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Regulatory T cells attenuate experimental periodontitis progression in mice

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Abstract

Aims: The aim of this study was to identify the presence and characterize the function of regulatory T cells (Tregs) in experimental periodontitis in mice.

Material and Methods: C57Bl/6 mice infected with *Actinobacillus actinomycetemcomitans*, treated or not with anti-glucocorticoid-inducible tumour necrosis factor receptor (anti-GITR) to inhibit Tregs function, were analysed regarding inflammatory cell and Tregs influx, alveolar bone loss and cytokine expression/production (analysed by real-time polymerase chain reaction and ELISA) throughout experimental periodontitis.

Results: *A. actinomycetemcomitans* inoculation in mice resulted in periodontal disease characterized by marked alveolar bone loss and an influx of inflammatory cells. Flow cytometry evaluation of inflammatory cells demonstrated an increased number of $CD4^+CD25^+$ and $CD4^+FOXp3^+$ cells, characterizing the presence of Tregs in the periodontal environment in a late stage after infection. Tregs-associated cytokines interleukin-10 (IL-10), cytotoxic T lymphocyte-associated molecule 4 (CTLA-4) and transforming growth factor-β (TGF-β) were found to be expressed/produced in a kinetics that resembles Tregs migration. Treatment with anti-GITR, which inhibits Tregs function, showed increased alveolar bone loss and inflammatory cell migration. A reduction in IL-10, CTLA-4 and TGF-β levels was also observed, while interferon-γ, tumour necrosis factor-α and receptor activator for nuclear factor κB ligand levels were increased. However, bacterial load and C-reactive protein serum did not show any differences.

Conclusion: Taken together, our results showed that the presence of Treg cells attenuates the severity of experimental periodontitis without impairment in the control of infection.

Key words: cytokine; FOXp3; periodontal disease; RANKL; regulatory T cells

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Periodontal diseases (PDs) are chronic inflammatory diseases characterized by the inflammatory bone resorption of the teeth-supporting structures, being the most prevalent form of bone pathology in humans and a modifying factor of the systemic health of patients (Tonetti & Claffey 2005, Albiger et al. 2007). Inflammatory immune reactions in response to periodontopathogens are thought to protect the host against the infectious agents, but the persistent release of inflammatory mediators results in the destruction of soft and mineralized periodontal tissues (Ebersole & Taubman 1994, Gemmell et al. 2001). Inflammatory mediators, such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), initiate tissue destruction through the generation of proteases that degrade the extracellular matrix, mainly matrix metalloproteinases (MMPs – a family of zinc- and calcium-dependent proteases), and the activation of mechanisms for bone resorption driven by receptor activator for nuclear factor κB ligand (RANKL), which leads to the differentiation and activation of osteoclasts through binding with receptor activator for nuclear factor κB (RANK) (Taubman et al. 2005, Garlet et al. 2006b). Recently, Th1- and Th17-type cytokines have also been described as

Conflict of interest and source of funding statement

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potentially destructive cytokines in view of its ability to upregulate pro-inflammatory cytokines and RANKL expression (Kotake et al. 1999, Teng et al. 2005, Sato et al. 2006, Beklen et al. 2007, Gao et al. 2007, Cardoso 2009 Garlet et al. 2008, Yago et al. 2009).

