. Por fim, a ausência de IL-17 resultou em lesões menos severas, associadas com o aumento da expressão de marcadores de reparo e citocinas anti-inflamatórias, em paralelo com a menor expressão de marcadores de destruição tecidual e citocinas pró-inflamatórias. De fato, observou-se menor concentração de osteoclastos, neutrófilos e células inflamatórias, na ausência de IL-17. Conclui-se, portanto, que as células T reguladoras são essenciais no controle da lesão periapical, enquanto as células Th17 acentuam a severidade dessas lesões. Comparando com outras estratégias clínicas, tais como a terapia anti-RANKL que perpetua a resposta inflamatória do hospedeiro levando a recidiva da lesão, a quimioatração de Treg bem como a inibição de Th17 podem ser estratégias promissoras para o manejo clínico das lesões periapicais.

Palavras-chaves: Granuloma periapical. Células T reguladoras. Células Th17. Cicatrização. RANKL

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1 INTRODUCTION

1 INTRODUCTION

Periapical lesions are alterations in the periradicular region resulting from infection of the root canal, involving bacteria of the oral flora and its products (STASHENKO, 1990). Microorganisms can reach the pulp chamber by exposing the tissue to the oral microflora, resulting in infection of the pulp tissue, which can develop into a necrotic process, making the endodontic region a conducive environment to the establishment of a mixed bacterial microflora, predominantly anaerobic and Gramnegative species with Fusobacterium, Porphyromonas, Prevotella, Eubacterium and Peptostreptococcus (NAIR, 1997; 2004). Although the presence of pathogenic microorganisms is necessary for the early development of periapical lesions, progression and severity of this process are highly dependent on the host immune and inflammatory response to bacteria or their products (GARLET, T.P. et al., 2010; GRAVES; OATES; GARLET, 2011; LETRA et al., 2013). At first, an acute inflammatory response occurs against microbial challenge; in which tissue injury is limited to the periodontal ligament, being related to early exsudative events of inflammation, such as hyperemia, vascular congestion, edema and initial neutrophil extravasation followed by monocytes migration (NAIR, 1997; 2004).

Due to persistence of immune function and inflammation, phagocytic activation and migration lead to the production of mediators, such as proinflammatory cytokines (IL-1 and TNF-α) and chemokines that act enhancing the immune response and local inflammation, which are associated with the degradation of extracellular matrix and subsequently bone resorption (ARAUJO-PIRES et al., 2014a; ARAUJO-PIRES et al., 2014b; GRAVES; OATES; GARLET, 2011; NAIR, 1997; 2004). Then, it tends to occur chronicity of inflammatory and immune process leading to a change in cell migration pattern which results in the predominance of lymphocytes and macrophages in generally surrounded area (if not encapsulated) by connective tissue (ARAUJO-PIRES et al., 2014b; STASHENKO, 1990; STASHENKO et al., 2007; STASHENKO et al., 1991).

The acute inflammatory response is defined as a series of tissue responses that can occur within the first few hours following injury. The earliest in vivo hallmark

of the acute inflammatory response is the adhesion of neutrophils (polymorphonuclear leukocytes, PMNs) to the vascular endothelium. Resolution of the inflammatory response requires killing of bacteria and remove their debris (SERHAN; WARD; GILROY, 2010).

The chronic inflammatory response is defined according to the nature of the inflammatory cells appearing in tissues. The definition of chronic inflammatory is not related to the duration of the inflammatory response. It is defined not as the persistence of acute inflammation but is defined morphologically by the presence of lymphocytes, macrophages, and plasma cells in tissues (SERHAN; WARD; GILROY, 2010).

This mononuclear infiltration (in chronic inflammatory) is associated with increased degradation of the extracellular matrix and also the generation and activation of osteoclasts responsible for bone resorption around the periapex and the lesions evolution. In fact, studies have shown that this effect occurs because of the modulating of RANKL (receptor activator of NFκB ligand) expression, main osteoblast gene factor that acts through binding to RANK present on the surface of osteoclasts precursors, inducing their differentiation and activation; this process is modulated by OPG (osteoprotegerin) that prevents binding RANKL/RANK and thus inhibits bone resorption (BOYCE; XING, 2007; BOYLE; SIMONET; LACEY, 2003). In this context, our group demonstrated that ratio RANKL/OPG appears to be linked to the state of activity or inactivity of injuries in human periapical granulomas (ARAUJO-PIRES et al., 2014b; MENEZES et al., 2008a; MENEZES et al., 2008b), as well as increased expression of MMPs, such as MMP-1, MMP-2, MMP-8 and MMP-9, the major enzymes involved in the degradation of extracellular matrix components in the periapical environment (ANDONOVSKA; DIMOVA; PANOV, 2008; CARNEIRO et al., 2009; DE PAULA-SILVA et al., 2009).

Increased expression of genes related to healing was reported in periapical granulomas when compared to healthy tissues (ARANHA et al., 2013; GARLET et al., 2012; MENEZES et al., 2008b). When comparing active and inactive granulomas, the expression of healing related genes was greatly pronounced in inactive granulomas, while in active lesions prevail the expression of inflammatory genes along a minor expression of healing markers (ARANHA et al., 2013; GARLET et al.,

2012; MENEZES et al., 2008b). This suggests that chronic inflammatory and immune process minimizes the possibility of tissue repair or limits the lesions progression verified in molecular terms in lesions characterized as inactive (GARLET et al., 2012).

Previous studies have shown that during the development of periapical lesions, different subtypes of lymphocytes and their characteristics cytokines may modulate the severity of lesions regulating the expression of RANKL and MMPs. In fact, different patterns of immune/inflammatory responses can be generated by the activity of different subpopulations of T helper (Th) responses being initially described as type 1 (Th1) and type 2 (Th2), characterized by expression of IFN-γ or IL-4, respectively (GARLET, G.P. et al., 2010b; GRAVES; OATES; GARLET, 2011). Analysis of human periapical lesions suggests that Th2 response prevails in human periapical lesions in regeneration, while in apical granulation tissue (granuloma) Th1 response is predominantly, suggesting that Th1/Th2 dichotomy could be a determinant of the activity or stability of lesions (COLIC et al., 2006; FUKADA et al., 2009; KABASHIMA et al., 2001; KABASHIMA et al., 2004). However, the Th1/Th2 paradigm has been questioned due to some controversial results in literature, and especially the discovery of new helper T lymphocyte subpopulations with important immunoregulatory properties in different infectious and inflammatory processes (GARLET, G.P. et al., 2010b; GRAVES, 2008; GRAVES; OATES; GARLET, 2011; NAKASHIMA; TAKAYANAGI, 2008).

In this context, regulatory T cells (Tregs) and Th17 cells have extremely interesting immunoregulatory roles (BETTELLI; KORN; KUCHROO, 2007; CARDOSO et al., 2008; GAFFEN; HAJISHENGALLIS, 2008), including control of cell migration and bone resorption (GARLET, G.P. et al., 2010b; GRAVES, 2008; GRAVES; OATES; GARLET, 2011; NAKASHIMA; TAKAYANAGI, 2008). These cells have been already identified in the environment of periapical lesions (ALSHWAIMI et al., 2009; ANDRADE et al., 2013; COLIC et al., 2007; FUKADA et al., 2009; MARCAL et al., 2010; YANG et al., 2014) but their roles in the pathogenesis of lesions remain unknown.

The differentiation of naive T cells into Th17 or Treg lineages is influenced by the level of expression of different cytokines such as transforming growth factor

(TGF)-β (CHEN et al., 2011; GAFFEN et al., 2006; YOSHIMURA; WAKABAYASHI; MORI, 2010; YU; GAFFEN, 2008). Low levels of TGF-β, IL-23 or IL-6 in the tissue microenvironment induce the development of Th17 cells (ZHOU; CHONG; LITTMAN, 2009), whereas high levels of TGF-β stimulate the differentiation of Treg cells (ZHOU et al., 2008). An imbalance between the differentiation of Th17 and Treg cells causes the loss of host immune homeostasis and the development of autoimmune diseases (CHEN et al., 2011; MARCAL et al., 2010).

Tregs comprise a CD4+CD25+ T cell subpopulation that specifically suppress the activation, proliferation and proinflammatory effective function of activated conventional T cells, preventing or attenuating deleterious effects of exaggerated responses and tissue damage (BELKAID, 2008; BELKAID; CHEN, 2010; BELKAID et al., 2002; CAMPANELLI et al., 2006; SAKAGUCHI, 2008; SAKAGUCHI et al., 2010; SAKAGUCHI et al., 2001).

Tregs characteristically express as phenotypic markers the transcription factor forkhead box P3 (FOXp3), CD103, the glucocorticoid-inducible TNF receptor (GITR), the inhibitory molecule cytotoxic T-lymphocyte-associated molecule 4 (CTLA-4) and cell surface TGF-β (FEHERVARI; SAKAGUCHI, 2004a; b; MIYARA et al., 2009; SHEVACH, 2002). In addition to the expression of CD4, CD25 and Foxp3, these cells are characterized by expressing CD5high, CD38, and CD45low.

Once activated, their function leads to suppression of the proliferation of other T cells, as well as it can block or decrease the production of inflammatory mediators by different leukocyte subpopulations. Although there is controversy about its mechanisms of action, it is believed that Tregs exert their functions dependently the cell-cell contact via the inhibitory molecule CTLA-4, or through the cytokines production such as TNF-β and IL-10 (RUDENSKY, 2011; SAKAGUCHI et al., 2001; SHEVACH et al., 2006; WAN, 2010).

A previous study demonstrates an increased frequency of Tregs in healthy tissues (ERNST et al., 2007), suggesting a role for Tregs in the maintenance of periodontal health. However, subsequent reports demonstrate an increased frequency of this T cell subset in disease tissues, suggesting that Tregs infiltration could reflect an attempt to control tissue destruction, but it also could be indicative of

destructive role for Tregs in periodontitis (CARDOSO et al., 2008; DUTZAN et al., 2009; NAKAJIMA et al., 2005).

Recent study reinforces this hypothesis, demonstrating that the coexistence of high levels of IL-4 (the prototypical Th2 cytokine), FOXp3 (the Tregs' master transcription factor) and IL-10 (a major product of Tregs) is associated with an inactive profile of osteolytic periapical lesions (ARAUJO-PIRES et al., 2014b). Such protective interplay seems not to be limited to periapical diseases, since a positive correlation between increased levels of IL-4 and IL-10 was described to be associated with the attenuation of rheumatoid arthritis (RA) severity (BROWNLIE et al., 2006). It is also worth mentioning that Tregs and Th2 cells appear to share the expression of the chemokine receptors, such as CCR4 and CCR8, making these cells similarly responsive in theory for chemokines such as CCL17 and CCL22 (GRIFFITH; SOKOL; LUSTER, 2014). Indeed, these chemokines/chemokine receptors have been identified in inflammatory osteolytic lesions, reinforcing their potential role in the immunoregulation of bone pathologies (GARLET, G.P. et al., 2010a).

Therefore, the migration of Tregs has been associated with the chemotactic cytokine CCL22 as well as decreased severity of experimental periapical lesions in rats (HE et al., 2015). Accordingly, CCL22 interaction with the receptor CCR4 appears to control Tregs migration into mice periodontal tissues and consecutively suppresses local inflammatory bone loss (ARAUJO-PIRES et al., 2015).

While Tregs appear as active immune suppressive agents in periapical granuloma pathogenesis, evidence suggests that other subpopulation of T cells called Th17 can play an antagonistic role, acting as potential mediators of periapical lesions progression.

Th17 cells comprise a subpopulation of CD4+T cells whose main secretion product is interleukin (IL)-17, a proinflammatory cytokine that exerts potent effects on different cell types of the innate immunity and is considered a molecular bridge between the innate and acquired immune systems (YU; GAFFEN, 2008). It plays an important role in the initiation and maintenance of proinflammatory responses, and it has been found to stimulate osteoclastic activation (VERNAL et al., 2005). Indeed,

IL-17 has been associated with the pathology of numerous autoimmune and inflammatory diseases, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, psoriasis and allograft rejection (HARRINGTON et al., 2005; WEAVER et al., 2006).

Th17 development relies on the action of a lineage-specific transcription factor, the orphan nuclear receptor gt (RORgt) (IVANOV et al., 2006). Transforming growth factor-β (TGF-β), IL-6, IL-21 and IL-23 are also required for the development and expansion of Th17 cells (KORN et al., 2009). Interestingly, IL-17 induces RANKL expression, which is crucial to osteoclastogenesis and bone destruction (KOTAKE et al., 1999). IL-17 protein is also present in periapical lesions (XIONG; WEI; PENG, 2009), suggesting that Th17 cells may contribute to pathogenic tissue destruction, including bone destruction in apical periodontitis.

The role of IL-17 in human oral diseases began to be investigated because its presence in the periodontal tissues was correlated with MMP expression, proinflammatory cytokines such as IL-1β and TNF-α, and induction of bone resorption, contributing to greater disease severity (CARDOSO, 2008) (SATO, SUEMATSU et al. 2006; BEKLEN, AINOLA et al. 2007; COLIC, VASILIJIC et al. 2007; SATO, 2008). Subsequent studies confirmed that this cytokine is strongly expressed in chronic periapical lesions (ALSHWAIMI et al., 2013; ANDRADE et al., 2013; COLIC et al., 2007; VERNAL et al., 2005; WEI et al., 2013; XIONG; WEI; PENG, 2009; YANG et al., 2014) being able to exacerbate inflammation (COLIC et al., 2009a; COLIC et al., 2007; OSEKO et al., 2009).

Th17 responses can be effectively controlled by regulatory T cell (Tregs) and Th1-specific factors. Recently, it was reported that IFN-g strongly inhibits T cell differentiation towards the Th17 cell lineage (DONG, C., 2009).

So, periapical lesion outcome is determined by the balance between pro- and anti-inflammatory cytokines, which differentially modulates proteolytic activity, bone resorption and healing mechanisms (COLIC et al., 2009b; FRANCISCONI, C. F. et al., 2016; GRAVES; OATES; GARLET, 2011; MARTON; KISS, 2014). In this context, adjunct therapies targeting pro-inflammatory cytokines, such as TNF, IL-1 and IL-17,

have been proposed to the clinical management of inflammatory osteolytic alterations (GRAVES; OATES; GARLET, 2011; MARTON; KISS, 2014).

The inhibition of TNF-α is a validated and favorable method for treating several important TNF-α associated diseases. Infliximab is a therapeutic mAb that was approved by the United States Food and Drug Administration to treat Crohn disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, and ulcerative colitis (LIANG et al., 2013). However, these therapies impact host response and increase individual susceptibility to infections, and therefore their applications in infectious conditions are still limited (BRZUSTEWICZ; BRYL, 2015)**.**

The identification of the RANK-RANKL-OPG system provided additional targets to treat bone diseases. Evidence from animal models showing that inhibition of the major osteoclastogenic factor RANKL attenuated bone loss associated with osteoporosis and arthritis, led to successful clinical trials in humans and the approval of anti-RANKL monoclonal antibodies for using in treatment of those conditions (LACEY et al., 2012; LEDER et al., 2015; TYAGI et al., 2014).

However, recent studies have shown that RANKL-blockage can impair Tregs function (LIN et al., 2016; MCCARTHY et al., 2015) and the therapeutic potential of short-term RANKL inhibition, as potentially needed during routine endodontic practice, remains undetermined. So, it is crucial to determine if RANKL-inhibition also impacts inflammatory/immune responsiveness at the periapical region.

Finally, the general aim of this study is to determine the influence of Tregs and Th17 cells in periapical lesions outcome to generate information potentially applicable not only in the specific field of endodontics/dentistry but in other models involving the interaction between bone and immune systems.

2 ARTICLES

2 ARTICLES

The articles presented in this Thesis were written according to the Journal of Endodontics instructions and guidelines for article submission.

- ARTICLE 2.1 – Francisconi CF, Vieira AE, Biguetti CC, Glowacki AJ, Trombone AP, Letra A, et al. Characterization of the protective role of regulatory T cells in experimental periapical lesion development and their chemoattraction manipulation as a therapeutic tool. Journal of Endodontics 2016;42(1):120-126.

- ARTICLE 2.2 – Anti-RANKL therapy limits periapical lesions progression but is prone to relapse due impaired immunoregulation and unremitting host pro-inflammatory response (Submitted in July 2016)

- ARTICLE 2.3 – Role of Th17 cells in experimental periapical lesions development (To be submitted)

ARTICLE 2.1

Basic Research—Biology

Characterization of the Protective Role of Regulatory T Cells in Experimental Periapical Lesion Development and Their Chemoattraction Manipulation as a Therapeutic Tool

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Abstract

Introduction: The pathogenesis of periapical lesions is determined by the balance between host proinflammatory immune response and counteracting anti-inflammatory and reparative responses, which include regulatory T cells (Tregs) as potential immunoregulatory agents. In this study, we investigated (in a cause-and-effect manner) the involvement of CCL22-CCR4 axis in Treg migration to the periapical area and the role of Tregs in the determination of outcomes in periapical lesions. **Methods:** Periapical lesions were induced in C57Bl/6 (wild-type) and CCR4KO mice (pulp exposure and bacterial inoculation) and treated with anti–glucocorticoid-induced TNF receptor family regulated gene to inhibit Treg function or alternatively with CCL22-releasing, polylactic-glycolic acid particles to induce site-specific migration of Tregs. After treatment, lesions were analyzed for Treg influx and phenotype, overall periapical bone loss, and inflammatory/immunologic and wound healing marker expression (analyzed by real-time polymerase chain reaction array). **Results:** Treg inhibition by anti–glucocorticoid-induced TNF receptor family regulated gene or CCR4 depletion results in a significant increase in periapical lesion severity, associated with upregulation of proinflammatory, T-helper 1, T-helper 17, and tissue destruction markers in parallel with decreased Treg and healing marker expression. The local release of CCL22 in the root canal system resulted in the promotion of Treg migration in a CCR4-dependent manner, leading to the arrest of periapical lesion progression, associated with downregulation of proinflammatory, T-helper 1, T-helper 17, and tissue destruction markers in parallel with increased Treg and healing marker expression. **Conclusions:** Because the natural and CCL22-induced Treg migration switches active lesion into inactivity phenotype, Treg chemoattractant may be a promising strategy for the clinical management of periapical lesions. (J Endod 2016;42:120–126)

Key Words

Apical lesions, cytokines, regulatory T cells, T helper, wound healing

Pathogenesis of periapical lesions involves a complex host inflammatory immune response to the bacterial infection of the root canal system, which ultimately drives the destruction of periapical tissue (1). The breakdown of soft and mineralized tissues surrounding the root apex is triggered by a series of host mediators, which independently or cooperatively mediate increased proteolytic activity and the activation of bone resorption mechanisms (1–5).

