RANKL Triggers Treg-Mediated Immunoregulation in Inflammatory Osteolysis

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Abstract

The chronic inflammatory immune response triggered by the infection of the tooth root canal system results in the local upregulation of RANKL, resulting in periapical bone loss. While RANKL has a well-characterized role in the control of bone homeostasis/pathology, it can play important roles in the regulation of the immune system, although its possible immunoregulatory role in infectious inflammatory osteolytic conditions remains largely unknown. Here, we used a mouse model of infectious inflammatory periapical lesions subjected to continuous or transitory anti-RANKL inhibition, followed by the analysis of lesion outcome and multiple host response parameters. Anti-RANKL administration resulted in arrest of bone loss but interfered in the natural immunoregulation of the lesions observed in the untreated group. RANKL inhibition resulted in an unremitting proinflammatory response, persistent high proinflammatory and effector CD4 response, decreased regulatory T-cell (Treg) migration, and lower levels of Treg-related cytokines IL-10 and TGF β . Anti-RANKL blockade impaired the immunoregulatory process only in early disease stages, while the late administration of anti-RANKL did not interfere with the established immunoregulation. The impaired immunoregulation due to RANKL inhibition is characterized by increased delayed-type hypersensitivity in vivo and T-cell proliferation in vitro to the infecting bacteria, which mimic the effects of Treg inhibition, reinforcing a possible influence of RANKL on Treg-mediated suppressive response. The adoptive transfer of CD4+FOXP3+ Tregs to mice receiving anti-RANKL therapy restored the immunoregulatory capacity, attenuating the inflammatory response in the lesions, reestablishing normal T-cell response in vivo and in vitro, and preventing lesion relapse upon anti-RANKL therapy cessation. Therefore, while RANKL inhibition efficiently limited the periapical bone loss, it promoted an unremitting host inflammatory response by interfering with Treg activity, suggesting that this classic osteoclastogenic mediator plays a role in immunoregulation.

Keywords: bone diseases, bone resorption, regulatory T-lymphocytes, t-lymphocytes, cytokines, mucosal immunity

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A supplemental appendix to this article is available online.

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Introduction

The balance between proinflammatory cytokines (which mediate increased proteolysis and bone resorption) and anti-inflammatory mediators (which counteract tissue degradation and stimulate healing) determines the outcome of infectious inflammatory osteolytic lesions (Graves et al. 2011; Francisconi et al. 2016). Indeed, leukocyte subsets such as Th2 and regulatory T cells (Tregs) mediate a natural immunoregulatory response that suppresses periapical and periodontal lesion development, which involves the downregulation of the primarily activated proinflammatory and osteoclastogenic pathways (Garlet et al. 2012; Araujo-Pires et al. 2014; Francisconi et al. 2016). In this context, inhibition of ultimate mediators of bone resorption, such as the master osteoclastogenic factor receptor activator of nuclear factor kappa-B ligand (RANKL), has been the basis of clinical treatment for conditions such as osteoporosis and arthritis (Lacey et al. 2012; Tyagi et al. 2014).