In contrast to the destructive pathway, the regulatory mechanisms involved in the control of PD severity remain relatively unknown. Anti-inflammatory cytokines and immunoregulatory factors, such as IL-10, transforming growth factor-β (TGF-β) and cytotoxic T lymphocyte-associated molecule 4 (CTLA-4), are expressed in diseased periodontal tissues and are thought to be associated with lower disease severity (Lappin et al. 2001, Garlet et al. 2004, Cardoso et al. 2008). In this context, it is reasonable to suppose that a T-cell subset with regulatory properties called regulatory T cells (Tregs) is potentially involved in PD pathogenesis (Nakajima et al. 2005, Ernst et al. 2007, Cardoso et al. 2008, Dutzan et al. 2009, Garlet et al. 2010). Tregs are $CD4^+CD25^+$ T cells that specifically regulate the activation, proliferation and effector function of activated conventional T cells determining the outcome of several immunological settings, ranging from infectious diseases to immunopathology and autoimmunity (Sakaguchi et al. 2001, Belkaid et al. 2002, Shevach 2002, Fehervari & Sakaguchi 2004, Campanelli et al. 2006, Cavassani et al. 2006). Tregs characteristically express as phenotypic markers the transcription factor forkhead box P3 (FOXp3), CD103, the glucocorticoid-inducible TNF receptor (GITR), the inhibitory molecule CTLA-4 and cell surface TGF-β (Dieckmann et al. 2001, Shevach 2002, Fontenot et al. 2003, Fehervari & Sakaguchi 2004, Miyara et al. 2009). In spite of some controversies, there is accumulating evidence indicating that Tregs' suppressive function can be mediated by CTLA-4, TGF-β and IL-10 (Dieckmann et al. 2001, Shevach 2002, Fontenot et al. 2003, Fehervari & Sakaguchi 2004, von Boehmer 2005, Miyara et al. 2009). Tregs have been identified in human periodontal lesions by the expression of the phenotypic markers FOXp3, CTLA-4, IL-10, TGF-β, GITR, CD103 and CD45RO (Nakajima et al. 2005, Ernst et al. 2007, Cardoso et al. 2008, Dutzan et al. 2009). A previous study demonstrated 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 have demonstrated an increased frequency of this T-cell subset in disease tissues, suggesting that Tregs infiltration could reflect an attempt to control tissue destruction, but could also be indicative of a destructive role for Tregs in periodontitis (Nakajima et al. 2005, Cardoso et al. 2008 Dutzan et al. 2009). Interestingly, Tregs can indeed play a harmful role because this T-cell subset can inconveniently impair the immune response against infectious agents (Belkaid et al. 2002, Joosten & Ottenhoff 2008), which would be potentially deleterious in a periodontal environment. However, the theoretical role played by Tregs in the control of periodontal infection and in the determination of disease outcome remains to be determined.

To clarify these questions, we investigated the kinetics of Tregs migration and the expression of phenotypic markers during the course of *Actinobacillus actinomycetemcomitans*-induced PD in mice. In addition, we inhibited Tregs action by means of anti-GITR treatment (whose signalling pathway inhibits Tregs function both in vitro and in vivo) (Kohm et al. 2004, Scumpia et al. 2007, Mariano et al. 2008) in order to evaluate its role and the possible mechanisms involved in the immunoregulation of experimental PD by Tregs.

Material and Methods

Periodontal infection

Experimental groups comprised 8-week-old male wild-type C57BL/6 mice bred and maintained in the animal facilities of the Department of Biochemistry and Immunology – FMRP/USP. Throughout the period of the study, the mice were fed with sterile standard solid mice chow (Nuvital, Curitiba, PR, Brazil) and sterile water. Bacterial culture and periodontal infection were performed as described previously (Garlet et al. 2005). In brief, the animals received an oral delivery of 1×10^9 CFU of a diluted culture of *A. actinomycetemcomitans* JP2 (grown anaerobically in a supplemented agar medium, TSBV), in 100 μl of phosphate-buffered saline (PBS) with 2% of carboxymethylcellulose, placed in the oral cavity of mice with a micropipette. After 48 and 96 h, this procedure was repeated. Negative controls included sham-infected mice, which received PBS with carboxymethylcellulose in solution without *A. actinomycetemcomitans*, and non-infected animals. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use.

Quantification of alveolar bone loss

Evaluation of the extent of alveolar bone loss was performed as described previously (Garlet et al. 2007). The maxillae were hemisected, exposed overnight in 3% hydrogen peroxide and defleshed mechanically. The palatal faces of the molars were photographed at $\times 20$ magnification using a dissecting microscope (Leica, Wetzlar, Germany), with the occlusal face of the molars positioned perpendicular to the base. The images were digitized and analysed using ImageTool 2.0 software (University of Texas Health Science Center, San Antonio, TX, USA). Quantitative analysis was used for the measurement of the area between the cement–enamel junction (CEJ) and the alveolar bone crest (ABC) in the three posterior teeth, in arbitrary units of area. At each time point, five animals were analysed, and for each animal, the alveolar bone loss was defined as the average of CEJ–ABC between the right and the left arch.