However, host response regulatory mechanisms activated along lesion development can convert active lesion into an inactive phenotype and consequently arrest or limit progression of tissue destruction (6). Protective mechanisms involve certain T helper (Th) subsets, mesenchymal stem cells, and suppressors of cytokine signaling, which collectively are thought to dampen the tissue destructive pathways while boosting healing mechanisms (6–10). Within the potentially protective Th subsets, accumulating evidence points to the involvement of regulatory T cells (Tregs) as potential determinants of lesion outcomes (6, 11–13).

Tregs comprise a CD4 +CD25+ T-cell subpopulation that specifically suppresses the activation, proliferation, and proinflammatory effector function of activated conventional T cells (6, 11–15). Tregs were identified in human and experimental periapical lesions by the expression of phenotypic markers FOXp3, glucocorticoid-induced TNF receptor family regulated gene (GITR), CD103, and CD45RO, as well as by the functional markers CTLA-4, interleukin (IL)-10, and transforming growth factor (TGF)-β, which are associated with Treg suppressive function (6, 11, 12, 16). Indeed, the presence of Tregs in periapical lesions accounts for the attenuation of local host inflammatory immune responses (8, 17–19). Accordingly, decreased expression of Treg phenotypic and functional markers and its impaired function because of increased FOXp3 methylation are characteristic features of progressive human periapical lesions (20). Migration of Tregs has been associated

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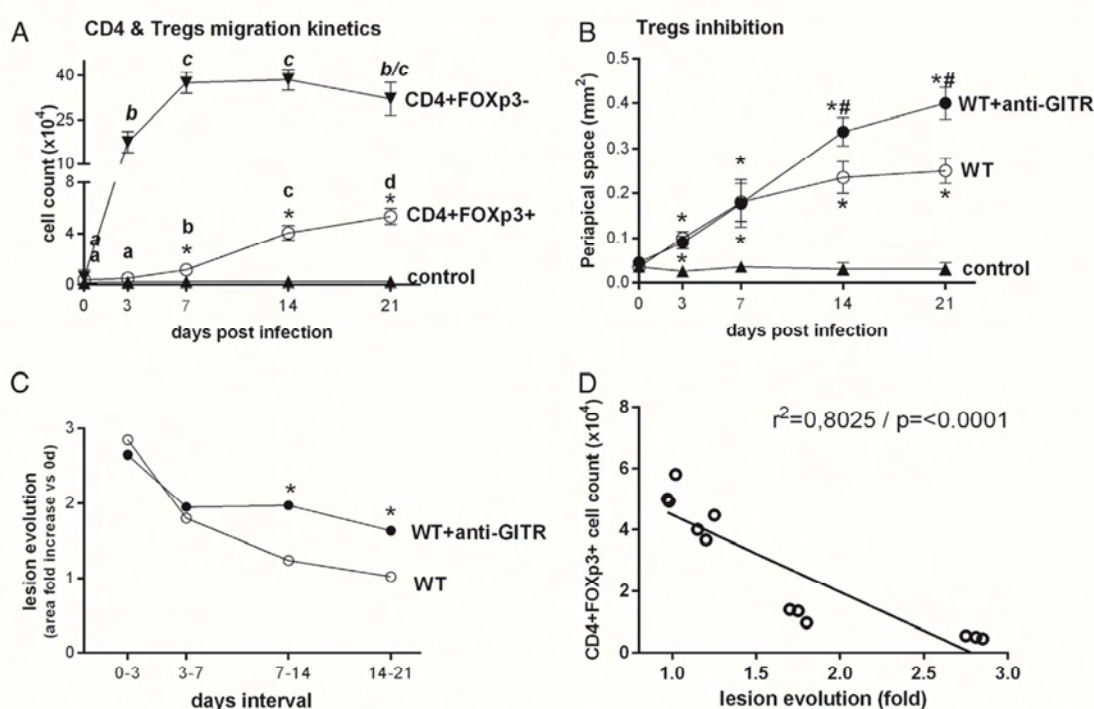
Figure 1. Treg migration kinetics and its impact on periapical bone loss and in the expression of inflammatory/immunologic and healing markers in experimental periapical lesions in mice. C57Bl/6 (WT) mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation) and treated (or not) with anti-GITR to inhibit Treg function. Samples from experimental and control groups were collected for histomorphometric and molecular analysis and evaluated for (*A*) Treg (CD4+FOXp3+) cell counts in periapical tissues analyzed by flow cytometry at 0, 3, 7, 14, and 21 days after infection, depicted as the cell number $\times 10^4$; (*B*) periapical lesion development, presented as periapical space area (mm^2) increase after induction of lesions, measured with ImageJ software in hematoxylin-eosin stained histologic sections, or presented as (*C*) the lesion evolution index (fold increase in specific time intervals); (*D*) correlation between the Treg (CD4+FOXp3+) cell counts and the lesion evolution index, performed with data from WT group; (*continued*)

with the chemotactic cytokine CCL22 as well as decreased severity of experimental periapical lesion in rats (21). Accordingly, CCL22 interaction with the receptor CCR4 appears to control Treg migration into mice periodontal tissues and consecutively suppresses local inflammatory bone loss (22).

Regardless, studies to date only support a theoretical protective role exerted by Tregs in the control of severity of periapical lesions, which remains to be definitely confirmed in a cause-and-effect manner. For this reason, we investigated the phenotypic features and kinetics of Tregs migration along experimental periapical lesion development in mice. In addition, the mechanisms underlying Tregs migration and function in periapical environment were investigated by their inhibition (with anti-GITR treatment) or chemoattraction (via CCL22/CCR4 axis).

Materials and Methods
Experimental Groups

Experimental groups comprised 8-week-old male C57BL/6 wild-type (WT) and CCR4 (CCR4KO) mice, treated with anti-GITR or with CCL22-releasing particles (22, 23). Anti-GITR antibodies were prepared from hybridomas grown in nude mice as previously described (24). CCL22-releasing particles were prepared by mixing an aqueous solution containing CCL22 and bovine serum albumin with polylactic-glycolic acid (PLGA), followed by sonication, homogenization, evaporation, and lyophilization as pre-

viously described (23). During the course of the study, the mice were maintained in the animal facilities of USP and fed with standard solid mice chow (Nuvital, Curitiba, PR, Brazil) and sterile water. The experimental protocol approved by the local Institutional Committee for Animal Care and Use follows the principles of the Guide for the Care and Use of Laboratory Animals and EU Directive 2010/63/EU for animal experiments.

Experimental Periapical Lesions and Treatments

Periapical lesion induction and quantification were performed as previously described (3, 7). Mice (N = 5/time/group) were anesthetized, and mandibular first molar dental pulp was exposed with a carbide bur in a slow-speed handpiece, followed with inoculation of endodontic pathogenic bacterial strains (*Porphyromonas gingivalis* ATCC33277, *Prevotella nigrescens* ATCC33563, *Actinomyces viscosus* ATCC91014, and *Fusobacterium nucleatum* ATCC10953) (3, 7). Treg function was inhibited by treatment with purified monoclonal antibody anti-GITR (or control rat immunoglobulin G) 500 μg/mouse intraperitoneal injection as previously described (25). Treg migration was induced by the CCL22-releasing PLGA microparticles (or control blank particles) (22, 23) that were injected (5 μL phosphate-buffered saline/CMC solution containing 25 mg/mL particles) in the root canal system at day 3 after bacterial inoculation. Animals were killed by cervical displacement after 0, 3, 7, 14, and 21 days of infection, the jaws were dissected, and independent samples were
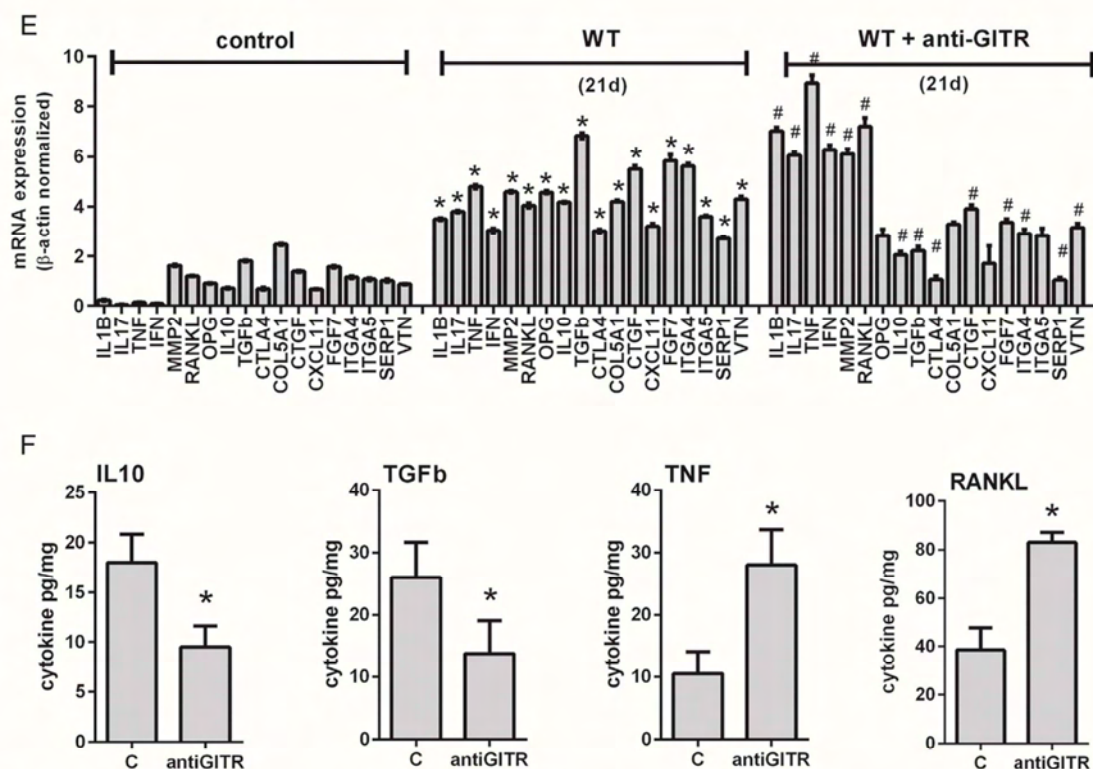
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Figure 1. *Continued.* (*E*) expression of inflammatory/immunologic and wound healing markers at 21-day time point, measured quantitatively by real-time PCR array, presented as fold change relative to the control group after normalization by constitutive genes (GAPDH, ACTB, Hprt1) expression levels; and (*F*) cytokine levels in periapical lesions, measured by ELISA, presented as cytokine pg/mg of periapical tissue. In (*A*), different letters represent statistically significant differences among the different time points within the respective groups ($P < .05$; one-way analysis of variance, Bonferroni post hoc test). In (*A*), (*B*), (*C*), (*E*), and (*F*), asterisks (*) represent statistically significant differences ($P < .05$; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs control group, and the hashtag (#) represents statistically significant differences ($P < .05$; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs WT group.

prepared for histomorphometric (right molars) or molecular (left molars) analysis. Results are depicted as the area of periapical space (9), which was measured in hematoxylin-eosin stained longitudinal 5-mm-thick sections by using ImageJ (NIH, Bethesda, MD); it is the area increase over time representative of lesion development.

Gene Expression and Enzyme-linked Immunosorbent Assay

Real-time polymerase chain reaction (PCR) array reactions were performed as previously described (7). The extraction of total RNA from periapical tissues was performed with the RNeasyFFPE kit (Qiagen Inc, Valencia, CA), followed by integrity analysis with 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA) and complementary DNA synthesis (Superscript III; Invitrogen Corporation, Carlsbad, CA); all were performed according to the manufacturers' instructions. Real-time PCR array was performed in a Viia7 instrument (Life Technologies, Carlsbad, CA) by using a custom panel for gene expression profiling (SABiosciences, Frederick, MD), analyzed by the RT2profiler software (SABiosciences) for normalizing target genes expression levels by constitutive genes (GAPDH, ACTB, Hprt1) and the control group. Gene expression levels are expressed as fold change relative to the control group as previously described (9). Measurements of cytokines

IL-10, TGF-β, tumor necrosis factor (TNF), and receptor activator of nuclear factor kappa B ligand (RANKL) in periapical lesions were performed by enzyme-linked immunosorbent assay (ELISA) as previously described (22) by commercially available kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results are expressed as picograms of cytokine ($\pm$ standard deviation [SD]) per milligram of periapical tissue.

Isolation and Analysis of Leukocytes from Periapical Tissues

Isolation and characterization of lesions' Tregs were performed as described previously (22). Whole periapical tissues of molar apex were initially incubated in RPMI-1640 with 0.28 Wunsch units/mL Liberase Blendzyme CI (Roche, Basel, Switzerland) and then processed with 0.05% DNase (Sigma-Aldrich, Steinhein, Germany) by using Medimachine (BD Biosciences, San Diego, CA), followed by cell viability analysis (trypan blue) and cell count (Neubauer chamber). Cells were incubated with optimal dilution fluorochrome-conjugated antibodies against CD4 (FITC, clone GK1.5, dilution 1:200), FOXp3 (Alexa488, R16-715, 1:100), CCR4 (PE, 1G1, 1:100), CCR5 (PE, C34-3448, 1:100), CCR7 (PE, 4B12, 1:50), CCR8 (PE, 1055c, 1:50), CXCR3 (PE, 1C6/CXCR3, 1:100), RANKL (PE, IK22-5, 1:100), IL-10 (PE,

JES5-16E3, 1:100), TGF-β (PE, TW7-16B4, 1:200), and CTLA-4 (PE, UC10-4F10-11, 1:200) (BD Biosciences) and then analyzed by flow cytometry (FACScan and CellQuest; BD Biosciences). Results represent the number of cells ± SD in the periapical tissues of each mouse, normalized by the tissue weight, or the number of positive cells for each marker in CD4+FOXp3+ subpopulation.

Statistical Analysis

Data are presented as means ± SD, and the statistical significance between the groups was analyzed by Kruskal-Wallis followed by Dunn post hoc test or by Mann-Whitney test; both were performed with Graph-Pad Prism 5.0 software (GraphPad Software Inc, San Diego, CA). PCR array data were analyzed by the Mann-Whitney test followed by Benjamini-Hochberg test. Values of $P < .05$ were considered statistically significant.

Results
Tregs Influence Experimental Periapical Lesions Outcome

The induction of experimental periapical lesions generated increasing Treg (CD4+FOXP3+) migration over time, whereas conventional T cells (CD4+FOXP3–) counts remained stable after 7-day time point (Fig. 1A). The development of experimental lesions in WT mice was similar to what was observed in previous reports (7), and inhibition of Tregs with anti-GITR resulted in a significant increase in the lesions at 14-day and 21-day time points (Fig. 1B), as well as increased lesion evolution rate (Fig. 1C). In addition, Treg counts negatively correlate with lesion evolution patterns (Fig. 1D). Treatment with anti-GITR was associated with increased expression of proinflammatory cytokines and tissue destructive mediators (IL-1B, IL-17, TNF-α, interferon (IFN)-γ, MMP2, RANKL) as well as decreased expression of Treg markers (IL-10, TGF-β, CTLA-4) and wound healing/inactive lesion markers (CTGF, FGF7, ITGA4, SERP1, VTN) (Fig. 1E). ELISA analysis confirmed the molecular analysis, showing the upregulation of TNF and RANKL in parallel with the downregulation of IL-10 and TFG-β by anti-GITR treatment (Fig. 1F).

Involvement of CCR4 and CCL22 in Treg Migration and Lesions Outcome

Phenotypic analysis demonstrated that periapical lesions' Tregs (CD4+FOXp3+cells) express high levels of the chemokine receptor CCR4 and its classic markers IL-10, TGF-β, and CTLA-4 and low but significant levels of CCR5 and CCR8 (Fig. 2A). In the absence of CCR4 (CCR4KO strain), the migration of Tregs was significantly impaired (Fig. 2B), and the severity of lesions increased (Fig. 2C). Molecular analysis of CCR4KO mice lesions demonstrated increased expression of proinflammatory cytokines and tissue destructive mediators (IL-1B, IL-17, TNF-α, IFN-γ, MMP2, RANKL) and decreased expression of Treg-associated markers (IL-10, TGF-β, CTLA-4) as well as markers of wound healing/inactive lesions (OPG, CTGF, CXCL11, FGF7, ITGA4, SERP1) (Fig. 2D). ELISA analysis confirmed data obtained via real-time PCR, suggesting that TNF and RANKL were upregulated in CCR4KO mice in parallel with the downregulation of IL-10 and TFG-β (Fig. 2E). The injection of CCL22-releasing particles in the root canal system appears to be effective in promoting Treg migration (Fig. 3A) and reducing lesion progression (Fig. 3B) in a CCR4-dependent manner. From the molecular viewpoint, CCL22 treatment resulted in decreased expression of proinflammatory cytokines and tissue destructive mediators (IL-1B, IL-17, IFN-γ, TNF-α, MMP2, RANKL) and increased the expression of Treg markers (IL-10, TGF-β, CTLA-4) and wound healing/inactive

lesion markers (OPG, CXCL11, FGF7, ITGA5, SERP1, VTN) (Fig. 3C). Similarly, ELISA analysis confirmed that CCL22-releasing particles mediate the downregulation of TNF and RANKL levels in parallel with the upregulation of IL-10 and TFG-β in the lesions (Fig. 3D).

Discussion

Pathogenesis of periapical lesions is determined by the balance between the host proinflammatory immune response and the counteracting anti-inflammatory and reparative responses (1, 5, 7, 8), which include Tregs as potential immunoregulatory agents. In this study, we investigated (in a cause-and-effect manner) the involvement of CCL22-CCR4 axis in migration of endogenous Tregs to periapical area and their subsequent role in the control of outcomes in periapical lesions.