Nevertheless, the effects of RANKL inhibition in the treatment of infectious inflammatory osteolytic conditions remain elusive. A few studies demonstrated that RANKL inhibition by OPG or anti-RANKL therapies can limit experimental periodontal and periapical bone loss in rodents (Jin et al. 2007; Lin et al. 2011; Aghaloo et al. 2014). In contrast, it can result in jaw osteonecrosis due to yet unknown mechanisms (Aghaloo et al. 2014). Importantly, such studies focused exclusively on bone loss (Jin et al. 2007; Lin et al. 2011; Aghaloo et al. 2014) and consequently did not provide any information about the potential effects of RANKL inhibition in the host-mediated immune response. In addition to the well-characterized control of bone homeostasis/pathology, recent studies demonstrated that the RANK-RANKL-OPG system can play important roles in the regulation of the immune system. RANKL, originally cloned from T cells, modulates primary and secondary lymphoid organs microenvironments and influences the induction and regulation of immune responses (Akiyama et al. 2012; Guerrini et al. 2015; Maharjan et al. 2016), thereby presenting immunostimulatory or immunosuppressive roles. In the first setting, RANKL enabled increased dendritic cell survival, cytokine production, antigen presentation, and T-lymphocyte trafficking (Guerrini and Takayanagi 2014; Guerrini et al. 2015), resulting in an enhanced immune response. Conversely, RANKL exerted immunosuppressive effects inducing the generation of Tregs, a CD4+CD25+ T-cell subset with critical immunoregulatory properties (Arpaia et al. 2015; Vasanthakumar and Kallies 2015). Furthermore, RANKL blockade resulted in impaired Treg response, concomitant with a reduction in its suppressive function and ability to attenuate the host response (McCarthy et al. 2015; Lin et al. 2016). Of note, such RANKL immunoregulatory effects were primarily investigated in the thymic environment, and its role in the regulation of peripheral responses remains unaddressed.

Interestingly, while RANKL peaks in a stage characterized by an exacerbated immune response with experimental periapical lesion development, suggesting a potential immunostimulatory role, such a peak is followed by a Treg-mediated response that restrains inflammation and bone loss, which

could suggest an immunosuppressive role (Araujo-Pires et al. 2015; Francisconi et al. 2016). However, in view of the purely associative nature of such data, it is not possible to draw definitive conclusions regarding the immunoregulatory role of RANKL, which require cause-and-effect experiments to enable understanding of the biological mechanisms of periapical disease and provide insights into future therapeutic approaches. In this context, we employed in this study an experimental periapical lesion mouse model, subjected to continuous or transitory anti-RANKL therapy, to evaluate periapical lesion outcomes and multiple host response parameters. Host response outcomes were mechanistically investigated with a focus on the possible link between 1) RANKL and Treg generation and function and 2) their impact on lesion outcome.

Material and Methods

Experimental Periapical Lesions

Periapical lesion induction was performed as previously described (Fukada et al. 2008; Francisconi et al. 2016). Briefly, 8-wk-old male C57BL/6 mice were anesthetized with mandibular first molar dental pulp exposed (carbide bur, slow-speed handpiece) and inoculated with 1×10^6 (colony-forming units) of each endodontic pathogenic bacterial strain: *Porphyromonas gingivalis* (ATCC33277), *Prevotella nigrescens* (ATCC33563), *Actinomyces viscosus* (ATCC91014), and *Fusobacterium nucleatum* (ATCC10953; Fukada et al. 2008). The experimental protocol (028/2012) was approved by the Institutional Committee for Animal Care and Use following the principles of the *Guide for Care and Use of Laboratory Animals* and the ARRIVE guidelines. Animals were euthanized by cervical displacement and samples prepared for analysis. Histomorphometric analysis (per analysis, $n = 5$ samples/group per time point) was performed as previously described (Fukada et al. 2008; Francisconi et al. 2016). Briefly, hematoxylin and eosin-stained mesiodistally oriented 5- μ m-thick serial cuts were prepared, and 5 sections per lesion (where the whole root canal, including apical foramen, could be seen) were analyzed. Periapical lesion development was defined as the increase of the periapical space area, as measured with ImageJ software (version 1.45; National Institutes of Health).

RANKL Inhibition

RANKL was inhibited with purified monoclonal antibody (mAb) anti-RANKL (OYC Americas) as previously described (Tyagi et al. 2014). Anti-RANKL mAb (300 μ g/kg) was applied via intraperitoneal injections with 48-h intervals, from day 3 postinfection until the 28-d endpoint (continuous treatment) or from day 14 postinfection until the 28-d endpoint (transient treatment; per analysis, $n = 5$ samples/group per time point). Control groups received anti-TNF purified mAb (1.0 mg/kg, infliximab [Remicade]; Janssen Biotech) and control IgG (20 μ g/dose; R&D Systems), both applied via intraperitoneal injections with 48-h intervals.