Isolation of inflammatory cells from periodontal tissues and flow cytometric analysis

The isolation and characterization of leucocytes present in the lesion site were performed as described previously (Garlet et al. 2007). The whole buccal and palatal periodontal tissues of the upper molars were collected, weighed and incubated for 1 h at 37°C, dermal side down, in RPMI-1640, supplemented with $NaHCO_3$, penicillin/streptomycin/gentamycin and 0.28 Wunsch units/ml of liberase blendzyme CI (F. Hoffmann-La Roche Ltd., Basel, Switzerland). The tissues of five mice, at each time point per group, were processed in the presence of 0.05% DNase (Sigma-Aldrich, Steinhein, Germany) using Medimachine (BD Biosciences PharMingen, San Diego, CA, USA), according to the manufacturer's instructions. After processing, cell viability was assessed by trypan blue exclusion, and the cell count was performed in a Neubauer chamber. For immunofluorescence staining, after cell counting, the cells were stained for 20 min. at 4°C

with the optimal dilution of each antibody, phycoerythrin- and fluorescein isothiocyanate-conjugated antibodies against CD4, CD25 and FOXp3 antibodies (analysed as CD4/CD25 and CD4/FOXp3 pairs), as well with respective isotype controls (BD Biosciences PharMingen). Cells were washed again and analysed by flow cytometry [fluorescence-activated cell sorter (FACScan)] and CellQuest software (BD Biosciences PharMingen). Results represent the number of cells ± SD in the periodontal tissues of each mouse, normalized by the tissue weight, for two independent experiments.

Table 1. Primer sequences and reaction properties

Target	Sense and anti-sense sequences	t_A (°C)	t_M (°C)	Bp
TNF-α	TGTGCTCAGAGCTTTCAACAA CTTGATGGTGGTGCATGAGA	58	80	124
IFN-γ	GCA TCT TGG CTT TGC AGC T CCTTTTTCGCCTTGCTGTTG	28	77	121
CTLA-4	AACCATGCCCGGATTCTGA CCAAGCTAACTGCGACAAGGA	59	80	51
IL-10	TGGACAACATACTGCTAACC GGATCATTTCCGATAAGGCT	58	85	127
TGF-β	GCTGAACCAAGGAGACGGAAT GCTGAT CCCGTTGATTTCCA	62	79	112
RANKL	CAGAAGATGGCACTCACTGCA CACCATCGCTTTCTCTGCTCT	65	73	203
β-actin	ATGTTTGAGACCTTCAACA CACGTCAGACTTCATGATGG	56	75	495

t_A, annealing temperature; t_M, melting temperature; bp, base pairs of amplicon size, TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ; CTLA-4, cytotoxic T lymphocyte-associated molecule 4; IL-10, interleukin-10; TGF-β, transforming growth factor-β; RANKL, receptor activator for nuclear factor κB ligand.

Real-time polymerase chain reaction (PCR) reactions

The extraction of total RNA from periodontal tissues was performed using the Trizol reagent following the protocol recommended by the manufacturer (Life Technologies, Rockville, MD, USA), and the complementary DNA was synthesized using 3 μg of RNA through a reverse transcription reaction (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA). For the quantification of *A. actinomycetemcomitans*, DNA extraction from periodontal tissue samples was performed with DNA Purification System (Promega Biosciences Inc., San Luis Obispo, CA, USA), as described previously (Garlet et al. 2007). Real-time PCR quantitative mRNA or DNA analyses were performed in a MiniOpticon system (BioRad, Hercules, CA, USA) using the SYBR-Green System (Applied Biosystems, Warrington, UK). SYBR-Green PCR MasterMix (Applied Biosystems), 100 nM specific primers (Table 1) and 2.5 ng of cDNA (or 5 ng of DNA) were used in each reaction. The standard PCR conditions were 95°C (10 min.), followed by 40 cycles of 94°C (1 min.), 56°C (1 min.) and 72°C (2 min.), and by the standard denaturation curve. For mRNA analysis, the relative level of gene expression was calculated with reference to β-actin expression in the sample, using the cycle threshold (C_t) method. For DNA analysis, gene expression levels were determined using the C_t method and normalized by the tissue weight. Negative controls without cDNA or DNA and without reverse transcriptase were also performed.