Our results demonstrate that migration of Tregs (CD4+FOXP3+ cells) into periapical lesions is temporally and functionally associated with the attenuation of the progression of these lesions. In WT mice, the increasing number of Tregs in periapical lesions is temporally associated with the conversion of the lesion phenotype from active into inactive, a previously described phenomenon (9). In addition, the inhibition of Treg function (with anti-GITR) prevents the establishment of the inactive/stable status, resulting in the exacerbation of severity of lesions. Accordingly, Tregs were previously suggested to attenuate inflammatory osteolysis in arthritis and periodontitis models (24, 26). Regarding the mechanisms underlying Treg protective function in periapical milieu, our data suggest that Treg inhibition results in decreased expression of IL-10, TGF-β, and CTLA-4, which is typically associated with Treg-mediated immunosuppressive properties (22, 23). Indeed, Treg inhibition appears to imbalance host response, as demonstrated by the increased expression of proinflammatory and Th1 and Th17 cytokines, previously associated with periapical lesion progression (1). Lesion exacerbation in the absence of functional Tregs is supposed to be due to the upregulation of catabolic factors such as MMP2 and the osteoclastogenic factor RANKL in parallel with decreased expression of OPG and wound healing related factors (COL5A1, CTGF, CXCL11, FGF7, ITGA4, ITGA5, SERP1, VTN). Accordingly, in the absence of Tregs the overall molecular profile of periapical lesions matches with the profile previously described in active lesions, whereas the gene expression profile of lesions populated by active Tregs resembles the profile of inactive/stable lesions (7, 10).

Considering that Treg migration into the periapex arrests progression of lesions, we evaluated the mechanism of chemoattraction of Tregs into the lesions. Our data demonstrate that Tregs extracted from the lesions express the chemokine receptor CCR4 but not CCR5, CCR7, CCR8, and CXCR3 (Fig. 2A). Interestingly, although Tregs can express different chemokine receptors varying according to the developmental stage and the environment where they undergo activation, CCR4 seems to be essential to its migration to mucosal and periodontal tissues (22, 27, 28). Indeed, our data demonstrate the essential role of CCR4 in Tregs migration because CCR4KO mice present a significant decrease in CD4+FOXP3+ cell counts in the aggravated periapical lesions observed in this strain. Similarly, CCR4KO mice develop an increased periodontitis severity in response to experimental infection because of impaired Treg migration (22). Importantly, the gene expression profile in CCR4KO-derived periapical lesions is very similar to that observed in the lesions from WT mice treated with anti-GITR (ie, increased expression of proinflammatory cytokines and tissue destructive mediators and decreased expression of Treg markers and wound healing markers), reinforcing that the compromised Treg migration accounts for the aggravated lesions of CCR4KO mice. Indeed, adoptive transfer
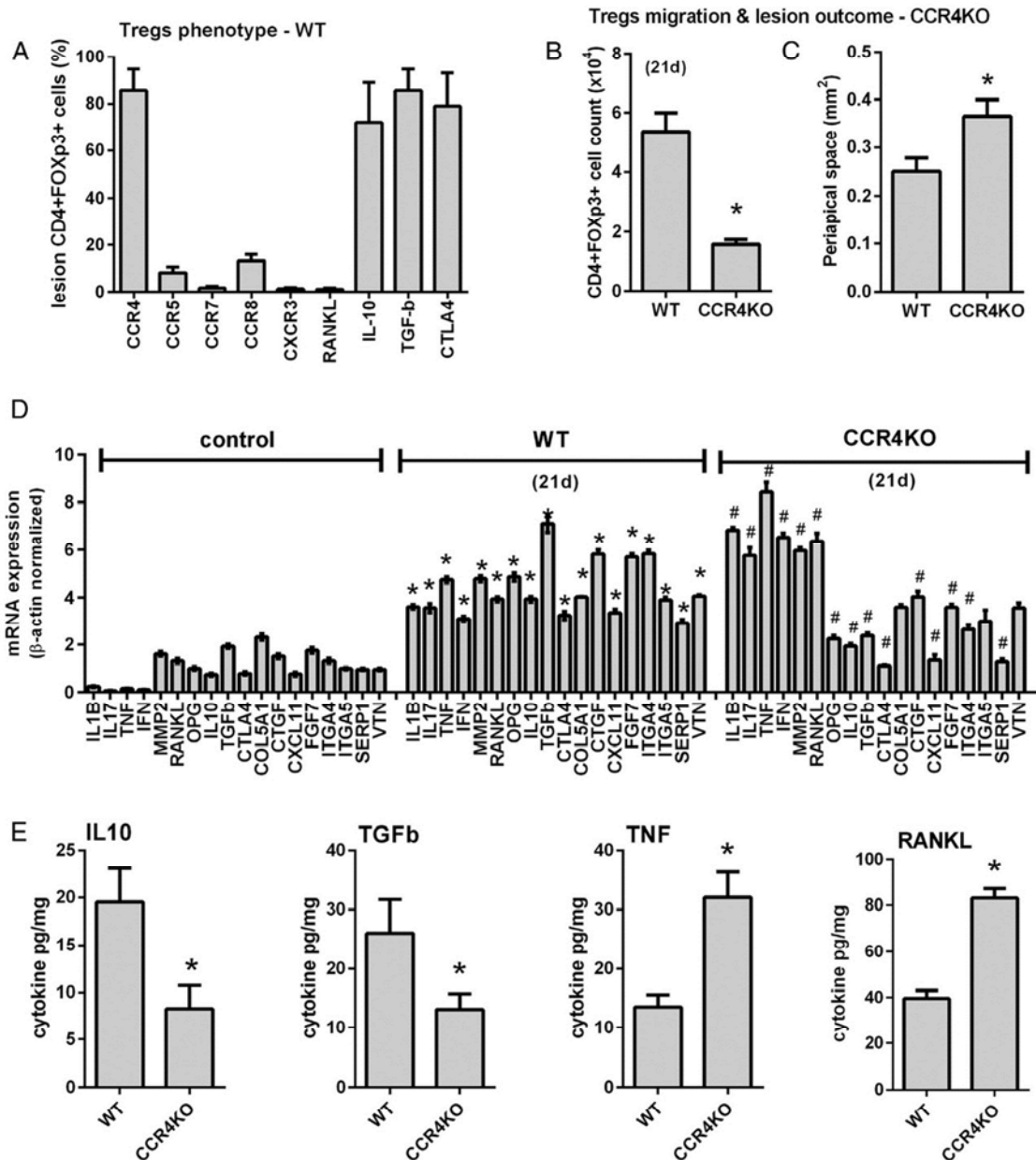
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Figure 2. Role of CCR4 in Treg migration kinetics and its impact on periapical bone loss and in expression of inflammatory/immunologic and healing markers in experimental periapical lesions in mice. C57Bl/6 (WT) and CCR4KO mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation). Samples from WT and CCR4KO groups were collected for histomorphometric and molecular analysis and evaluated for (*A*) phenotype of Tregs (CD4+FOXp3+) from periapical lesions, evaluated by flow cytometry and depicted as the number of positive cells for each marker; (*B*) Treg (CD4+FOXp3+) cell counts in periapical tissues analyzed by flow cytometry at 21 days after infection, depicted as the cell number $\times$ 10^4; (*C*) periapical lesion development, presented as periapical space area (mm^2) increase after induction of lesions, measured with ImageJ software in hematoxylin-eosin stained histologic sections; (*D*) expression of inflammatory/immunologic and wound healing markers at 21-day time point, measured quantitatively by real-time PCR array, presented as fold change relative to control group after normalization by constitutive genes (GAPDH, ACTB, Hprt1) expression levels, and (*E*) cytokine levels in periapical lesions, measured by ELISA, presented as cytokine pg/mg of periapical tissue. Asterisks (*) represent statistically significant differences ($P < .05$; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs control group, and hashtag (#) represents statistically significant differences ($P < .05$; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs WT group.
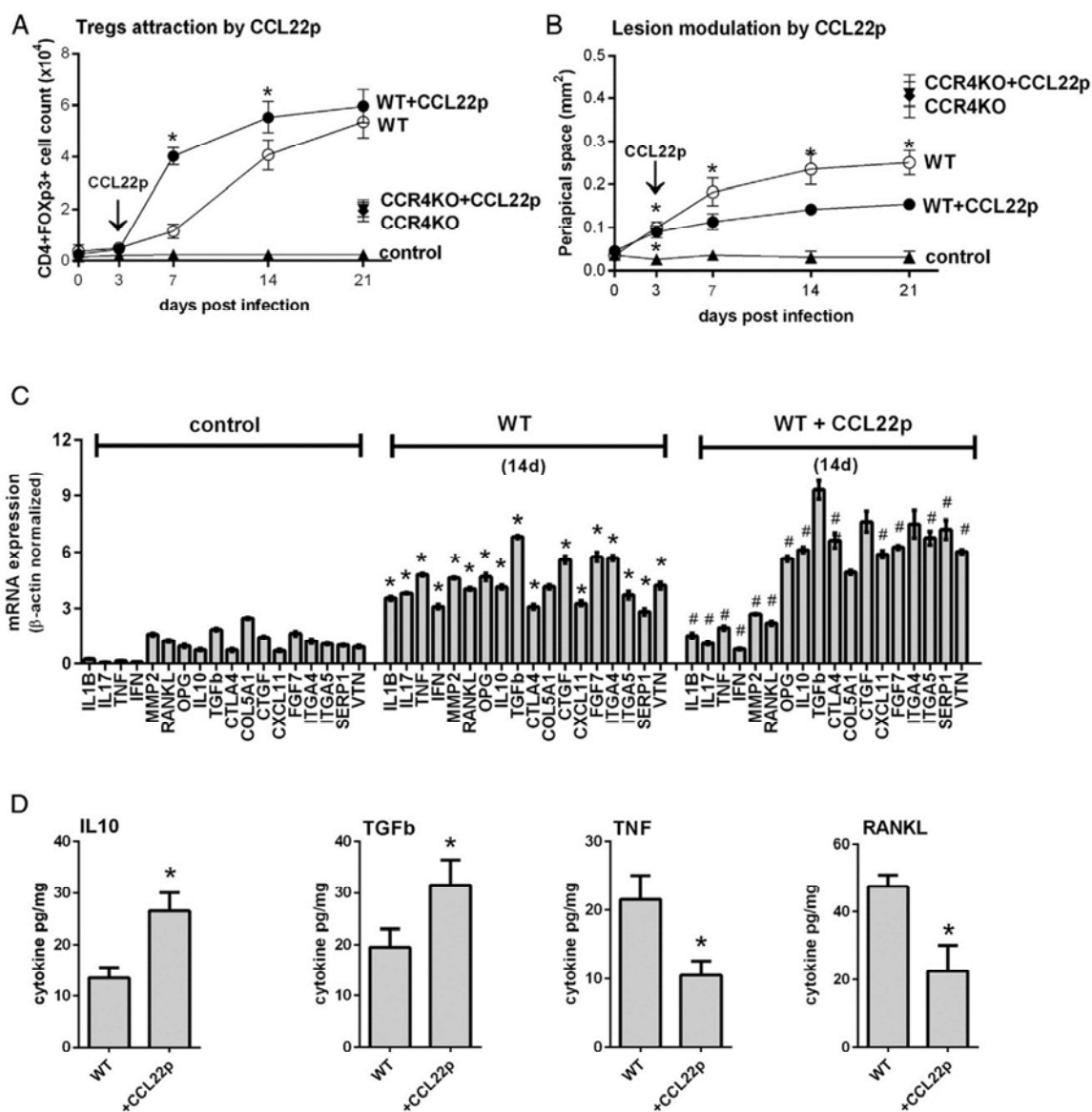
Figure 3. Involvement of CCL22 in Treg migration kinetics and its impact on periapical bone loss and in expression of inflammatory/immunologic and healing markers in experimental periapical lesions in mice. C57Bl/6 (WT) and CCR4KO mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation) and treated (or not) with CCL22-releasing PLGA particles to induce Tregs migration by direct injection of the particles at root canal system at 3-day time point. Samples from WT and CCR4KO strains from control and experimental groups were collected for histomorphometric and molecular analysis and evaluated for (*A*) Treg (CD4+FOXp3+) cell counts in periapical tissues analyzed by flow cytometry at 0, 3, 7, 14, and 21 days after infection, depicted as the cell number $\times$ 10^4; (*B*) periapical lesion development, presented as periapical space area (mm^2) increase after induction of lesions, measured with ImageJ software in hematoxylin-eosin stained histologic sections; (*C*) expression of inflammatory/immunologic and wound healing markers at 21-day time point, measured quantitatively by real-time PCR array, presented as fold change relative to control group after normalization by constitutive genes (GAPDH, ACTB, Hprt1) expression levels, and (*D*) cytokine levels in periapical lesions, measured by ELISA, presented as cytokine pg/mg of periapical tissue. Asterisks (*) represent statistically significant differences ($P < .05$; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs control group, and hashtag (#) represents statistically significant differences ($P < .05$; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs WT group.

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of CCR4+ Tregs restores original host response phenotype of CCR4KO mice in experimental periodontitis (22). Similarly, in rats' experimental periapical lesions, the chemokine CCL22, a CCR4 ligand, was chronologically alleged to mediate Treg appearance and regulatory activity (21). Our results confirm this hypothesis, because CCL22 release in the root canal system (by means of previously developed degradable PLGA microparticles containing recombinant CCL22 [29]) effectively promotes Treg migration and arrests progression of the lesions. Importantly, the protective effect of CCL22-releasing particles was proven to be CCR4-dependent, because such treatment was ineffective in CCR4KO strain.

Interestingly, although the CCL22/CCR4/Tregs axis compromises anti-tumoral immunity and its inhibition with anti-CCR4 antibody (mogamulizumab) can improve therapeutic protocols (30, 31), chemoattraction of Tregs via CCL22/CCR4 was demonstrated to be a promising therapeutic strategy in different chronic inflammatory conditions (23, 32, 33). Indeed, it is important to consider that the approval for polymeric controlled release systems for clinical use in humans, along with the biological effectiveness of extremely miniscule amounts of chemokine (ng/kg total body weight), reinforces the potential safety and affordability of the proposed strategy and may enable the translation to clinical reality (34, 35).

Therefore, considering that the CCL22-mediated Treg migration switches active periapical lesions into an inactivity phenotype, Treg chemoattractant may be a promising strategy for the clinical management of periapical lesions.

Conclusion

Our results demonstrate that Tregs are active immunosuppressive (and even pro-healing) agents in periapical granuloma pathogenesis. Treg chemoattraction to the periapical area is mediated by the CCL22/CCR4 axis. Treatments oriented on recruitment of endogenous Tregs can be potentially explored as a clinical strategy for management of periapical lesions.

Acknowledgments

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ARTICLE 2.2

Anti-RANKL therapy limits progression of periapical lesions but is prone to relapse due to impaired immunoregulation and unremitting host pro-inflammatory response

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ABSTRACT

Introduction: Development of periapical lesions involves the production of host inflammatory immunological mediators, which activate pathways that ultimately drive the breakdown of periapical tissues. Adjunct therapies targeting host inflammatory and osteoclastogenic mediators have been proposed for the clinical management of osteolytic alterations, but their potential application in endodontic practice remains unknown. In this study, we investigated the impact of anti-RANKL therapy on inflammatory cell influx and bone loss during periapical lesion development and its possible effect on the host immunological responses. **Methods:** Experimentally-induced periapical lesions in C57Bl/6 (WT) mice were treated with anti-RANKL using continuous or intermittent protocols, and with anti-TNF therapy as a control. Lesions were assessed for leukocytes influx, periapical bone loss, and the levels of inflammatory, immunological and healing markers expression (RealTimePCRarray, ELISA). **Results:** Anti-RANKL treatment arrested lesion development, but prompted a continuous inflammatory response characterized by unremitting elevated expression of pro-inflammatory cytokines and tissue destructive mediators, and decreased expression of Tregs and wound healing markers levels. Conversely, untreated lesions undergo an immunoregulatory process that determine an inactive/stable status in the equivalent time period. Anti-RANKL treatment triggered lesion development relapse and was associated with high T CD4 effector/suppressor cells ratio and active lesions gene expression signature. Anti-TNF treatment limits lesions progression and does not drive lesions relapse upon cessation, being associated with attenuated host response. **Conclusions:** Anti-RANKL treatment efficiently limits periapical bone loss, although perpetuates the host inflammatory response prompting lesion relapse.

Keywords: periapical granuloma, regulatory T cells, T-Lymphocytes, cytokines, wound healing, RANKL.

INTRODUCTION

Development of periapical lesions is triggered by infection of the root canal system and the subsequent production of host inflammatory immunological mediators, which in turn activate pathways that drive the breakdown of periapical tissues (1). Periapical lesion outcome is determined by the balance between pro- and anti- inflammatory cytokines, which differentially modulates proteolytic activity, bone resorption and healing mechanisms (1-4).

In this context, adjunct therapies targeting pro-inflammatory cytokines, such as TNF, IL-1 and IL-17, have been proposed to the clinical management of inflammatory osteolytic alterations (1, 2). Tumor necrosis factor α (TNF α) is an inflammatory cytokine that plays a central role in acute inflammation and is responsible for a diverse range of signaling events within cells that triggers necrosis or apoptosis (AN, 2010; IDRISS; NAISMITH, 2000). Its inhibition by Infliximab was approved by the United States Food and Drug Administration to treat Crohn disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, and ulcerative colitis. However, these therapies impact host response and increase individual susceptibility to infections, and therefore their applications in infectious conditions are still limited (5).

The identification of the RANK-RANKL-OPG system provided additional targets to treat bone diseases. Evidence from animal models showing that inhibition of the major osteoclastogenic factor RANKL attenuated bone loss associated with osteoporosis and arthritis, led to successful clinical trials in humans and the approval of anti-RANKL monoclonal antibodies for use in treatment of those conditions (6-8)

The application of RANKL-inhibition based therapies to manage the bone loss associated with infectious inflammatory diseases remains elusive. Only a few studies demonstrate that RANKL inhibition by its decoy receptor OPG or anti-RANKL limits experimental periodontal bone loss in rats (9, 10). RANKL inhibition was also effective in preventing periapical osteolysis in a long-term treatment mimicking osteoporosis therapy, but resulted in jaw osteonecrosis (11, 12). However, the therapeutic potential of short-term RANKL inhibition, as potentially needed during routine endodontic practice, remains undetermined.