Host Response Readouts

The impact of RANKL inhibition on the host inflammatory immune response was evaluated by multiple readouts, such as the quantification and phenotypic analysis of inflammatory cells from the periapical lesion, enzyme-linked immunosorbent assay (ELISA), and real-time polymerase chain reaction (PCR) array, and by the analysis of DTH (delayed type hypersensitivity), as well as in vitro by T-cell proliferation responses (per analysis, $n = 5$ samples/group per time point). In brief, for the isolation and characterization of leucocytes (Araujo-Pires et al. 2015), the periapical tissues surrounding the lesions were mechanically/enzymatically processed, followed by cell viability analysis via trypan blue and counting in a Neubauer chamber. For flow cytometry analysis, cells were stained with the optimal dilution of each antibody and analyzed by FACScan and CellQuest software (BD Biosciences). For the measurements of cytokines, periapical lesions were processed for protein extraction, followed by the analysis of IL-10, TGF- β , TNF, and RANKL concentrations via ELISA (R&D Systems; Garlet et al. 2012). For gene expression analysis, the extraction of total RNA from periapical tissues was performed with the RNeasy Plus Mini kit (Qiagen), followed by an RNA integrity check (Bioanalyzer; Agilent). Real-time PCR array was performed with mRNA (2 μ g) with a custom gene expression profiling panel in a Vii7 sequence detection instrument (Life Technologies) and analyzed with RT2 profiler software (SABiosciences). The patterns of immunologic hypo- and hyperresponsiveness to *Streptococcus mitis* (ATCC 6249) and *P. gingivalis* (ATCC 33277) were evaluated by the analysis of DTH and T-cell proliferation via standard protocols (Sosroseno and Herminajeng 2002; Williamson et al. 2002; Allen 2013). DTH was measured in vivo by left-hind footpad swelling after intradermal challenge (heat-killed *S. mitis*/*P. gingivalis* suspension in saline, 5 μ L [100 μ g/mL]). The Ag-specific proliferative capacity of the submandibular lymph node cells was examined by culturing T cells (2×10^5 cells per well) in the presence of heat-killed *S. mitis* or *P. gingivalis* (4×10^6 cells per well), followed by cell proliferation analysis via [3 H]thymidine incorporation. Standard protocols of tolerization to control antigen ovalbumin (OVA; Williamson et al. 2002), performed previously (OVA.OTpre) or with anti-RANKL treatment (OVA.OT), followed by the DTH response to OVA, were used as controls. Additional details are provided in the Appendix.

Treg Adoptive Transfer and Inhibition

Tregs were isolated from the peripheral blood mononuclear cells of mice submitted to periapical lesion induction at the 28-d time point, with a magnetic bead-based Treg Isolation Kit (Miltenyi Biotec) as previously described (Araujo-Pires et al. 2015). Purified single-cell suspensions (CD4+CD25+ >95%, 1×10^6 cells per mouse) were adoptively transferred to anti-RANKL-treated mice at 3- or 14-d time points via tail vein injection. Treg function was inhibited in vivo by treatment with anti-GITR (DTA-1, 500 μ g per mouse, 7-d time point; Araujo-Pires et al. 2015).

Statistical Analysis

Data are presented as means $\pm$ SD; the statistical significance between the groups was analyzed by 1-way analysis of variance, followed by the Bonferroni posttest; and the Mann-Whitney and Benjamini-Hochberg tests were used to analyze PCR array data (GraphPad Software, version 7). $P < 0.05$ was considered statistically significant.