Protein extraction and cytokine ELISA

Measurements of cytokines in periodontal tissues were performed as described previously (Garlet et al. 2006b). For protein extraction, palatal periodontal tissue was homogenized in PBS pH 7.4, centrifuged at 100 *g* at 4°C and the supernatants were stored at −70°C. The concentrations of cytokines in periodontal extracts were determined by ELISA using commercially available kits (R&D Systems, Minneapolis, MN, USA) as follows: IL-10 (> 4 pg/ml), TGF-β1 (> 1.7 pg/ml), TNF-α (> 3.4 pg/ml), RANKL (> 5 pg/ml) and interferon-γ (IFN-γ) (> 2 pg/ml). All assays were carried out according to the manufacturer's instructions. The results were expressed as picograms of cytokine (± SD) per milligram of periodontal tissue, for one experiment representative of three.

Antibody and treatment

The anti-GITR (DTA-1) hybridomas were grown i.p. in mineral oil-injected nude mice. The antibodies were purified from ascites by precipitation using ammonium sulphate (45%, w/v), and subsequently purified by a G protein column (Amersham Biosciences, Piscataway, NJ, USA), as described previously (Mariano et al. 2008). Protein was quantified using the bicinchoninic method. The in vivo blockage of GITR molecules was performed by i.p. injecting 500 μg/mice of purified mAb anti-GITR diluted in PBS, and was performed at 15, 30 and 45 days after infection. Control mice received 500 μg of normal rat IgG diluted in PBS at the same time points.

Statistical analysis

Data are presented as means ± SD, and the statistical significance between the experimental groups was analysed by ANOVA, followed by the Bonferroni post-test, or by the unpaired *t*-test, both performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Results

Tregs migration and markers' expression in response to *A. actinomycetemcomitans* infection

In order to confirm the establishment of experimental PD, we initially evaluated alveolar bone loss and inflammatory reactions after *A. actinomycetemcomitans* infection. We verified the presence of progressive alveolar bone loss and inflammatory cell influx into periodontal tissue in *A. actinomycetemcomitans*-infected mice when compared with control mice 15 days post-infection (pi) (Fig. 1a and b). The number of $CD4^+CD25^+$ cells was found to be increased 15, 30, 45 and 60 days pi (Fig. 1c). However, our results also demonstrate that Tregs ($CD4^+FOXp3^+$ cells) only migrate to gingival tissues of *A. actinomycetemcomitans*-infected mice 30 days pi (Fig. 1d). No significant increase in the CEJ–ABC area or in the number or leucocytes extracted from periodontal tissues was found in the control group throughout the experimental period analysed.

We next investigated the expression of CTLA-4, TGF-β and IL-10, characteristically described as Tregs makers and also to mediate its suppressive activity (Fig. 2). Our data demonstrated a significant expression of IL-10 in *A. actinomycetemcomitans*-infected mice 30 pi (Fig. 2a). Similarly, CTLA-4 was detectable in significant levels at 15 pi, but 30 days pi, its expression was significantly increased. TGF-β presented a kinetics of expression similar to that of CTLA-4; however, in spite of a trend towards an increased expression in the latter time points, this increase was not statistically significant. The levels of both IL-10 ($p = 0.004$, $r^2 = 0.6663$) and CLTA-4 ($p = 0.012$, $r^2 = 0.5624$) were found to be positively correlated with $CD4^+FOXp3^+$ cell counts when analysed by linear regression, while no positive correlation was found between Tregs counts and TGF-β mRNA levels ($p = 0.062$, $r^2 = 6208$). These findings demonstrate that $CD4^+$ $FOXp3^+$ cells migrate into the periodontal environment in response to experimental infection, and that IL-10 and CTLA-4 expressions are potential Tregs products involved in the immunoregulation of periodontal lesions.