Indeed, additionally to regulate bone homeostasis/pathology, the RANK-RANKL-OPG system plays a key role in the regulation of lymphoid organ microenvironment, antigen presentation, induction and regulation of immune responses (13-16). Since

the nature and extent of the host response is a critical determinant of periapical lesion outcome (1, 2, 17, 18), it is crucial to determine if RANKL-inhibition also impacts inflammatory/immune responsiveness at the periapical region. This hypothesis is reinforced by recent studies linking RANKL-blockage to impaired Tregs function (19, 20). Tregs have been shown to have a protective role in determining periodontal and periapical lesion inactivity, arresting bone loss and upregulating the expression of repair-associated factors (3, 21, 22).

In this study, we investigated the effects of anti-RANKL therapy on inflammatory cell influx and bone loss during periapical lesion development, as well its possible impact on the host regenerative and immune responses.

MATERIAL AND METHODS

Experimental periapical lesions & treatments - Experimental groups comprised 8-week-old male C57BL/6 wild-type (WT) mice maintained in the animal facilities of USP and fed ad libitum with standard solid mice chow (Nuvital, Curitiba, PR, Brazil) and sterile water. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use following the Guide for the Care and Use of Laboratory Animals principles. Periapical lesion induction and quantification was performed as previously described (3, 18). Mice (N=5/time/group) were anesthetized, and the dental pulps of mandibular first molars were exposed with a carbide bur, and inoculated with: P. gingivalis ATCC33277, P. nigrescens ATCC33563, A. viscosus ATCC91014, and F. nucleatum ATCC10953. RANKL function was inhibited by treatment with purified mouse anti-RANKL mAb (OYC Americas, Andover, MA, USA) injected i.p. at 300µg/kg once every 48h (7). Another group were treated with anti-TNF therapy (1.0mg/kg/48h i.p.) (Infliximab/Remicade-Janssen Biotech) (7) and an additional group for endpoint evaluation of T cell subsets comprised CCL22 releasing particles (CCL22p) (3, 22). Treatment protocols were initiated 3 days after periapical lesion induction and sustained up to 21 days (continuous protocol) or it was initiated at 3 days time point and sustained until the 14 days, followed additional 21 days and 28 days time points analysis (intermittent protocol). In the control group there was no induction of periapical lesion. Animals were killed by cervical displacement after 0, 3, 7, 14, 21 and 28 days of infection, the jaws were dissected and independent samples

were prepared for histomorphometric (right molars) or molecular (left molars) analysis. Results are depicted as the area of periapical space, measured in HE stained longitudinal 5-µm-thick sections by using ImageJ (NIH, Bethesda, MD), being the area increase over time representative of lesion development.

Gene Expression and ELISA – Total RNA extraction from periapical lesions was performed using RNeasyFFPE (Qiagen Inc, Valencia, CA, USA), followed by cDNA synthesis (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA). Gene expression analyses were performed as previously described (3), in RealtimePCR using a custom gene expression profiling panel (SABiosciences, Frederick, MD, USA) Viia7 sequence detection instrument (LifeTechnologies, Carlsbad, CA, USA). Array data was analyzed using the RT2profiler software (SABiosciences), and the results are expressed as the fold change of target genes relative to the control (0h) group, after normalization by constitutive genes (GAPDH, ACTB, Hprt1). The levels of IL-10, TGF-b, TNF and RANKL in periapical lesions were assessed using ELISA (R&S Systems, Minneapolis, USA), as previously described (23). The results are expressed as picograms of cytokine/mg of periapical tissue.

Isolation and analysis of leukocytes from periapical tissues - Isolation and characterization of periapical lesions' cells was performed as described elsewhere (23). In brief, periapical tissues were incubated in RPMI-1640/blendzyme (Roche, Basel, Switzerland) and processed with 0.05% DNase (Sigma-Aldrich, Steinhein, Germany) using Medimachine (BDbiosciences, San Diego, CA, USA) and followed by cell viability analysis (Trypan blue) and cell counting (Neubauer chamber). Cells were incubated with optimal dilution PE- and FITC-conjugated CD4 and FOXp3 antibodies (BD Biosciences) and then analyzed by flow cytometry (FACScan and CellQuest; BD Biosciences). Results represent the number of cells ± SD in the periapical tissues of each mouse, normalized by the tissue weight.

Statistical analysis - Data are presented as means ± SD, and the statistical significance between the groups was analyzed by Kruskal-Wallis followed by Dunn's post test, or Mann-Whitney test, as implemented in GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). PCRarray data was analyzed by the

Mann-Whitney test followed by Benjamini-Hochberg test. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Anti-RANKL treatment influences periapical lesion progression but not host inflammatory immune response. Continuous anti-RANKL treatment arrested periapical bone loss when compared to untreated controls (Fig.1A), despite the similar number of inflammatory cells in the periapical region of both experimental and untreated control groups. The only exception was at 21d when anti-RANKL group presented increased inflammation (Fig.1B). The anti-TNF control group presented a significant decrease in lesion development (Fig.1A) and inflammatory cell count (Fig.1B). Lesions from untreated and anti-RANKL groups presented a similar gene expression signature at 7d (Fig.1C), characterized by increased expression of pro-inflammatory and tissue destructive mediators (IL1B, IL17, TNF, MMP2, RANKL) and decreased Tregs (TGF-b, CTLA-4) and wound healing markers (OPG, COL5A1, CTGF, FGF7, ITGA4/5, SERP1) levels. At 21d, while untreated lesions presented a switch in this pattern, anti-RANKL group remained with a gene expression signature of active lesions (24, 25). The anti-TNF treatment resulted in an overall decrease of pro-inflammatory and healing markers, and increase in Tregs markers (Fig.1D). Higher TNF and RANKL, and lower IL-10 and TFG-b levels were also detected by ELISA in the anti-RANKL treated group at 21d, in contrast with untreated and anti-TNF groups (Fig. 1E).

Cessation of anti-RANKL therapy results in immediate lesion expansion associated with sustained inflammatory immune response. While the transient (3-14d) anti-RANKL treatment arrested periapical bone loss, treatment cessation resulted in lesion relapse (Fig.2A, C), characterized by increased bone loss and inflammatory cell counts (Fig.2B, D), high TNF and RANKL and low IL-10 and TGF-b levels (Fig.2E, F). In the corresponding period, untreated lesions remained stable presenting lower severity than RANKL-treated lesions at the endpoint (28d) (Fig.2A-D), whereas anti-TNF treatment suppression resulted in a minor increase in lesion severity (Fig.2A-D). These findings are supported by the molecular data; anti-RANKL group lesions presented a gene expression signature characteristic of active lesions

(3, 24, 25), in contrast with untreated and anti-TNF groups (Fig. 2E, F). Analysis of T CD4 cells demonstrated a sustained and increased migration of effector T cells (CD4+FOXp3-) with Th1 and Th17 features (Fig.3A, C), along with limited Tregs (CD4+FOXp3+) counts (Fig.3B, D) in the anti-RANKL group, resulting in a higher effector/suppressor ratio (Fig.3E, F) correlated with the lesion evolution pattern (Fig.3D). Anti-TNF and CCL22p treatments limited the infiltration of effector T cells and increased Tregs counts (Fig.3A, B).

DISCUSSION

Host pro-inflammatory mediators, such as TNF, mediates periapical bone loss by the activation of bone resorption mechanisms via the osteoclastogenic factor RANKL (1). Therefore, the inhibition of host factors responsible for tissue breakdown has been suggested as a potential therapeutic strategy for the clinical management of periapical lesions. Our results demonstrated that the inhibition of RANKL effectively prevents the progression of periapical bone loss, as predicted by previous studies on osteoporosis, arthritis and periodontitis (7, 9). Importantly, the short-term (18d) treatment did not result in pathological consequences such as jaw osteonecrosis, often seen after long-term (12w) anti-RANKL treatment (11). However, RANKL inhibition impaired the natural immunoregulatory process generally observed in untreated lesions.

Untreated lesions present initially an active/progressive pattern that temporally evolves to an inactive/stable status, both characterized by unique and opposing patterns of immune-inflammatory and wound healing markers expression (23, 24). Our data demonstrate that at 7 days (when the periapical lesions are considered active) (23, 24), untreated and anti-RANKL-treated lesions presented similar patterns of inflammatory immune response. However, while untreated lesions acquire an inactive/non-progressive phenotype over time, despite the absence of bone loss, anti-RANKL treated lesions still presented a gene expression signature compatible with an active lesion phenotype (3, 23, 24). Lesions from anti-RANKL group featured high levels of pro-inflammatory (TNF, IL1B and IL17) and low levels of anti-inflammatory (IL-10 and TGF-b) mediators. Accordingly, recent studies demonstrate that RANKL inhibition does not prevent inflammation in bowel disease and arthritis

models (26). Conversely, the anti-TNF treatment used as control, resulted in a quick attenuation of the host inflammatory immune reaction and bone loss, resulting in lesions with inactive phenotypes also from the gene expression viewpoint (3, 23, 24), in accordance with previous studies (27-29).

Importantly, the exacerbated inflammatory immune response derived from the anti-RANKL treatment seems to contribute to the progression of lesions by limiting the tissue repair/regeneration attempts (24), as demonstrated by the decreased expression of healing markers in this group. Accordingly, RANKL inhibition was associated with sustained inflammation and delayed healing of palatal lesions in mice (30). Corroborating previous findings (31, 32), anti-TNF treatment resulted in a decreased expression of healing markers in the lesions.

In order to simulate a clinical endodontic scenario, where an eventual anti-RANKL adjunct therapy would be temporary (as opposed to long-term therapies as in osteoporosis and arthritis) (11), we examined the outcomes of the persistent and exacerbated host response upon cessation of the anti-RANKL therapy. We found that the interruption of anti-RANKL treatment prompted lesion relapse, with increased severity compared to untreated controls. Accordingly, previous studies have demonstrated that discontinuation of anti-RANKL therapy resulted in a quick reestablishment of bone resorptive function (12). Indeed, since the pro-resorptive environment high inflammatory cell counts (including pre-osteoclasts) and high levels of pro-osteoclastogenic mediators (including TNF and RANKL) was sustained in the periapical region during the anti-RANKL therapy, (33), the quick relapse of the lesions seems predictable. Conversely, interruption of anti-TNF treatment resulted in a slight increase in inflammatory cell infiltration and in the periapical space, resulting in a lesion significantly less severe than in the untreated and anti-RANKL treated groups.

Interestingly, the stability phenotype of untreated and anti-TNF treated lesions presents high IL-10 and TGF-b levels as a common feature, meanwhile lower levels of these cytokines characterize the unremitting lesions after anti-RANKL treatment. Untreated lesions naturally remain in a stable/inactive status through the concerted action of MSCs and Tregs, whose immunoregulatory properties are mediated mainly by IL-10 and TGF-b (3, 23). Indeed, while the inhibition of Tregs leads to continuous lesion progression in mice (3) its chemoattraction by CCL22-releasing particles (CCL22p) prompts its switch to an inactive status and arrests lesion progression (3).

Importantly, our results demonstrate that anti-RANKL therapy, even upon cessation, significantly decrease and delay Tregs migration into the periapex, leading to an increased T CD4 effector/suppressor ratio, which was found to be positively correlated with lesion evolution in the present and in previous studies (1, 21, 23). Further, recent studies demonstrate that RANKL plays a role in the generation of FOXp3+ regulatory T cells, and that its inhibition may increase effector T cell responses (19, 34). Importantly, gene expression and ELISA data suggest a trend towards the conversion of anti-RANKL group lesions into an inactive phenotype 2 weeks after therapy cessation. While an extended analysis would be required to determine if the consequences of anti-RANKL therapy on the immunoregulation process are actually reversible, our data demonstrate that the immunological consequences of RANKL blockage lasts longer than the quickly reversible effects on bone.

Contrarily, anti-TNF treatment resulted in early Tregs migration and decreased CD4 effector/suppressor ratio. Previous studies have demonstrated that TNF inhibition may increase Tregs suppressive function (7, 35), and may interfere with the effector/suppressor ratio, such as in the Th17/Treg balance (7). Interestingly, the anti-TNF treatment resulted in T CD4 effectors/suppressors counts and ratio similar to those observed in response to CCL22 releasing particles, which was effective in attenuating periapical lesion progression via Tregs chemoattraction (3).

In conclusion, our results demonstrate that while anti-RANKL treatment efficiently limits the periapical bone loss, RANKL blockage interferes with the natural immunoregulatory process, leading to an unremitting destructive host inflammatory response prompt to lesion relapse upon the discontinuation of anti-RANKL therapy. Therefore, despite the existence of RANKL inhibitors approved for clinical use, its potential impact in the Treg-mediated protective host response may limit its application as an adjunct therapy for the treatment of periapical lesions.

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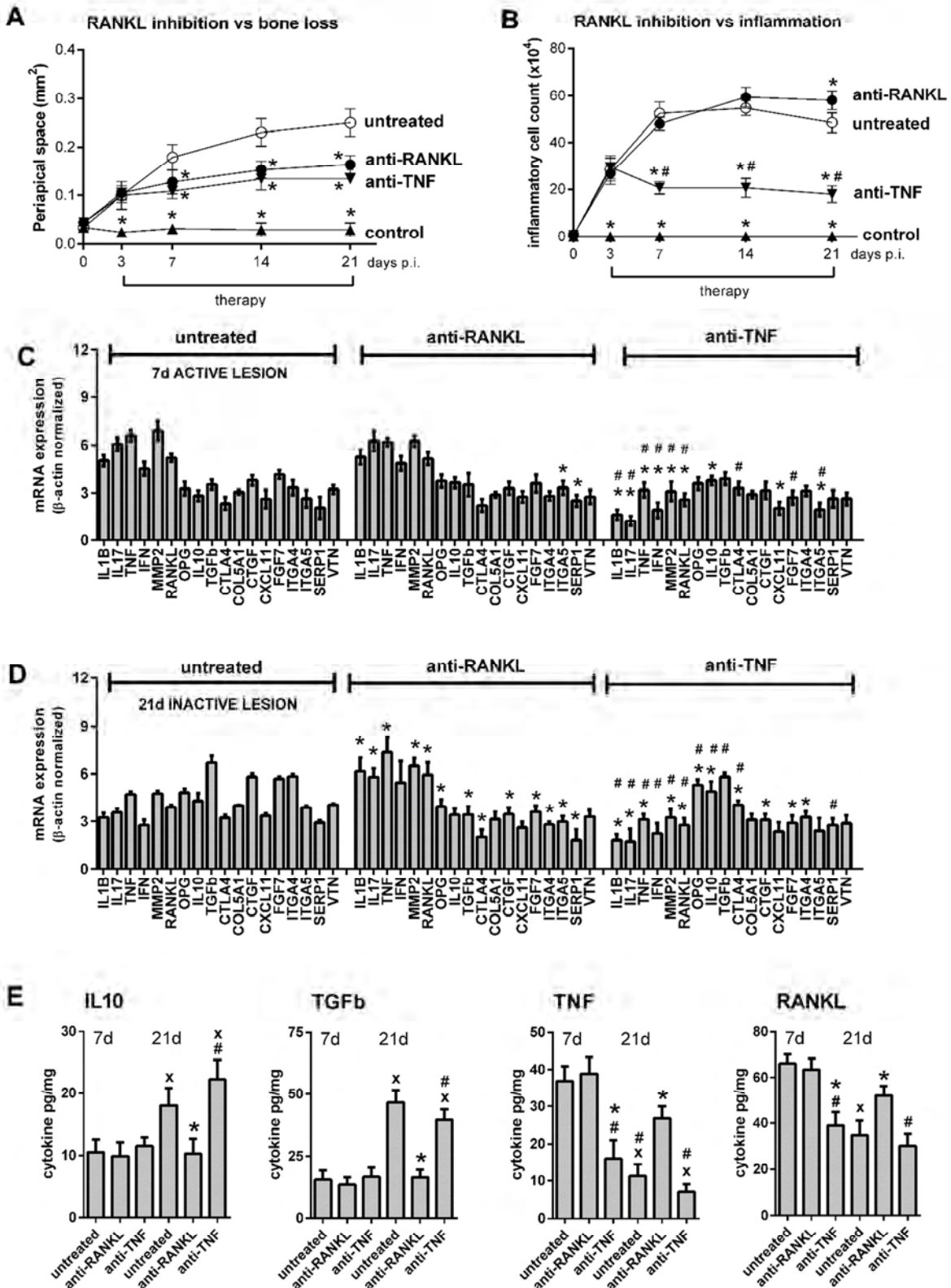
Figure 1. Impact of anti-RANKL treatment on periapical bone loss and expression of immunological markers in experimental periapical lesions in mice. C57Bl/6 (WT) mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation) and treated (or not) with anti-RANKL (300μg/kg/48h i.p.) or anti-TNF (0.75mg/kg/48h i.p.). Treatment protocols were initiated at 3d and sustained up to 21d. Samples from experimental and control groups were collected for histomorphometric and molecular analysis, and evaluated for: (A) periapical lesion development, presented as periapical space area (mm^2) increase after lesions induction, measured with ImageJ software in HE stained histological sections; (B) inflammatory cell counts in periapical tissues analyzed by flow cytometry, depicted as the cell number $x10^4$; (C-D) the expression of inflammatory/immunological and wound healing markers at 7d (C) and 21d (D) time points, measured quantitatively by RealTimePCRarray, presented as fold change relative to the control group after normalization by constitutive genes (GAPDH, ACTB, Hprt1) expression levels; and (E) cytokine levels in periapical lesions, measured by ELISA, presented as cytokine pg/mg of periapical tissue. The asterisks (*) represents statistically significant differences between the indicated group vs untreated group, the hashtag (#) represents statistically significant differences between anti-RANKL and anti-TNF groups, the 'x' represents statistically significant differences in each group when 7d and 21d time points are compared (P<0.05; One-way ANOVA, Bonferroni post-test for A, B and E; Mann-Whitney test followed by Benjamini-Hochberg test for C and D).