Results

Effects of Anti-RANKL in Periapical Bone Loss and Host Response at Periapex

Periapical lesion development was characterized by bone loss around the periapical region of the treated tooth with inflammatory cell influx (Fig. 1). Continuous anti-RANKL treatment (3 to 28 d) significantly decreased the observed periapical bone loss in comparison to the untreated group (Fig. 1A) and resulted in sustained inflammation at 21 and 28 d (Fig. 1B). Real-time PCR array results demonstrated similar gene expression patterns in untreated and anti-RANKL-treated groups at 7 d (Fig. 1C), characterized by increased mRNA expression of proinflammatory and osteoclastogenic mediators (*Il1b*, *Il17*, *Tnf*, *Mmp2*, *Rankl*) and decreased Tregs (*Il10*, *Tgfb*, *Ctla4*) and wound-healing markers (*Opg*, *Col5a1*, *Ctgf*, *Fgf7*, *Itga4*, *Itga5*, *Serp1*). A similar gene expression profile was observed in the anti-RANKL group at 21 d, while untreated lesions presented with an opposing gene expression pattern at this time point (Fig. 1D). The results of the ELISA confirm the molecular data, suggesting a natural immunoregulation in the untreated lesions over time, while the anti-RANKL group presented high TNF and RANKL and low IL10 and TGF β levels at both time points (Fig. 1E).

The analysis of T CD4 subset influx at the periapex in the anti-RANKL group revealed the predominance of effector CD4+FOXP3 $^-$ cells, which coexpress to a large extent IFN γ , IL-17, and RANKL (Fig. 2A, C). Conversely, untreated lesions showed a decrease in the number of CD4+FOXP3 $^-$ effector cells with a concomitant increase in the suppressor CD4+FOXP3 $^+$, characterized by the coexpression of CCR4, IL-10, TGF- β , and CTLA-4 in the late stage and by minimal bone loss progression over time (Fig. 2A–D).

Effects of Anti-RANKL in T-cell Responses In Vivo and In Vitro

Untreated mice presented low in vivo DTH and in vitro T cell-proliferative responses to the commensal bacteria *S. mitis* at all time points, while DTH and proliferative responses to *P. gingivalis* were significantly increased at 14 d, followed by a significant decrease in both responses over time (Fig. 2E, F). For the anti-RANKL group, the hyporesponsiveness to *S. mitis* remained unaltered, but the high responsiveness to *P. gingivalis* remained high, resembling the effect of Treg inhibition by anti-GITR (Fig. 2E, F). Similar results were obtained in

Figure 1. Impact of anti-RANKL treatment on periapical bone loss, inflammation, and host response in experimental periapical lesions in mice. C57Bl/6 mice were submitted to experimental periapical lesion induction and treated with anti-RANKL or anti-TNF, from day 3 postinfection until the 28-d endpoint, and evaluated for **(A)** periapical lesion development, presented as periapical space area (mm²) increase; **(B)** inflammatory cell counts in periapical tissues; **(C, D)** the expression of bone, inflammatory/immunologic, and wound-healing marker mRNA (real-time polymerase chain reaction array); and **(E)** IL-10, TGF- β , TNF, and RANKL levels (ELISA) in periapical lesions. * $P < 0.05$ (indicated group vs. untreated group). # $P < 0.05$ (anti-RANKL vs. anti-TNF groups). x $P < 0.05$ (intragroup differences; 7 d vs. 21 d). Values are presented as mean $\pm$ SD.

Figure 2. Impact of anti-RANKL treatment on T CD4 migration and in vivo/in vitro responses. C57Bl/6 mice were submitted to experimental periapical lesion induction and treated with anti-RANKL or anti-TNF, from day 3 postinfection until the 28-d endpoint, and evaluated for (A) T CD4 effector cells (CD4+FOXP3-) and (B) T CD4 suppressor cells (Tregs, CD4+FOXP3+) in periapical tissues (flow cytometry); the phenotype of T CD4 (C) effector cells (CD4+FOXP3-) and (D) suppressor cells (Tregs, CD4+FOXP3+) from periapical lesions (flow cytometry); (E) the DTH response to *Streptococcus mitis* and *Porphyromonas gingivalis*, as measured by the footpad swelling after challenge; and (F) the proliferation of the submandibular lymph node CD4 T cells in response to *S. mitis* or *P. gingivalis* (thymidine incorporation). * $P < 0.05$ (indicated group vs. untreated group). # $P < 0.05$ (anti-RANKL vs. anti-TNF groups). (E, F) * $P < 0.05$ (differences vs. 0-h time point in each group). * $P < 0.05$ (intragroup differences; 14 d vs. 28 d). # $P < 0.05$ (indicated group vs. anti-RANKL group in each time point). Values are presented as mean $\pm$ SD.