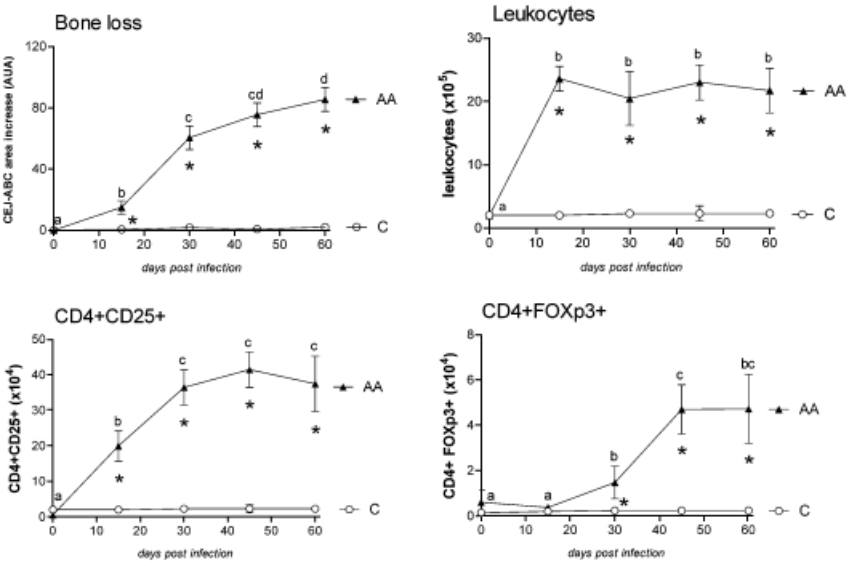

Fig. 1. Alveolar bone loss and influx of inflammatory cells from *Actinobacillus actinomycetemcomitans* (AA)-infected periodontal tissues. C57BL/6 mice were inoculated orally with AA and periodontal samples were taken. Alveolar bone level was evaluated and inflammatory cells were phenotypically characterized from AA-infected periodontal tissues. Alveolar bone loss quantification was performed through the measurements of the cement–enamel junction–alveolar bone crest area in the palatal face of maxillary molars, with ImageToll2.0 software (a). Values (mean+SD) obtained from five animals at each time point, from three independent experiments. Inflammatory cells were isolated and the cell viability was assessed by trypan blue exclusion and the total leucocytes were counted in a Neubauer chamber (b). Frequency of $CD4^+CD25^+$ and $CD4^+FOXp3^+$ cells as determined by flow cytometry (c and d). The values (mean+SD) obtained from three animals at each time point are shown from two independent experiments. Different letters indicate the differences within the experimental groups; $^*p < 0.05$ *versus* C group; one-way ANOVA, Bonferroni post-test.

Determination of Treg functions in experimental PD by means of anti-GITR treatment

In order to confirm the involvement of Treg cells in experimental PD outcome, we treated infected mice with anti-GITR antibodies, which characteristically inhibit Tregs function (Kohm et al. 2004, Scumpia et al. 2007, Mariano et al. 2008). Our results demonstrated a significant and progressive increase of alveolar bone loss 30 days pi in anti-GITR-treated mice (Fig. 3a), and similarly, an increased number of inflammatory cell influx were detected in the latter times of *A. actinomycetemcomitans* infection (Fig. 3b). Because Tregs only migrate to periodontal tissues 30 days pi, hanges in PD severity markers were expected only after this time point. In view of the potential dual role of Tregs (i.e. attenuation of host response and interference with antimicrobial activity), our next step was to evaluate the infection pattern in GITR-treated mice. Interestingly, it was observed that anti-GITR treatment did not impair the control of periodontal infection, as demonstrated by the similar levels of *A. actinomycetemcomitans*