A anti-RANKL cessation vs bone loss

B anti-RANKL cessation vs inflammation

C Patterns of lesion evolution

D Patterns of inflammation evolution

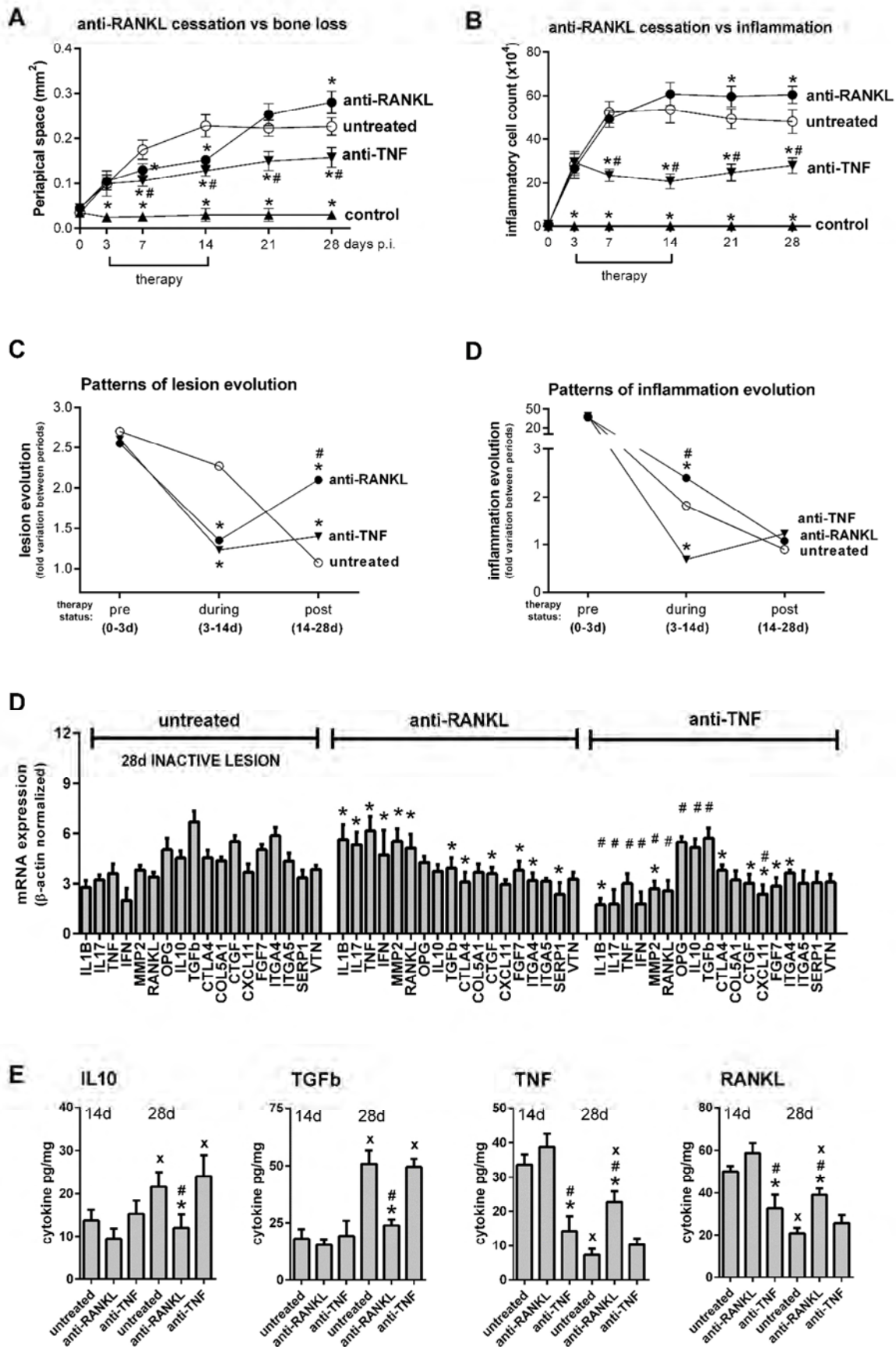**D** 28d INACTIVE LESION

E IL10 TGFb TNF RANKL

Figure 2. Impact of anti-RANKL treatment cessation on periapical bone loss and the expression of immunological markers in experimental periapical lesions in mice. C57Bl/6 (WT) mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation) and treated (or not) with anti-RANKL (300µg/kg/48h i.p.) or anti-TNF (0.75mg/kg/48h i.p.). Treatment protocols were initiated at 3d time point and sustained until the 14d, followed additional 21d and 28d time points analysis. Samples from experimental and control groups were collected for histomorphometric and molecular analysis, and evaluated for: (A) periapical lesion development, presented as periapical space area (mm^2) increase after lesions induction, measured with ImageJ software in HE stained histological sections; (B) inflammatory cell counts in periapical tissues analyzed by flow cytometry, depicted as the cell number $x10^4$; (C) the lesion evolution index, depicted as the fold increase in specific time intervals pre, during and post-treatment; (D) the inflammation evolution index depicted as the fold increase in specific time intervals pre, during and pos-treatment; (E-F) the expression of inflammatory/immunological and wound healing markers at 28d (E) time point, measured quantitatively by RealTimePCRarray, presented as fold change relative to the control group after normalization by constitutive genes (GAPDH, ACTB, Hprt1) expression levels and (F) cytokine levels in periapical lesions, measured by ELISA, presented as cytokine pg/mg of periapical tissue at 14d and 28d time point. The asterisks (*) represents statistically significant differences between the indicated group vs untreated group, the hashtag (#) represents statistically significant differences between the anti-RANKL and anti-TNF groups ($P<0.05$; One-way ANOVA, Bonferroni post-test for A, B, C, D, E, F; Mann-Whitney test followed by Benjamini-Hochberg test for D).
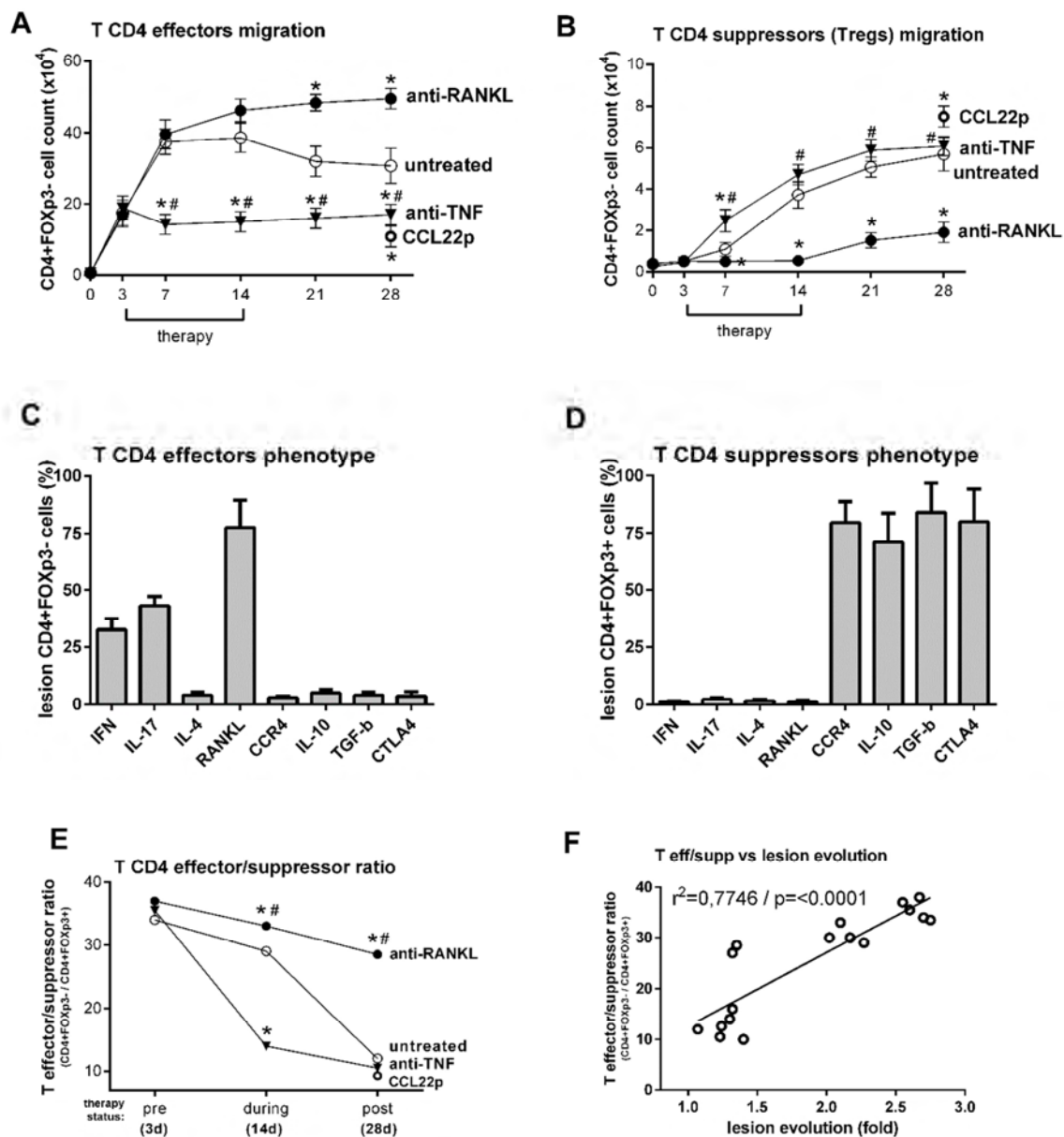
Figure 3. Involvement of effector and suppressor T cells on periapical lesions relapse and/or stability after cessation of anti-RANKL therapy. C57Bl/6 (WT) mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation) and treated (or not) with anti-RANKL (300µg/kg/48h i.p.) or anti-TNF (0.75mg/kg/48h i.p.).Treatment protocols were initiated at 3d time point and sustained until the 14d, followed additional 21d and 28d time points analysis. Samples from experimental and control groups were collected for histomorphometric and molecular analysis, and evaluated for: (A) T CD4 effector cells (CD4+FOXp3-) cells count in periapical tissues analyzed by flow cytometry,

depicted as the cell number x10^4; (B) T CD4 suppressor cells (i.e. Tregs, CD4+FOXp3+) cells count in periapical tissues analyzed by flow cytometry, depicted as the cell number x10^4; (C) the phenotype of T CD4 effector cells (CD4+FOXp3-) cells from periapical lesions, evaluated by flow cytometry and depicted as the number of positive cells for each marker; (D) the phenotype of T CD4 suppressor cells (i.e. Tregs, CD4+FOXp3+) cells from periapical lesions, evaluated by flow cytometry and depicted as the number of positive cells for each marker; (E) the T CD4 effector/suppressor ratio in different stages of treatments (pre, during and post treatment); the (F) correlation between the T CD4 effector/suppressor ratio and the lesion evolution index. The asterisks (*) represents statistically significant differences between the indicated group vs untreated group, the hashtag (#) represents statistically significant differences between the anti-RANKL and anti-TNF groups (P<0.05; One-way ANOVA, Bonferroni post-test).

ARTICLE 2.3

Role of Th17 cells in experimental periapical lesions development.

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ABSTRACT

Introduction: the presence of pathogenic microorganisms is necessary to start the development of periapical lesions, but its progression and severity are highly dependent on the host immune and inflammatory response. In this context, different subtypes of lymphocytes have been implicated in periapical lesion pathogenesis, and Th17 has been correlated with the expression of proinflammatory cytokines and bone resorption induction, contributing to greater severity of disease. In this study, we investigated in a cause-and-effect manner the involvement of Th17 and its products in the progression of experimental periapical lesions. **Methods:** C57Bl/6 (WT) and IL-17KO mice were submitted to experimental periapical lesions induction (pulp exposure and bacterial inoculation), and analyzed regarding Th17 influx, periapical bone loss via µCT, histomorphometric analysis and inflammatory/immunological and healing markers expression (analyzed by RealTimePCRarray). **Results:** the absence of IL-17 results in a significant decrease in periapical lesions severity, associated with upregulation of healing markers and anti-inflammatory cytokines, in parallel with decreased expression of tissue destruction markers and proinflammatory cytokines. The histomorphometric analysis proved a lower severity of injuries in IL-17KO mice showing lower concentration of osteoclasts, neutrophils and mononuclear cells in periapical lesions without IL-17. **Conclusion:** the absence of IL-17 in periapical lesions promoted less severe lesions with mild inflammatory character and therefore less bone resorption. Thus, possible strategies focusing on Th17 inhibition may be a promising strategy for the clinical management of periapical lesions.

Keywords: Periapical Granuloma, Th17 Cells, Cytokines.

INTRODUCTION

Periapical lesions are common inflammatory osteolytic diseases of the jaw (ANDRADE et al., 2013). They happen due to the host response to continuous antigenic stimulation in the infected canals (STASHENKO; TELES; D'SOUZA, 1998). The constant presence of infiltrating bacteria and bacterial noxious substances evokes host immunologic reactions. Inflammatory cells could be recruited from nearby bone marrow, producing proinflammatory cytokines, activating osteoclasts, and leading to adjacent bone destruction (ALSHWAIMI et al., 2009; XIONG; WEI; PENG, 2009).

Periapical lesions are histologically characterized by fibrous and granulation tissue, proliferating epithelium or cyst infiltrated by different inflammatory cells (DE OLIVEIRA RODINI; BATISTA; LARA, 2004). Among infiltrating leukocytes, neutrophil granulocytes are the first line of defense which stimulate the migration of monocytes and lymphocytes. Mononuclear cell infiltrates, composed of antigen-presenting cells (APC), T and B lymphocytes and their effectors are characteristic of chronic periapical processes (MARTON; KISS, 1993).

In these context, the role of the immune response in the formation of chronic periapical lesions has been extensively studied (COLIC et al., 2007; MARCAL et al., 2010; OSEKO et al., 2009; STASHENKO; TELES; D'SOUZA, 1998) and Th17 cells were described in the maintenance of inflammatory process (ANDRADE et al., 2013; WEI et al., 2013; XIONG; WEI; PENG, 2009). Th17 cells comprise a subpopulation of CD4+T cells whose main secretion product is interleukin (IL)-17, a proinflammatory cytokine that exerts potent effects on different cell types of the innate immunity and is considered a molecular bridge between the innate and acquired immune systems (YU; GAFFEN, 2008). It plays an important role in the initiation and maintenance of proinflammatory responses, and has been found to stimulate osteoclastic activation (VERNAL et al., 2005).

The functions of IL-17 have been examined in many contexts. In vitro, IL-17 has been shown to activate fibroblast, epithelial cells, endothelial cells, and osteoblasts to produce proinflammatory cytokines such as IL-6, IL-8, granulocyte colony-stimulating factor, and matrix metalloproteinases (WITOWSKI; KSIAZEK; JORRES, 2004). IL-17 plays an essential, nonredundant role in neutrophil activation, maturation, and homeostasis (KOLLS; LINDEN, 2004). It could also promote bone resorption by stimulating osteoblasts to produce the receptor activator of nuclear factor-kappa B

(RANKL) that affects the activity and formation of osteoclasts (GAFFEN, 2004). In humans, IL-17 has been associated with the pathology of numerous autoimmune and inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus, multiple sclerosis, psoriasis and allograft rejection (DONG, 2006). Furthermore, the presence of IL-17 has also been documented in periodontitis patients, suggesting that this cytokine might mediate inflammation in periodontal diseases (TAKAHASHI et al., 2005; VERNAL et al., 2005).

Taken together, these data led us to believe that Th17 cells and their related factors may contribute to tissue inflammation and bone destruction of periapical lesions. However, the actions of Th17 in periapical lesions are not fully understood. The aim of this study was to evaluate the role of IL-17 in periapical lesions of WT mice through the use of IL-17KO mice.

Materials and Methods

Experimental periapical lesions & treatments. Experimental groups comprised 8-week-old male C57BL/6 wild-type (WT) and IL-17 knockout (IL-17KO) mice maintained in the animal facilities of USP and fed with standard solid mice chow (Nuvital, Curitiba, PR, Brazil) and sterile water. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use following the Guide for the Care and Use of Laboratory Animals principles. Periapical lesion induction and quantification was performed as previously described (FRANCISCONI, et al., 2016). Mice (N=5/time/group) were anesthetized, upper first molars dental pulp was exposed with a carbide bur followed by bacterial inoculation (P.gingivalis ATCC33277, P.nigrescens ATCC33563, A.viscosus ATCC91014 and F.nucleatum ATCC10953). Animals were killed by cervical displacement after 0, 3, 7, 14 and 21 days of infection, the maxillae were scanned by the Skyscan 1174 System (Skyscan, Kontich, Belgium) being the area increase over time representative of lesion development. After that, the maxillae were dissected and independent samples were prepared for histomorphometric or molecular analysis.

Micro-computed tomography (µCT) assessment

µCT analysis is a valid method for the diagnosis of periapical lesions in mice (OOKUBO et al., 2016; OSEKO et al., 2009). The maxillae samples were scanned by the Skyscan 1174 System (Skyscan, Kontich, Belgium) at 50 kV, 800 µA, with a 0.5 mm aluminium filter and 15% beam hardening correction, ring artifacts reduction, 180 degrees of rotation and exposure range of 1 degree. Images were captured with 1304x1024 pixels and a resolution of 14µm pixel size. Projection images were reconstructed using the NRecon software and three-dimensional images obtained by the CT-Vox software. Based on prior study (KANG et al., 2013) a cylindrical region of interest (ROI) was determined by segmenting the space of root apex and the alveolar bone over 6 tomographic cuts in mesial root region, comprising approximately 0.15 mm deep and 10 tomographic slices (0.2 mm) at the distal root. This analysis included the tissue volume (TV), bone volume (BV), bone volume fraction (BV/TV, %), the width of the space periapical ligament and the length of the mesial root.

Histomorphometric Analysis

Serial sections (5 semi-serial sections of each maxillae, with a 5µm thickness for each section) were obtained using a microtome (Leica RM2255, Germany) and stained with H.E. (hematoxylin and eosin). Morphometric measurements were performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100x immersion objective and a Zeiss kpl 8X eyepiece containing a Zeiss II integration grid (Carl Zeiss Jena GmbH, Jena, Germany) with 10 parallel lines and 100 points in a quadrangular area. In the morphometric analysis, points were counted coinciding with the images of the following components of periapical space: blood vessels, fibroblasts, collagen fibers, mononuclear cells, neutrophils, osteoclasts and other components (empty space left by the inflammatory exudate, intercellular liquid or bone matrix). It is important to note that the dark-stained cells with a multilobed, horseshoe-shaped nucleus were deemed neutrophils (DE ROSSI; ROCHA; ROSSI, 2008; IRIE et al., 2008). The results are presented as the volume density (mean) for each evaluated structure.