response to *F. nucleatum* in vivo and in vitro (data not shown). The treatment with control unrelated antibody did not influence any of the parameters evaluated (data not shown).

Differential Outcomes of Early or Late Anti-RANKL Administration

The early anti-Rankl treatment (3 to 14 d) resembled the effects of continuous (3 to 28 d) inhibition with regard to inflammatory cell counts and CD4+FOXP3[−] and CD4+FOXP3⁺ cell influx (Fig. 3B–D). The results of the ELISA confirmed the observed impaired immunoregulation, characterized by sustained high TNF and RANKL levels and low IL10 and TGFb levels even after cessation of anti-RANKL therapy (Fig. 3E) and by sustained high DTH response to *P. gingivalis* (Fig. 3F). Additionally, cessation of anti-RANKL therapy resulted in a severe bone loss relapse (Fig. 3A). Conversely, late anti-RANKL administration (14 to 28 d) did not result in any alteration in lesion phenotype or host response readouts in comparison with the untreated group (Fig. 3A–F). To exclude for possible confounding effects of a differential *P. gingivalis* and *S. mitis* antigenicity in the results, the prototypic antigen OVA was tested via standard protocols of immunologic tolerization. The anti-RANKL treatment prevented the development of immunologic tolerance, as demonstrated by the high DTH response of the OVA.OT group, but it did not modify the DTH when the immunologic tolerance was previously induced (OVA.OTpre group; Fig. 3G) or the response to OVA immunization (data not shown). Treg inhibition increased the DTH response to OVA in all the groups (Fig. 3G). The analysis of T-cell–proliferative responses to OVA in vitro, performed with cells from these same groups, replicated the in vivo DTH data (data not shown).

Treg Adoptive Transfer Reverted the Anti-RANKL-Induced Impaired Immunoregulation

The adoptive transfer of Tregs at 3 d prevented bone loss relapse (Fig. 4A) and triggered the natural immunoregulatory response, characterized by a reduction in the number of inflammatory cells, increased IL10 and TGFb levels, decreased TNF and RANKL expression, and low DTH response to *P. gingivalis* (Fig. 4B, E, F). Importantly, despite a similar phenotype, the adoptive transfer of Tregs from control naïve/noninfected mice did not exert the immunoregulatory effects observed upon the transfer of Tregs from infected mice (Fig. 4A–F). The blockage of Treg function by anti-GITR reverted the immunoregulatory effect of Treg transfer (Fig. 4F). The analysis of T cell–proliferative responses to *S. mitis* and *P. gingivalis* in vitro, performed with cells from the mice receiving adoptive Treg transfer, replicated the in vivo DTH data (data not shown).

Discussion

The chronic inflammatory immune response triggered by root canal infection results in the local upregulation of RANKL,

which acts as the ultimate mediator of periapical bone loss. However, while RANKL has a well-characterized role in the control of bone homeostasis/pathology, it can play important roles in the regulation of the immune system, although its role in infectious inflammatory osteolytic conditions remains largely unknown. In the present study, we confirm previous findings and demonstrate that RANKL inhibition predictably arrested periapical bone loss, and we show for the first time that RANKL blockade altered the natural immunoregulatory process observed in untreated periapical lesions.

