# 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)-$\beta$, 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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0099-2399/$ - see front matter



http://dx.doi.org/10.1016/j.joen.2015.09.022

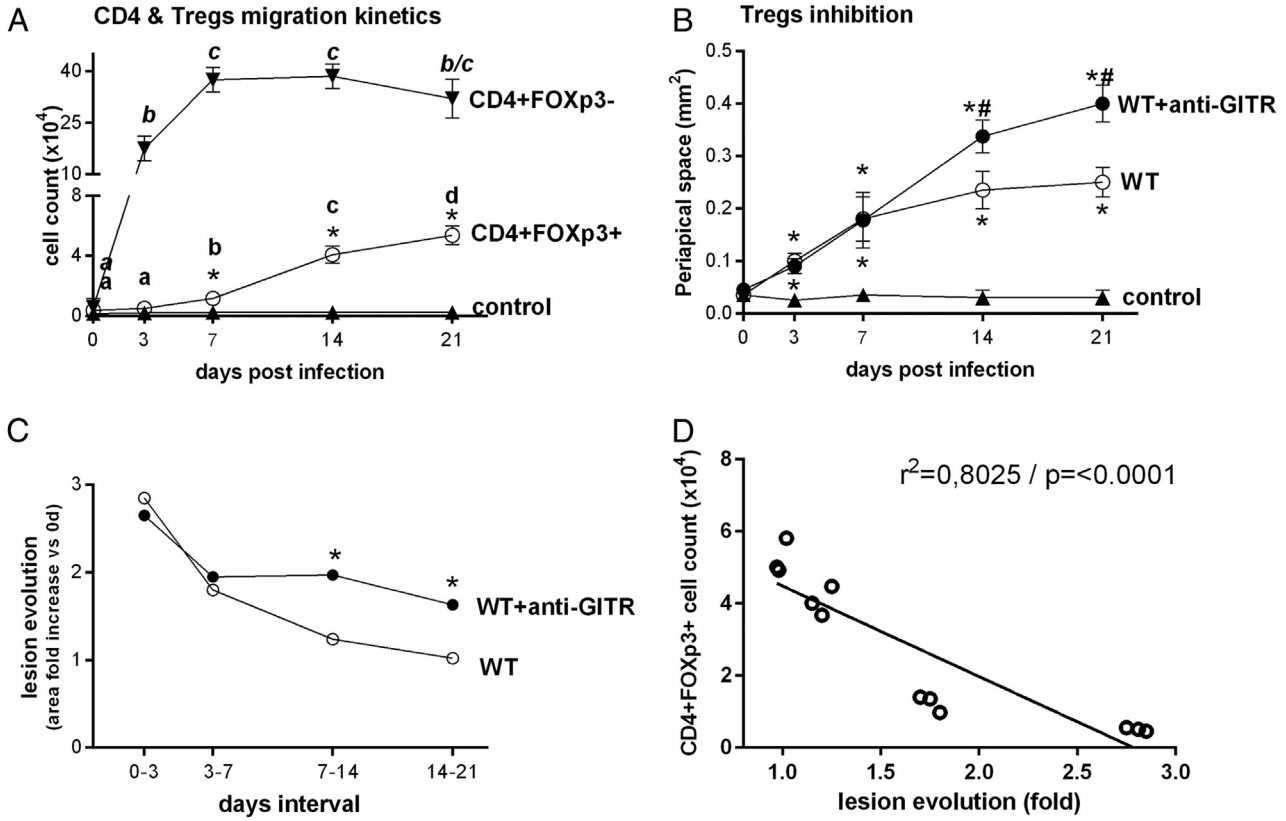


**Figure 1.** Treg migration kinetics and its impact on periapical bone loss and in the expression of inflammatory/immunologic and healing markers in experimental periapical lesions in mice. C57Bl/6 (WT) mice were submitted to an experimental periapical lesion inducing protocol (pulp exposure and bacterial 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 (CCL22p) (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 previously 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 $\mu$g/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 $\mu$L 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