Gene Expression and Enzyme-linked

Immunosorbent Assay

Real-time polymerase chain reaction (PCR) array reactions were performed as previously described (ARAUJO-PIRES et al., 2014a). Periapical tissues total RNA extraction was performed with RNeasyFFPE (Qiagen Inc, Valencia, CA, USA), followed by 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) analysis and cDNA synthesis (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA), according to the manufacturers' instructions. Realtime PCR array was performed in a Viia7 instrument (Life Technologies, Carlsbad, CA) by using a custom panel for gene expression profiling (SABiosciences, Frederick, MD), analyzed by the RT2profiler software (SABiosciences) for normalizing target genes expression levels by constitutive genes (GAPDH, ACTB, Hprt1) and the control group. The results are expressed as picograms of cytokine (± standard deviation [SD]) per milligram of periapical tissue (ARANHA et al., 2013). Measurements of cytokines IL-10, TGF-b, tumor necrosis factor (TNF), interferon (IFN) and receptor activator of nuclear factor kappa B ligand (RANKL) in periapical lesions were performed by enzyme-linked immunosorbent assay (ELISA) as previously described (ARAUJO-PIRES et al., 2015) by commercially available kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results are expressed as picograms of cytokine (± standard deviation [SD]) per milligram of periapical tissue.

Isolation and analysis of leukocytes from periapical tissues - Isolation and characterization of lesions' cells were performed as described previously (ARAUJO-PIRES et al., 2014a; ARAUJO-PIRES et al., 2014b). Periapical tissues were incubated in RPMI-1640/blendzyme (Roche, Basel, Switzerland) and then processed with 0.05% DNase (Sigma-Aldrich, Steinhein, Germany) using Medimachine (BDbiosciences, San Diego, CA, USA); followed by cell viability analysis (Trypan blue) and cell count (Neubauer chamber). Cells were incubated with optimal dilution fluorochrome-conjugated antibodies against CD4 (FITC, clone GK1.5, dilution 1:200), CCR4 (PE, 1G1, 1:100), CCR5 (PE, C34-3448,1:100), CCR7 (PE, 4B12, 1:50), CCR6 (PE, 1055c, 1:50), CXCR3 (PE, 1C6/CXCR3, 1:100), RANKL (PE, IK22-5, 1:100), IL-10 (PE, JES5-16E3, 1:100), TGF-b (PE, TW7-16B4, 1:200) and CTLA-4 (PE,UC10-4F10-11, 1:200) (BD Biosciences) and then analyzed by flow cytometry

(FACScan and CellQuest; BD Biosciences). Cells were incubated with optimal dilution PE- and FITC-conjugated CD4 and IL-17 antibodies (BD Biosciences) and then analyzed by flow cytometry (FACScan and CellQuest; BD Biosciences). Results represent the number of cells ± SD in the periapical tissues of each mouse, normalized by the tissue weight.

Statistical Analysis

Data are presented as means ± SD, and the statistical significance between the groups was analyzed by Kruskal-Wallis followed by Dunn post hoc test or by Mann-Whitney test; both were performed with Graph-Pad Prism 5.0 software (GraphPad Software Inc, San Diego, CA). PCR array data were analyzed by the Mann-Whitney test followed by Benjamini-Hochberg test. Values of $P < 0.05$ were considered statistically significant.

Results

Kinetics of Th17 cells migration and its phenotype

The induction of experimental periapical lesions generated increasing Th17 cells (CD4+IL17+) migration over time, however there was a higher concentration of Th17 cells from 7 days (Fig. 1A). Phenotypic analysis demonstrated that periapical lesions' Th17 (CD4+IL17+cells) express high levels of the chemokine receptor CCR6 and RANKL (Fig. 1B).

Th17 cells contribute to more severe periapical lesions

Since the migration of Th17 cells occurs mainly after 7 days of experimental periapical lesion (Fig. 1A), we choose experimental periods of 7, 14 and 21 days post-infection to analyze via micro-computed tomography, the bone volume, the width of periapical space and the length of the mesial root in WT and IL-17KO mice (Fig. 2A, 2B, 2C, 2D, 2E).

Analyzing the ratio of bone volume between control side and experimental side (pulp exposure and bacterial inoculation), it has been observed that WT mice showed larger bone loss in periapical area in mesial root than IL-17KO mice (Fig. 2A), mainly

on 21 days of experimental periapical lesion (p<0.05). Similar results were observed for distolingual and distobuccal roots, during the later periods of the progression of periapical lesions (Fig. 2B, C). Periapical space increased was observed in WT mice over time, but in the period of 14 days p.i. there was significant difference between groups (p<0.05) (Fig. 2D).

Hypothesizing the apical inflammatory process could be related with the root resorption, we measured the length of the mesial root. Both groups showed root resorption time-dependent, but no difference between them was observed (Fig. 2E).

Th17 cells increase the concentration of inflammatory cells in periapical lesions

In order to confirm the greater severity of periapical lesions in WT mice (Fig. 2A), we compared the composition of periapical space in control side and experimental side.

The periapical region was intact, and neither inflammation nor bone resorption could be observed in the controls groups in all experimental periods in both lineages (Fig. 3A, B). However, in the experimental groups, we observed the presence of inflammatory infiltrates and increased periapical space in all periods, but this increase was greater in the 14-day period (Fig. 3C, D, E, F).

Indeed, the inflammatory process was more exacerbated in mesial root of WT animals, since on days 7 and 14 we observed higher concentration of blood vessels, mononuclear inflammatory cells and neutrophils in such animals (Fig. 4C, D, E). IL-17KO mice presented higher concentrations of fibers and fibroblasts in these periods (Fig. 4A, B).

Similar results were observed for the distobuccal root, where animals IL-17KO showed lower concentrations of mononuclear inflammatory cells, neutrophils and osteoclasts compared to WT animals (Fig. 5D, E, F).

Furthermore, as in prior study (XIONG; WEI; PENG, 2009), after periapical lesion induction, neutrophils could be readily seen in the inflamed periapical regions on day 7 and subsequently increased on day 14, mainly in WT mice (Fig. 4E, 5E). In the distobuccal root, there was a significant difference in the number of neutrophils in each group compared with the other time group (Fig. 5E).

These results confirm μCT results, showing a less severe inflammatory process in the absence of IL-17, and consequently, less periapical bone resorption.

Th17 changes the gene expression profile in periapical lesions

Molecular analysis of IL17-KO mice lesions demonstrated decreased expression of proinflammatory cytokines and tissue destructive mediators (IL-1B, TNF, RANKL) and increased expression of wound healing/inactive lesions markers (CTLA4, CXCL11, ITGA4, SERP1) (Fig. 6A). However, it is important to highlight that in absence of IL-17, there was a significant increase of IFN levels, the prototypical Th1 cytokine, and probably the responsible for the progression of periapical lesion even in the absence of IL-17. Indeed, ELISA analysis confirmed data obtained via realtime PCR, suggesting that TNF and RANKL were downregulated in IL17-KO mice in parallel with the upregulation of IFN (Fig. 6B).

Discussion

Our first objective was to determine the kinetics of Th17 migration and its phenotype. Other studies has shown that IL-17 is expressed at high levels in the expansion stage of periapical lesions but not in the chronic stage, indicating that IL-17 may be responsible for active osteoclast induction and periapical bone destruction (WEI et al., 2013). It is interesting to note that the kinetics of Th17 migration do not show directly correlated with the phenotype of lesions. Whereas a significant infiltration of Th17 cells in early stages of development of the lesions shows temporally associated with the largest progression phase of lesions considered active phase (ARANHA et al., 2013; ARAUJO-PIRES et al., 2014a), their number remains relatively constant over time, even with the conversion of the phenotype of lesions for a pattern of relative stability, or significantly slower progression (Fig. 1A).

Analyzing the Th17 cells phenotype we observed coexpression of CCR6 and RANKL (Fig. 1B). In fact, a previous study shows Th17 expressing RANKL, which directly act on osteoclasts precursors to induce osteoclastogenesis (SATO et al., 2006).

Once observed the presence of Th17 in periapical lesions, we analyzed its role in this lesion progression through micro-computed tomography. This is a strong tool for evaluation of the periapical lesions measurement and has been implicated in mice experimental models (DE OLIVEIRA et al., 2015; OOKUBO et al., 2016; OSEKO et al., 2009).

We observed increased periapical space in WT mice as a result of increased bone resorption (Fig. 2D). Indeed, most studies show that IL-17, by stimulating the

production of IL-8, may play a role in exacerbating inflammation in periapical lesions (COLIC et al., 2007). IL-17 primarily acts on stromal endothelial and epithelial cells, as well as on a subset of monocytes, to induce the secretion of proinflammatory mediators. These include IL-8, CXC ligand 1, TNF-α, IL-1, IL-6, and GM-CSF (JOVANOVIC et al., 1998), which promote rapid neutrophil recruitment to the site of the infection. Moreover, IL-17 may induce osteoclastogenesis and bone resorption, supporting cells to induce RANKL (SATO et al., 2006), which is correlated with periapical lesion expansion. IL-17 induces RANKL in mesenchymal cells and promotes osteoclastogenesis in vitro (KOTAKE et al., 1999).

In addition, we observed, via μCT, the association of periapical lesions and root resorption in this experimental model (Fig. 2E). Similar results were observed in human periapical lesions, but a significant large size associated with root resorption was observed in cystic lesions compared with granulomatous lesions (OOKUBO et al., 2016).

Morphometric results confirmed the presence of more severe periapical lesion in WT mice. Indeed, in different rodent RA models, IL-17 was elevated in synovial fluid (KOTAKE et al., 1999; SATO et al., 2006; VAN BEZOOIJEN et al., 1999) and blocking of IL-17 reduced inflammation and bone damage, whereas IL-17 excess led to disease exacerbation (LUBBERTS et al., 2002). In human periapical lesions, higher IL-17 levels and greater numbers of neutrophils were observed in symptomatic lesions compared to asymptomatic lesions (COLIC et al., 2009b), as well as our results (Fig. 4E, 5E).

The functional role of IL-17 in periapical lesions has been assessed using IL-17KO mice (OSEKO et al., 2009), and showed significantly less bone loss in infected IL-17KO mice than infected WT littermates. However, in a previous study, the extent of bone loss in IL-17RAKO was significantly increased compared to WT controls (ALSHWAIMI et al., 2013). These basic findings observed in gene modified mouse models seem to be contradictory and are not always accordant with the clinical findings. One possible reason for this discordance is pathogen-dependent alteration of macrophage activation. Another possible reason is that IL-17F compensates for the role of IL-17A via IL-17RA in IL-17KO mice. IL-17F utilizes IL-17RA and has similar biological effect compared to IL-17A (IWAKURA et al., 2011).

In another study, IL-17KO mice were resistant to develop experimental periapical lesions (OSEKO et al., 2009) but, in our study, IL-17KO mice developed apical periodontitis, but less severe when compared to WT animals.

Finally, we analyzed the gene expression profile of periapical lesions in both groups. Lesions of IL17-KO mice showed a pattern of production/expression of mediators compatible with a lower severity (lower levels of proinflammatory cytokines and higher levels of anti-inflammatory cytokines when compared to WT mice), but increased levels of IFNg, a major Th1-type cytokine, was significant at both mRNA (RealTimePCR) as the protein level (ELISA) (Fig. 6A, B).

Indeed, IL-17 produced in periodontal lesions may be involved in Th1 modulation and enhance inflammatory reactions via gingival fibroblast-derived mediators in periodontal disease (XIONG; WEI; PENG, 2009). However it was reported also that IFN-g strongly inhibits T cell differentiation towards the Th17 cell lineage (DONG, CHEN, 2009).

Interferon gamma (IFN-g)[+] Th1 cells are strongly associated with enhanced alveolar bone loss during periodontal infections (BAKER et al., 1999) and RANKL is often highly co-expressed in Th1 cells (JOSIEN et al., 1999).

Analyzing how the exogenous addition of a particular Th subset cytokine modulates the production of other Th subsets' cytokines, it was observed that IFN-g inhibited the production of IL-17 by PL-MNC, whereas IL-17 augmented the production of IFN-g (COLIC et al., 2009b).

Indeed, the involvement of Th1 and Th17 subsets and its prototypic cytokines in inflammatory osteolysis have been demonstrated in a cause-and-effect manner in PD and arthritis (RA) models, which share several immunopathological phenotypical characteristics (GARLET, G.P. et al., 2010a).

In this context, complementing the experiments in this study, we will accomplish experiments in order to understand the possible crosstalk inhibition of Th1 and Th17 cells, and possible compensatory involvement of these cytokines in the absence of any of them.

Conclusion

Our results demonstrate that Th17 cells are related to periapical bone destruction and controlling IL-17 synthesis or function, blocking Th17, may be a therapeutic target to prevent periapical lesion expansion.

Acknowledgments

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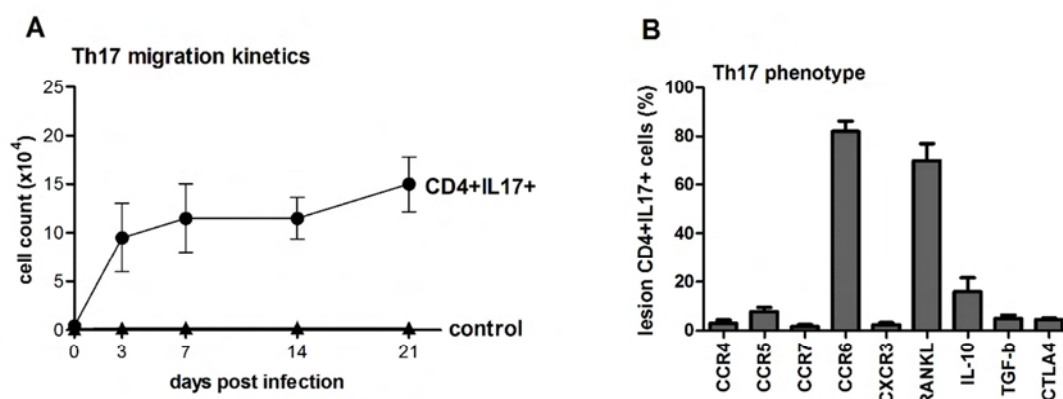
FIGURE LEGENDS