Periapical lesions present an initial active stage characterized by progressive bone loss and prominent expression of pro-inflammatory and pro-osteoclastogenic mediators, including RANKL (Araujo-Pires et al. 2014). Such initial host response naturally evolves over time to an inactive status, characterized by a decline in osteolytic activity, increased anti-inflammatory mediators, and wound-healing marker expression (Garlet et al. 2012). Importantly, such natural immunoregulation takes place without endodontic-like treatment of the root canal system, which is not possible due to the limited size of mouse molars. Despite the expected modulation of bone resorption, untreated and anti-RANKL-treated lesions presented similar immune-inflammatory features at early active stages (Garlet et al. 2012; Araujo-Pires et al. 2014). In addition, despite reducing bone destruction, RANKL inhibition did not modify the local inflammation microenvironment in experimental arthritis (Kong et al. 1999; Stolina et al. 2009; Ferrari-Lacraz and Ferrari 2011). However, our results demonstrate that RANKL inhibition was associated with persistent inflammation over time, with gene expression profiles compatible with an active lesion phenotype (Araujo-Pires et al. 2014; Francisconi et al. 2016). The cytokine profile in the anti-RANKL group (high proinflammatory and low anti-inflammatory) at early and late stages matched an active lesion expression profile (Araujo-Pires et al. 2014; Francisconi et al. 2016). RANKL inhibition was associated with sustained inflammation and delayed healing of palatal lesions in mice (Kuroshima et al. 2016). These findings suggest that anti-RANKL administration may contribute to lesion progression by limiting the tissue repair/regeneration attempts, as demonstrated by the decreased expression of healing markers in this group. Conversely, anti-TNF control treatment resulted in a rapid attenuation of the host inflammatory immune reaction and bone loss, as expected in the view of the central role of TNF in the control of cell migration and osteoclastogenesis (Garlet et al. 2007; Goncalves et al. 2014).

The sustained and persistent host response due to RANKL inhibition is also accompanied by an alteration in effector/suppressor T-cell dynamics. Our results revealed that effector (CD4+FOXP3[−]) T cells with a proinflammatory and pro-osteoclastogenic phenotype (i.e., CD4+RANKL⁺/CD4+IFN- γ +/CD4+IL-17+), previously correlated with lesion evolution (Graves et al. 2011; Yang et al. 2014), remained prominent in the lesions of the anti-RANKL group while decreased in untreated mice. Additionally, despite presenting an usual phenotype, the influx of Tregs (CD4+FOXP3⁺) into the periapex over time was reduced in the anti-RANKL group, which is accordance with the lower levels of IL-10, TGF- β , and

Figure 3. Impact of early or late anti-RANKL treatment on periapical bone loss, inflammation, and host response in experimental periapical lesions in mice. C57Bl/6 mice were submitted to experimental periapical lesion induction and treated with anti-RANKL from day 3 post infection until 14 d (early treatment) or from 14 d until the 28-d endpoint (late treatment), and evaluated for (A) periapical lesion development, presented as periapical space area (mm²) increase; (B) inflammatory cell counts in periapical tissues; (C) T CD4 effector cells (CD4+FOXP3[−]) and (D) T CD4 suppressor cells (Tregs, CD4+FOXP3⁺) in periapical tissues (flow cytometry); (E) the DTH response to *Streptococcus mitis* and *Porphyromonas gingivalis*, measured by the footpad swelling after challenge; (F) the proliferation of the submandibular lymph node CD4 T cells in response to *S. mitis* or *P. gingivalis* (thymidine incorporation). **P* < 0.05 (indicated group vs. untreated group). #*P* < 0.05 (early vs. late anti-RANKL therapy groups in each time point). (E, F) **P* < 0.05 (differences vs. 0-h time point in each group). **P* < 0.05 (intragroup differences; 14 d vs. 28 d). Values are presented as mean ± SD.