Figure 1. Th17 migration kinetics and its phenotype in experimental periapical lesions in mice. C57Bl/6 (WT) mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation). Samples from experimental and control sides were collected for histomorphometric and molecular analysis, and evaluated for: (A) Th17 (CD4+IL17+) cells count in periapical tissues analyzed by flow cytometry, at 0, 3, 7, 14 and 21 days post infection; depicted as the cell number $x10^4$; and (B) the phenotype of Th17 (CD4+IL17+) from periapical lesions, evaluated by flow cytometry and depicted as the number of positive cells for each marker.
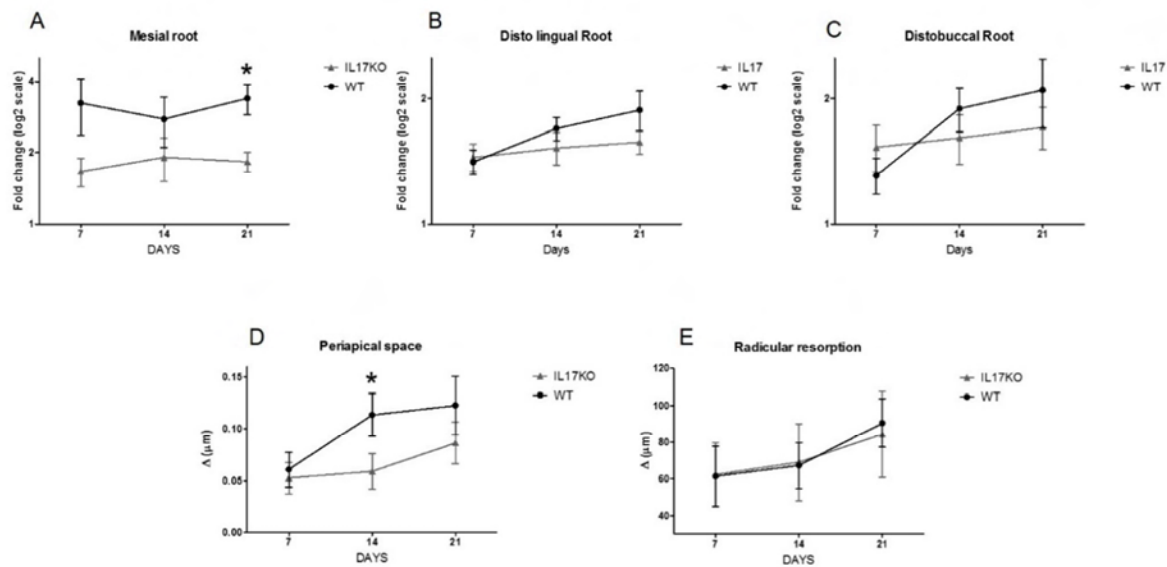
Figure 2. Th17 cells contribute to more severe periapical lesions. Micro-computed tomography (μCT) analysis of experimental periapical lesion in mice. Samples from 8-week-old male wild-type (WT) C57BL/6 and IL17-KO mice were scanned with the μCT System (Skyscan 1174; Skyscan, Kontich, Belgium): control (maxilla without pulp exposure) and at 7, 14 and 21 days post periapical lesion inducing protocol (pulp exposure and bacterial inoculation) to evaluate: (A) the ratio of bone volume between control side and experimental side in mesial root; (B) distolingual root, and (C) distobuccal root; (D) the width of periapical space; and (E) the radicular resorption of mesial root. Asterisks (*) represents statistically significant differences ($P < 0.05$; unpaired t test) between the indicated group/time point vs control group.
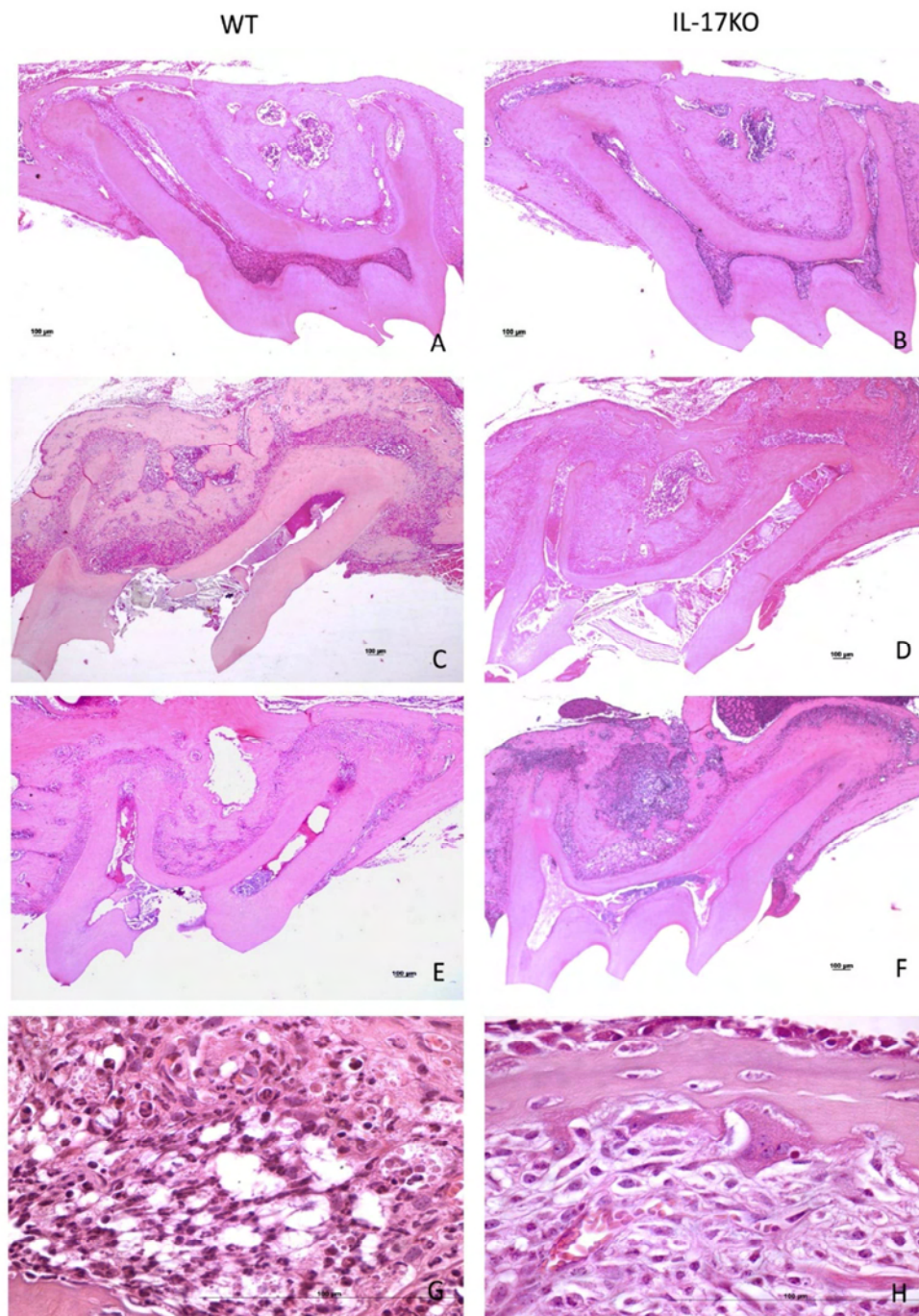
Figure 3. Th17 cells increase the concentration of inflammatory cells in periapical lesions. Histological aspects of the mesial and distobuccal root of first upper molar in (A, B) non-infected control and (C, D, E, F, G, H) infected mice. A periapical lesion was determined by Hematoxylin-Eosin stain on day 14 (C, D) and 21 (E, F) after infection. WT mice exhibited large periapical lesion with severe inflammatory cell infiltration and elevated osteoclasts compared to IL17-KO mice (G, H). HE staining, original magnification 5x and 100x. Bar = 100 μm.
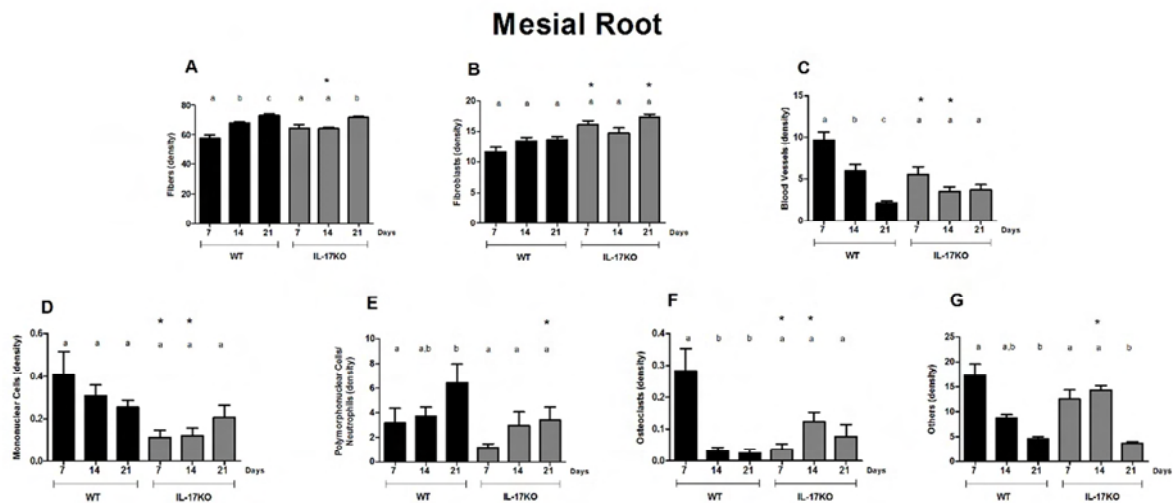
Figure 4. Histomorphometric analysis of apical periodontitis progression after pulp exposure and bacterial inoculation in mesial root. Results are presented as the means (±SEM) of density for each structure of the periapical space: (A) collagen fibers, (B) fibroblasts, (C) blood vessels, (D) mononuclear cells, (E) neutrophils, (F) osteoclasts and (G) other components (empty space left by the inflammatory exudate, intercellular liquid or bone matrix). Different letters indicate a statistically significant difference (p < 0.05) between the different time periods (p<0.05; one-way analysis of variance, Bonferroni post hoc test) and asterisks (*) represents statistically significant differences (P < 0.05; unpaired t test) between the indicated group/time point vs control group.
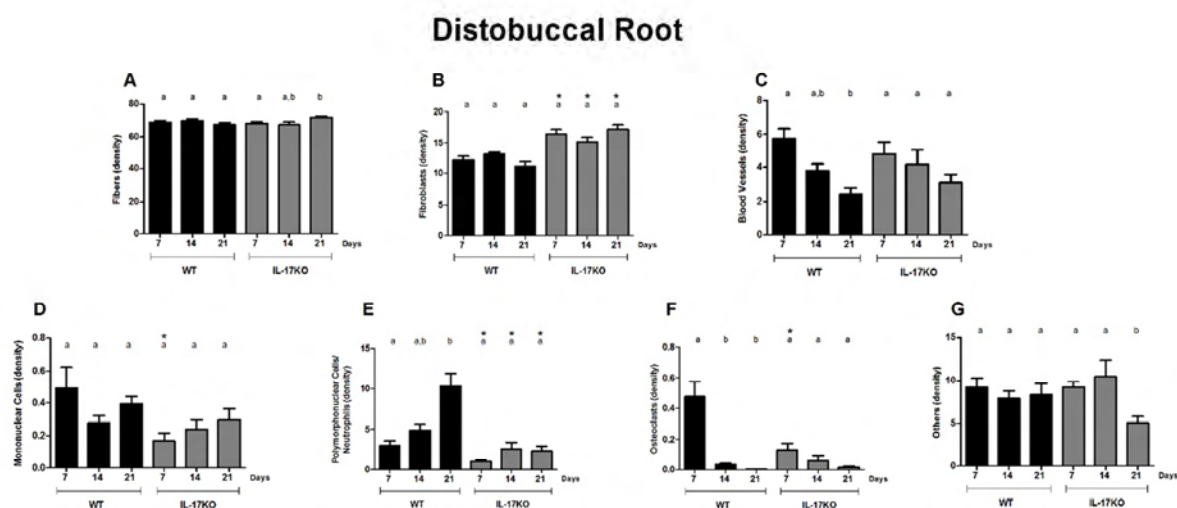
Figure 5. Histomorphometric analysis of apical periodontitis progression after pulp exposure and bacterial inoculation in distobuccal root. Results are presented as the means (±SEM) of density for each structure of the periapical space: (A) collagen fibers, (B) fibroblasts, (C) blood vessels, (D) mononuclear cells, (E) neutrophils, (F) osteoclasts and (G) other components (empty space left by the inflammatory exudate, intercellular liquid or bone matrix). Different letters indicate a statistically significant difference ($p < 0.05$) between the different time periods ($p<0.05$; one-way analysis of variance, Bonferroni post hoc test) and asterisks (*) represents statistically significant differences ($P < 0.05$; unpaired t test) between the indicated group/time point vs control group.
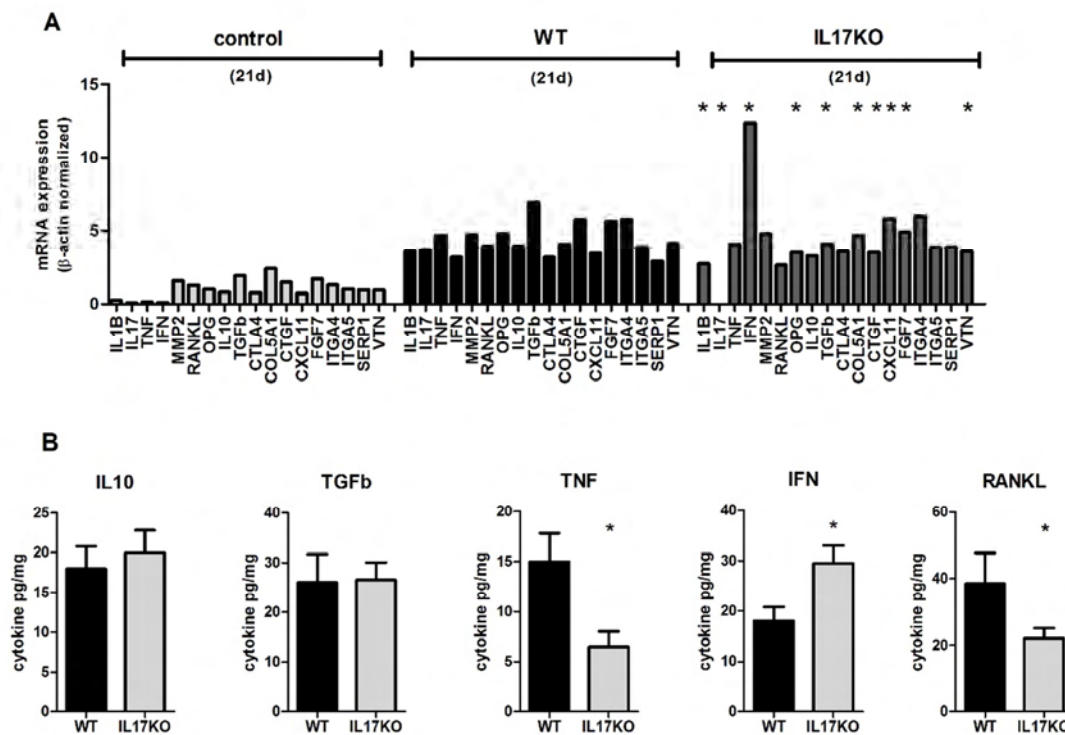
Figure 6. Th17 changes the gene expression profile in periapical lesions. C57Bl/6 (WT) and IL-17KO mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial inoculation). Samples from WT and IL-17KO groups were collected for molecular analysis and evaluated for: (A) expression of inflammatory/immunologic and wound healing markers at 21-day time point, measured quantitatively by real-time PCR array, presented as fold change relative to control group after normalization by constitutive genes (GAPDH, ACTB, Hprt1) expression levels; asterisks (*) represents statistically significant differences (P < 0.05; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs control group. (B) Cytokine levels in periapical lesions, measured by ELISA, presented as cytokine pg/mg of periapical tissue (P < 0.05; unpaired t test); asterisks (*) represents statistically significant differences (P < 0.05; one-way analysis of variance, Bonferroni post hoc test) between the indicated group/time point vs control group.

3 DISCUSSION

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Apical periodontitis is a consequence to endodontic infection and manifests itself as the host defense response to microbial challenge in root canal system. Periapical granulomas consist of a granulomatous tissue with inflammatory cells, fibroblasts, and a well-developed fibrous capsule. If the antigenic stimulus persists, the epithelial cell rests of Malassez can be stimulated by cytokines and growth factors to undergo division and proliferation, an event that may lead to the development of a radicular cyst (NAIR, 2004). A retrospective study in a large case series of radiolucent jaw lesions revealed that granulomas and radicular cists occur at similar rates totaled 73% of all lesions (KOIVISTO; BOWLES; ROHRER, 2012).

Periapical lesion outcome (cyst or granuloma) is determined by the balance between pro-and anti-inflammatory cytokines, which differentially modulates proteolytic activity, bone resorption and healing mechanisms (COLIC et al., 2009b; FRANCISCONI, C. F. et al., 2016; GRAVES; OATES; GARLET, 2011; MARTON; KISS, 2014). However, little is known about how the set of cytokines involved in the development and maintenance of chronic inflammatory processes is regulated and how the profile of these cytokines is correlated with the composition of the inflammatory infiltrate and clinical presentation of these lesions (COLIC et al., 2007).

Therefore, the aim of this study was to analyze, in a cause-and-effect manner, the role of regulatory T cells (Tregs), Th17 and their products in immunomodulation of experimental periapical lesions, since it was previously identified the presence of IL-17–positive cells in rat periapical lesions and the Foxp3 mRNA expression in mice lesions (ALSHWAIMI et al., 2009; XIONG; WEI; PENG, 2009). Besides that, we analyzed the impact of different therapies in the progression of experimental periapical lesions. For this aim, we used knockout mice as experimental model, being this model already established in the literature (ALSHWAIMI et al., 2013; ARAUJO-PIRES et al., 2015; FRANCISCONI, C. F. et al., 2016; OSEKO et al., 2009). We didn´t use medication after the periapical lesion induction, since these drugs may alter the periapical inflammatory process and thus masking our results. In this study,

as in previous studies, mice had no behavioral changes or weight loss after the periapical lesion induction without any treatment (GARLET, T.P. et al., 2010).

Th17 cells are a distinct CD4+ effector lineage and play important roles in host defense against a variety of pathogens as well as in the pathogenesis of several inflammatory conditions. While regulatory T cells have been shown to attenuate both Th1 and Th2 responses (ZHOU et al., 2008).

The balance between these two cell populations is essential for immune homeostasis and dysregulation of this balance has been implicated in a variety of inflammatory conditions including autoimmunity, allograft rejection, tumorigenesis, (CHADHA et al., 2011; DILLER et al., 2016; WANG et al., 2012) nasal polyposis and rheumatoid arthritis (DENG et al., 2010; SHEN et al., 2011).

Indeed, diminished Tregs counts and suppressor function often accompany Th17-mediated autoimmunity, propagating inflammation and tissue destruction. Th17/Tregs ratio are elevated in patients with active rheumatoid arthritis compared to healthy controls, highlighting a role for Th17/Tregs imbalance in autoimmune-related pathology (WANG et al., 2012).

Regulatory T cells have been shown to be important in controlling or preventing the development of autoimmunity and other deleterious inflammatory reactions. Really, evidences from human PD demonstrate that Tregs are present in periodontal tissues (CARDOSO et al., 2008; ERNST et al., 2007; NAKAJIMA et al., 2005; YANG et al., 2014), and they are associated with IL-10, TGF-b and CTLA-4 production and downregulation of proinflammatory and osteoclastogenic factors (GARLET, G.P. et al., 2010a).

As regards the periapical lesions, previous studies suggest that Tregs are associated with a possible reduction in the severity of human lesions (FUKADA et al., 2009; MARCAL et al., 2010), as well as in experimental lesions in mice (ALSHWAIMI et al., 2009). The presence of Tregs in these lesions has been associated with the attenuation of local host inflammatory immune responses (ARAUJO-PIRES et al., 2014b; MARCAL et al., 2010; PEIXOTO et al., 2012; YANG et al., 2014).

Indeed, our study showed that Tregs counts were negatively correlated with lesion evolution patterns. Furthermore, Tregs inhibition by anti-GITR treatment or CCR4 depletion promoted more severe periapical lesion, as seen in increased expression of proinflammatory cytokines and tissue destructive mediators and decreased expression of Tregs markers and wound healing markers (FRANCISCONI, C. F. et al., 2016).

Similar to previous results (ALSHWAIMI et al., 2009), flow cytometry demonstrated an increase in the number of FOXP3+ Tregs beginning between day 7 and day 14 after infection, and increased to day 21. These results demonstrate that Treg cells are induced to infiltrate into periapical lesions by pulpal infection, and suggest that they increase in a time-dependent manner.

Tregs seemed to be attracted to the periodontal environment by the chemokines CCL17 and CCL22, ligands of the chemokine receptor CCR4 characteristically expressed by Tregs (CARDOSO et al., 2008). CCR4 receptor is expressed at high levels in Tregs, and it is the primarily responsible for its selective migration to different sites (FAUSTINO et al., 2013; IELLEM et al., 2001).

So, the analysis of CCR4-KO mice showed Tregs migration significantly impaired, and increased severity of lesions. Molecular analysis demonstrated increased expression of proinflammatory cytokines and tissue destructive mediators and decreased expression of Treg-associated markers as well as markers of wound healing/inactive lesions, in the absence of CCR4 (FRANCISCONI, C. F. et al., 2016).