Figure 4. Treg adoptive transfer restores the immunoregulatory activity impaired by anti-RANKL and prevents inflammatory osteolytic lesion relapse. C57Bl/6 mice were submitted to experimental periapical lesion induction, treated with anti-RANKL from day 3 postinfection until 14 d, received the adoptive transfer of Tregs from untreated infected or control mice at the 3-d time point, and evaluated for **(A)** periapical lesion development, presented as periapical space area (mm²) increase; **(B)** inflammatory cell counts in periapical tissues; **(C)** phenotype and **(D)** frequency of T CD4 suppressor cells (Tregs, CD4+FOXP3+); **(E)** IL-10, TGF-β, TNF, and RANKL levels (ELISA) in periapical lesions; and **(F)** the DTH response to *Streptococcus mitis* and *Porphyromonas gingivalis*, measured by the footpad swelling after challenge. **P* < 0.05 (indicated group vs. untreated group). #*P* < 0.05 (early vs. late anti-RANKL therapy groups in each time point). (E) #*P* < 0.05 (differences vs. anti-RANKL). (F) **P* < 0.05 (differences vs. 0-h time point in each group). **P* < 0.05 (intragroup differences, 14 d vs. 28 d). Values are presented as mean ± SD.

CTLA-4, molecules known to mediate Treg immunoregulatory properties (Burr et al. 2013; Araujo-Pires et al. 2014). Accordingly, recent studies demonstrated that RANKL contributed to the generation of FOXP3⁺ Tregs in thymic and neoplastic microenvironments and that its inhibition consequently increased effector T-cell responses (Demoulin et al. 2015; Lin et al. 2016).

Looking for mechanistic insights regarding the impaired immunoregulation due to RANKL inhibition, we evaluated DTH and T-cell proliferation *in vivo* and *in vitro* surrogates for CD4 hyper- and hyporesponsiveness. The results demonstrate a strong response to *P. gingivalis* after experimental infection, which was attenuated over time in a Treg-dependent manner, since Treg inhibition with anti-GITR results in sustained host response over time. Although the mechanisms of Treg generation after periapical infection remains unclear, induced Tregs (iTregs) are usually generated in response to infectious agents and subsequently determine immunologic hyporesponsiveness in an antigen-specific manner (Shevach and Thornton 2014). Interestingly, the T CD4 hyporesponsiveness developed over time may suggest the development of immunologic peripheral tolerance, a complex hyporesponsiveness state where iTregs exert a key role (Weiner et al. 2011). Importantly, RANKL contributes to immunologic tolerance induction, which in turn limits DTH and T-cell proliferation in an antigen-specific manner (Williamson et al. 2002; Izawa et al. 2007).

Given these observations, we hypothesize that RANKL triggers immunoregulatory feedback via Treg induction, which in turn acts as a suppressive element. Our data support this notion based on the observed RANKL inhibition at only the early disease stages, when Tregs are theoretically in development (Araujo-Pires et al. 2014). Indeed, early RANKL inhibition recapitulated the features of continuous anti-RANKL administration, characterized by the sustained proinflammatory response, impaired immunoregulation, and bone loss relapse after withdrawal of anti-RANKL administration, which progressed at a similar rate as seen in active lesions. Accordingly, previous studies demonstrated that anti-RANKL therapy discontinuation resulted in a quick reestablishment of bone resorptive function (Aghaloo et al. 2014). Despite the theoretically inactive state of RANKL due to anti-RANKL mAb binding, RANKL levels remained high throughout the duration of anti-RANKL therapy, suggesting that the biologically active RANKL may be rapidly available right after discontinuation of the anti-RANKL administration, which could support the prompt relapse of the lesions with the potential preosteoclasts in the persistent inflammatory infiltrate. Importantly, while the lesion relapse may be considered predictable due to the absence of treatment of the root canal system, the natural course of the untreated lesions leads to a nonprogressive status mediated by immunoregulatory mechanisms (Graves et al. 2011; Lacey et al. 2012). Indeed, the intent of the RANKL blockade is to unravel the possible regulatory effect of this cytokine and not to mimic a possible clinical scenario where anti-RANKL administration would take place in parallel with the usual endodontic treatment.

Interestingly, RANKL inhibition did not modify the previously established and Treg-dependent hyporesponsiveness to the commensal bacteria *S. mitis*. These results support the antigen specificity of an iTreg response (Shevach and Thornton 2014) and suggest that previously existing Tregs are not affected by RANKL inhibition. Furthermore, RANKL inhibition at later disease stages (14 to 18 d), when Tregs are already developed (Araujo-Pires et al. 2014; Garlet et al. 2014), did not modify the host response profile, reinforcing that RANKL inhibition did not interfere with the role of preexisting Tregs. Importantly, while no previous studies evaluated the effects of RANKL inhibition on preexisting Tregs, clinical trials describe an anti-RANKL therapy safety profile as excellent, without evidences of major alterations in the overall immune response (e.g., autoimmune reactions or changes in infection susceptibility), thereby indirectly supporting the concept that preexisting Tregs are not affected by RANKL inhibition (Tarantino et al. 2013; Costa and Bilezikian 2015). Our additional experiments with standard immunization protocols with OVA confirmed those findings and strengthen the notion that RANKL inhibition interferes with new Treg generation and in the subsequent Treg-related immunologic outcomes, such as DTH response (Atkinson et al. 2016).

Therefore, to demonstrate a cause-and-effect relationship that RANKL immunoregulatory effects are in fact mediated by Tregs, we adoptively transferred Tregs to hosts/animals undergoing anti-RANKL therapy. Treg transfer proficiently prevented lesion relapse, promoting the local immunoregulation and restraining inflammation and bone loss, further supporting the role of Tregs as mediators of RANKL-induced immunoregulation and emphasizing that RANKL inhibition did not impair the preexisting Treg function (Demoulin et al. 2015; Lin et al. 2016). Notably, despite presentation of a similar phenotype, the adoptive transfer of Tregs from control (naïve/noninfected) mice did not revert the hyperresponsiveness state, highlighting the antigen specificity of iTregs developed after infection (Izawa et al. 2007; Shevach and Thornton 2014). Notwithstanding, a weak immunoregulatory activity was observed upon the transfer of control/naïve Tregs, probably due to a bystander tolerance effect (Paiatto et al. 2017).

Importantly, after cessation of anti-RANKL administration, a small increase in the number of Tregs within the lesions was observed, suggesting that the alterations in immunoregulation are potentially reversible but may last longer than the quickly reversible effects observed on bone (Guerrini and Takayanagi 2014). The long-lasting effect over the immune system may be due to the complexity of the immunologic tolerance system, in which RANKL initially determines a tolerogenic profile on dendritic cells, which subsequently participate of Treg induction. Noteworthy, this RANKL–dendritic cell tolerance was recently described in thymic and neoplastic environments (Demoulin et al. 2015; Atkinson et al. 2016; Lin et al. 2016; Ahern et al. 2017), and our study represents the first demonstration in infectious inflammatory conditions in mucosal tissues. Furthermore, this study is the first to identify Treg induction by RANKL as an immunoregulatory feedback mechanism.

In conclusion, our results suggest an immunoregulatory role for RANKL, mediated by the induction of Treg development and resulting in an antigen-specific immunologic hyporesponsiveness and the arrest of periapical lesion progression.

Author Contributions

C.F. Francisconi, A.E. Vieira, contributed to data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; M.C.S. Azevedo, A.P. Tabanez, A.C. Fonseca, contributed to data acquisition and analysis, critically revised the manuscript; A.P.F. Trombone, S.R. Little, contributed to conception, design, data analysis, and interpretation, critically revised the manuscript; A. Letra, contributed to conception, data analysis, and interpretation, critically revised the manuscript; R.M. Silva, contributed to conception, data acquisition, and interpretation, critically revised the manuscript; C.S. Sfeir, contributed to conception, design, data acquisition, and interpretation, critically revised the manuscript; G.P. Garlet, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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