Other way to inhibit the Tregs functions is via anti-GITR antibodies (MARIANO et al., 2008; SHIMIZU et al., 2002). As it could be expected (GARLET, G.P. et al., 2010a), anti-GITR treatment results in a significant decrease in the levels of Tregs markers IL-10, CTLA-4 and TGF-b, besides increased bone resorption and proinflammatory cytokines levels.

Finally, IL-4/CCL22/CCR4 axis is involved in the migration of Tregs to osteolytic lesion sites, and attenuates development of lesions by inhibiting inflammatory migration and the production of proinflammatory and osteoclastogenic mediators (ARAUJO-PIRES et al., 2015). Indeed, the injection of CCL22-releasing particles in the root canal system showed to be effective in promoting Tregs

migration and reducing lesion progression in a CCR4-dependent manner (FRANCISCONI, C. F. et al., 2016).

Considering that the CCL22-mediated Tregs migration switches active periapical lesions into an inactivity phenotype, Tregs chemoattractant may be a promising strategy for the clinical management of periapical lesions.

In this context, adjunct therapies targeting host inflammatory and osteoclastogenic mediators have been proposed for the clinical management of osteolytic alterations (GRAVES; OATES; GARLET, 2011; MARTON; KISS, 2014). Some of antiresorptive drugs include bisphosphonates, raloxifene (a selective estrogen receptor modulator), denosumab (a humanized monoclonal antibody against receptor activator of NF-kB ligand [RANKL]) infliximab, which neutralizes TNF-a (PINKERTON; THOMAS; DALKIN, 2013; TYAGI et al., 2014) and OPG-fc (JIN et al., 2007).

Neutralizing RANKL action by denosumab has been approved by the FDA (Food and Drug Administration) for postmenopausal women (MILLER, 2011), since this therapy prevents fragility fractures in osteoporosis, skeletal complications of malignancy, and potentially bone erosions in RA (FERRARI-LACRAZ; FERRARI, 2011).

There is a report describing that a robust osteoclastogenic effect of soluble RANKL was suppressed by anti-RANKL in mice, suggesting that it has potent osteoclast specific action (TOMIMORI et al., 2009). However, our results showed that continuous anti-RANKL treatment presented a gene expression signature at 7d characterized by increased expression of proinflammatory and tissue destructive mediators (IL-1B, IL-17, TNF, MMP2, RANKL) and decreased Tregs (TGF-b, CTLA-4) and wound healing markers (OPG, COL5A1, CTGF, FGF7, ITGA4/5, SERP1) levels. These results are in agreement with recent studies linking RANKL-blockage to impaired Tregs function (LIN et al., 2016; MCCARTHY et al., 2015).

The effect of anti-RANKL on immune cells, such as Th17 and Tregs, has been little investigated. Our results, however, demonstrate that anti-RANKL therapy, even upon cessation, significantly decrease and delay Tregs migration into the periapex, leading to an increased T CD4 effector/suppressor ratio, which was found to be

positively correlated with lesion evolution in the present and in previous studies (ARAUJO-PIRES et al., 2014a; GRAVES; OATES; GARLET, 2011; YANG et al., 2014). Further, recent studies demonstrate that RANKL plays a role in the generation of FOXp3+ regulatory T cells, and its inhibition may increase effector T cell responses (DEMOULIN et al., 2015; LIN et al., 2016).

Another disadvantage of the anti-RANKL therapy is that RANKL blockage interferes with the natural immunoregulatory process, leading to an unremitting destructive host inflammatory response prompt to lesion relapse upon the discontinuation of anti-RANKL therapy. Therefore, despite the existence of RANKL inhibitors approved for clinical use, its potential impact in the Treg-mediated protective host response may limit its application as an adjunct therapy for the treatment of periapical lesions.

In this context, there are some reports showing that infliximab (anti-TNF therapy) suppresses Th17 cell differentiation and induces T regulatory cells (Tregs) in patients with uveitis (SUGITA et al., 2012), ulcerative colitis (DAHLEN et al., 2013) and RA (SHEN et al., 2010). Moreover, previous studies have demonstrated that TNF inhibition may increase Tregs suppressive function (BITON et al., 2011; TYAGI et al., 2014), and may interfere with the effector/suppressor ratio, such as in the Th17/Treg balance (TYAGI et al., 2014). Interestingly, the anti-TNF treatment resulted in T CD4 effectors/suppressors counts and ratio similar to those observed in response to CCL22 releasing particles, which was effective in attenuating periapical lesion progression via Tregs chemoattraction (FRANCISCONI, C. F. et al., 2016). So, treatments oriented on recruitment of endogenous Tregs can be potentially explored as a clinical strategy for management of periapical lesions.

Furthermore, the anti-TNF treatment resulted an overall decrease of proinflammatory and healing markers, similar to a previous study which demonstrated that infliximab had significant anti-inflammatory and bone-protective effects in Wistar rats challenged by periodontitis (GONCALVES et al., 2014). Indeed, TNF-blocking agents combine a strong anti-inflammatory potential leading to direct protection of bone and cartilage (SCHETT et al., 2011).

Finally, a previous study showed that blocking IL-17 prevented bone loss in estrogen deficiency-induced osteopenia and increased differentiation of Th17 cells. The anti-IL-17 treatment presented the best immunoprotective effects when compared to anti-RANKL and anti-TNF treated groups (TYAGI et al., 2012).

Thereby, we analyzed the role of Th17 in experimental periapical lesions development and its possible use as a therapeutic target to prevent this inflammatory bone resorption.

The presence of Th17 cells and significantly higher levels of IL-17 expression had been found in periodontal lesions, with especially high levels observed adjacent to the sites of bone destruction (OHYAMA et al., 2009). Really, IL-17 has a potential role in the etiopathogenesis of periodontal disease (TAKAHASHI et al., 2005). The amounts of cytokine IL- 17 in gingival crevicular fluid samples and in the culture supernatants of gingival cells are significantly increased in periodontal disease (VERNAL et al., 2005).

In addition, several reports demonstrate the destructive role of Th17 cells in inflammatory periapical bone resorption (ANDRADE et al., 2013; COLIC et al., 2009b; OSEKO et al., 2009; YANG et al., 2014) and a previous study defined the presence of IL-17-positive cells in rat periapical lesions (XIONG; WEI; PENG, 2009).

We analyzed, at first, the Th17 migrations kinetics after induction of experimental periapical lesion and we observed an increasing Th17 cells (CD4+IL17+) migration over time, with a higher concentration of Th17 cells for 7 days. Indeed, in a previous study (YANG et al., 2014) the number of IL-17–positive cells markedly increased from day 7 to day 35 in periapical lesions in rat. After that, we proved to be Th17 cells through the analysis of their phenotype. Our results were also in accordance with other report where mononuclear cells, isolated from periapical lesions, produce significant amounts of IL- 17 on additional stimulation in vitro (COLIC et al., 2007).

The possible destructive role of IL-17 is supported by recent in vivo findings that show that IL-17KO mice have significantly reduced infection stimulated periapical bone resorption compared with wild-type animals (OSEKO et al., 2009).

Indeed, our results demonstrated a larger bone loss in WT than in IL-17KO mice, mainly on 21 days of experimental periapical lesion.

Although IL-17 might be one of the responsible factors for stimulating the expression of inflammatory effectors, impacting the bone metabolism and promoting osteoclastogenesis in alveolar bone, IL-17 is important for neutrophil recruitment and activation. So, despite its apparent pathologic role in promoting inflammatory response, IL-17 might play a protective role by preventing against infection-stimulated alveolar bone loss in periapical disease (XIONG; WEI; PENG, 2009). Indeed, our results demonstrated a higher concentration of neutrophils in WT animals, mainly especially during the later periods of periapical lesion.

At least, we observed that Th17 changes the gene expression profile in this disease. Molecular analysis of IL17-KO mice lesions demonstrated decreased expression of proinflammatory cytokines and tissue destructive mediators and increased expression of wound healing/inactive lesions markers. In absence of IL-17, there was a significant increase of IFN levels, the prototypical Th1 cytokine, and probably the responsible for the progression of periapical lesion even in the absence of IL-17.

The relationship between the production of IFN-g and IL-17, has not been studied yet in periapical lesions, however the levels of IFN-g and IL-17 in cultures of symptomatic lesions, but not in other types of lesions, correlated positively (COLIC et al., 2009b). This suggests that both cytokines are important for the exacerbation of inflammation within periapical lesions. Further, the relationship between IFN-g and IL-17 at the level of individual cells was studied by analysing intracellular cytokine production. Unexpectedly, it was found that most Th17 cells co-expressed IFN-g (COLIC et al., 2009b), but such results are generally in agreement with data already published, that in humans, as many as half of all IL-17+ cells are also IFN-g+ (ACOSTA-RODRIGUEZ et al., 2007; GARLET, 2010). It is as yet unclear if these cells represent a stable phenotype or a transitional phase, undergoing a switch from Th17 to Th1 or vice versa. Although there are no data on the specific role of these double-positive cells, it can be hypothesized that both cytokines, as important mediators of inflammation, contribute to pathogenesis of periapical lesions.

In conclusion, it is worth mentioning that we must be very cautious in the interpretation of data from experimental models, which resemble a series of characteristics of human disease but also differ from them in a number of aspects. Therefore, further studies are required to confirm the exact role of Tregs and Th17 in the outcome of human periapical lesions and their possible use as therapeutic tools in inflammatory bone resorption. Another perspective that we have, in this sense is accomplish experiments in order to understand the possible crosstalk inhibition of Th1 and Th17 cells, and possible compensatory involvement of these cytokines in the absence of any of them.   Besides, we would bring our results to clinical practice through pre-clinical tests.

4 CONCLUSION

4 CONCLUSION

Analyzing the role of Tregs and Th17 in the development of periapical lesions and hence the possible therapies for modulating the periapical bone loss, we conclude:

- Treg chemoattraction to the periapical area by the CCL22/CCR4 axis could be potentially explored as a clinical strategy for management of periapical lesions.

- Anti-RANKL treatment efficiently limits the periapical bone loss, but interferes with the natural immunoregulatory process, impacting in the Treg-mediated protective host response, limiting its application as an adjunct therapy for the treatment of periapical lesions.

- Th17 is correlated with periapical lesion severity and its blockade may be a therapeutic tool to prevent periapical lesion expansion.

Therefore, we conclude that regulatory T cells are essential in the control of apical periodontitis, while Th17 cells accentuate the lesions severity. Compared with other clinical strategies, such as anti-RANKL therapy, which perpetuates the host inflammatory response prompting lesion relapse, chemoattraction of Treg as well as inhibition of Th17 may be promising strategies for the clinical management of periapical lesions.

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APPENDIXES

APÊNDICE A – Autorização da Editora para publicação de artigo aceito ou publicado na tese

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APÊNCIDE A - DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS
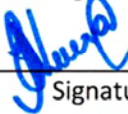
We hereby declare that we are aware of the article (Characterization of the Protective Role of Regulatory T Cells in Experimental Periapical Lesion Development and Their Chemoattraction Manipulation as a Therapeutic Tool) will be included in (The role of Th17 and regulatory T cells (TREGs) in immunomodulation of experimental periapical lesions) of the student (Carolina FávaroFrancisconi) was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 20 de Julho de 2016.

Carolina Favaro Francisconi
Author
Signature

Andreia Espindola Vieira
Author
Signature
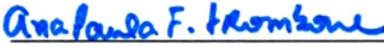
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Andrew J. Glowacki
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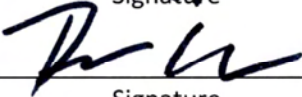
Ana Paula Favaro Trombone
Author
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Ariadne Letra
Author
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Renato Menezes Silva
Author
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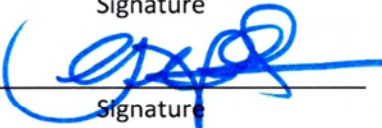
Charles S. Sfeir
Author
Signature

Steven R. Little
Author
Signature

Gustavo Pompermaier Garlet
Author
Signature

APÊNDICE B – Autorização da Editora para publicação de artigo aceito ou publicado na tese

APÊNCIDE B - DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS
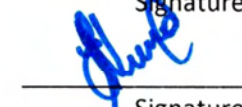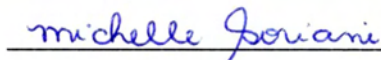
We hereby declare that we are aware of the article (Anti-RANKL therapy limits periapical lesions progression but is prone to relapse due impaired immunoregulation and unremitting host pro-inflammatory response) will be included in (The role of Th17 and regulatory T cells (TREGs) in immunomodulation of experimental periapical lesions) of the student (Carolina FávaroFrancisconi) was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 20 de Julho de 2016.

Carolina Favaro Francisconi
Author
_____
Signature

Andreia Espindola Vieira
Author
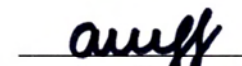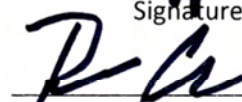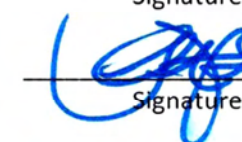_____
Signature

Angelica Cristina Fonseca
Author
_____
Signature

Michelle de Campos Soriani Azevedo
Author
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Signature

Ana Paula Favaro Trombone
Author
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Ariadne Letra
Author
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Signature

Renato Menezes Silva
Author
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Charles S. Sfeir
Author
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Steven R. Little
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Gustavo PompermaierGarlet
Author
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ANNEXES

ANEXO A - Ofício de aprovação do projeto de pesquisa pela Comissão de Ética no Ensino e Pesquisa em Animais da FOB/USP

Universidade de São Paulo
Faculdade de Odontologia de Bauru

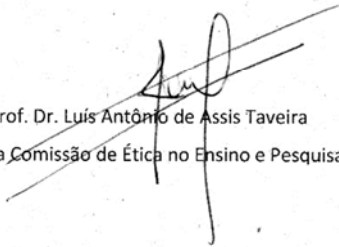Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 028/2012

Bauru, 19 de outubro de 2012.

Senhor Professor,

O projeto de pesquisa encaminhado a esta Comissão de Ética no Ensino e Pesquisa em Animais, denominado **Papel das células TH17 e T regulatórias (TREGs) na imunomodulação de lesões periapicais experimentais**, de sua autoria foi enviado ao relator para avaliação e considerado **APROVADO** *"ad referendum"* desta Comissão, nesta data.

Atenciosamente,

Prof. Dr. Luís Antônio de Assis Taveira

Vice-Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Prof. Dr. Gustavo Pompermaier Garlet

Docente do Departamento de Ciências Biológicas

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 028/2012

Bauru, 14 de fevereiro de 2014.

Senhor Professor,

Autorizamos a inclusão da doutoranda Carolina Fávaro Francisconi, no projeto de pesquisa **Papel das células TH17 e T regulatórias (TREGs) na imunomodulação de lesões periapicais experimentais**, desenvolvido sob sua orientação.

Lembramos que qualquer alteração na pesquisa seja comunicada a esta Comissão, e que, ao final seja enviado um Relatório com os resultados obtidos, para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Prof. Dr. Luís Antônio de Assis Taveira
Vice-Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Prof. Dr. Gustavo Pompermaier Garlet

Docente do Departamento de Ciências Biológicas

Anexo B - Autorização para inclusão do artigo "Characterization of the Protective Role of Regulatory T Cells in Experimental Periapical Lesion Development and Their Chemoattraction Manipulation as a Therapeutic Tool" em sua íntegra na presente Tese.

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ELSEVIER ORDER DETAILS

Jul 04, 2016

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Licensed Content Publication	Journal of Endodontics
Licensed Content Title	Characterization of the Protective Role of Regulatory T Cells in Experimental Periapical Lesion Development and Their Chemoattraction Manipulation as a Therapeutic Tool
Licensed Content Author	Carolina Favaro Francisconi,Andreia Espindola Vieira,Claudia Cristina Biguetti,Andrew J. Glowacki,Ana Paula Favaro Trombone,Ariadne Letra,Renato Menezes Silva,Charles S. Sfeir,Steven R. Little,Gustavo Pompermaier Garlet
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Title of your thesis/dissertation	The role of Th17 and regulatory T cells (Treg) in immunomodulation of experimental periapical lesions
Expected completion date	Oct 2016
Estimated size (number of pages)	75
Elsevier VAT number	GB 494 6272 12
Requestor Location	Carolina Francisconi Av. Alameda Octávio Pinheiro Brisola Bauru, 17012-901 Brazil Attn: Carolina Francisconi
Total	Not Available

ANEXO C- Carta de submissão do artigo "Anti-RANKL therapy limits progression of periapical lesions but is prone to relapse due to impaired immunoregulation and unremitting host proinflammatory response" ao periódico Journal of Endodontics.

Letter with Signatures

SÃO PAULO UNIVERSITY
SCHOOL OF DENTISTRY OF BAURU - **FOB/USP**

DEPARTMENT OF BIOLOGICAL SCIENCES

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru - SP – Brasil - CEP 17012-901
Phone +55 (14) 3235-8274 - Fax +55 (14) 3235-8274

Dear Dr. Kenneth Hargreaves,
Editor-in-Chief Journal of Endodontics

It is a pleasure to submit the files comprising the manuscript entitled **"Anti-RANKL therapy limits progression of periapical lesions but is prone to relapse due to impaired immunoregulation and unremitting host pro-inflammatory response"**, by Carolina F Franscisconi et al *(Garlet GP as the corresponding author, please access the full information and address at the end of this letter)* et al to be considered for publication in the **Journal of Endodontics**.

The authors warrant that the article is original, does not infringe upon any copyright or other proprietary right of any third party, is not under consideration by another journal, has not been previously published, and includes any product that may derive from the published journal, whether print or electronic media. In consideration of the editors of the Journal of Endodontics taking action in reviewing and editing this submission, the authors undersigned hereby transfer, assign or otherwise convey all copyright ownership to the AAE in the event that such work is published in that Journal. All the authors affirm that have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years. Any other potential conflict of interest is disclosed. Also, the authors affirm that the informed consent of all human subjects who participated in the experimental investigation reported or described in this manuscript was obtained after the nature of the procedure and possible discomforts and risks had been fully explained.

I hope the Editorial Board of **Journal of Endodontics** considers it to be suitable to publication.

Sincerely, on behalf of all the co-authors

Gustavo P. Garlet – *Corresponding author on behalf of all the co-authors*
Associated Professor – Dept of Biological Sciences
School of Dentistry of Bauru of São Paulo University – FOB/USP

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