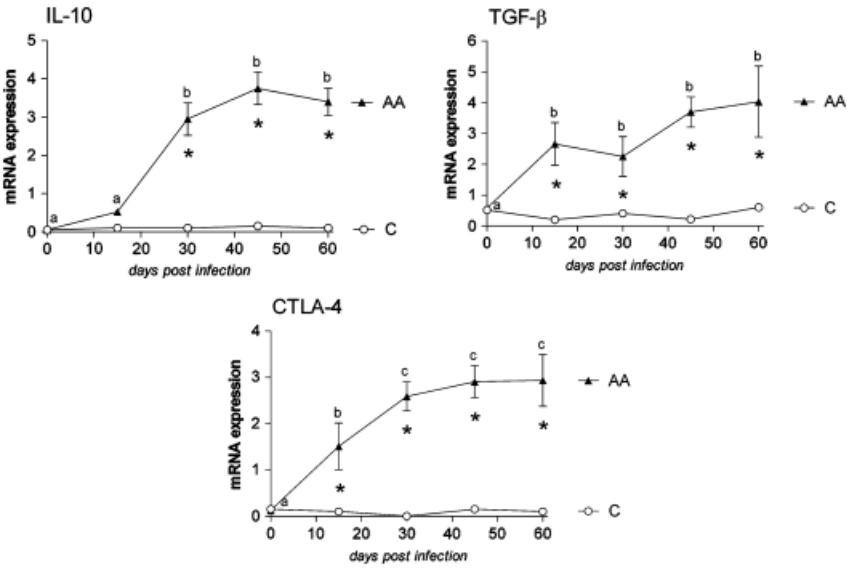

Fig. 2. Quantitative expression of transforming growth factor-β (TGF-β), interleukin-10 (IL-10) and cytotoxic T-lymphocyte antigen-4 (CTLA-4). Periodontal tissues of C57BL/6 mice inoculated orally with *Actinobacillus actinomycetemcomitans* (AA) were harvested from 0 (before infection) until 60 days of infection. The RNA was extracted, and the levels of TGF-β (a), IL-10 (b) and CTLA-4 (c) mRNA were measured quantitatively using the real-time polymerase chain reaction SYBR-Green System. The results are presented as the target mRNA level normalized to β-actin, using the cycle threshold (C_t) method. Each point represents the mean of triplicate measurements of each sample. Different letters indicate the differences within experimental groups; $^*p < 0.05$ *versus* C group, one-way ANOVA, Bonferroni post-test.

DNA in periodontal tissues of GITR-treated and control (infected but not untreated) mice (Fig. 3c). Accordingly, similar levels of serum C-reactive protein were found irrespective of GITR treatment, reinforcing the finding that Tregs attenuate experimental PD progression without interfering with the control of periodontal infection.

Determination of the mechanisms involved in the immunoregulation of experimental PD by Tregs by means of anti-GITR treatment

In order to investigate the possible mechanisms involved in the immunoregulation of experimental PD by Tregs, we next evaluated the pattern of Tregs markers and cytokines linked previously to the PD outcome in anti-GITR-treated mice. The quantitative analysis of IL-10 and CTLA-4 mRNA expression in periodontal tissues showed a significant reduction in their expression in anti-GITR-treated mice during the course of disease (Fig. 4). Investigating the expression of TGF-β mRNA, our real-time PCR results demonstrated that anti-GITR treatment resulted in a minor decrease of TGF-β expression, which only reached statistical significance at the time point of 45 days pi. ELISA analyses confirmed that the IL-10 and TGF-β levels in periodontal tissues were decreased by anti-GITR treatment 45 days pi. This results support the hypothesis that Tregs may act in a periodontal environment by means of CTLA-4, TGF-β and IL-10. Investigating the potential interference of Tregs and/or its products in the cytokine milieu in periodontal tissues, we also found that the levels of IFN-γ, TNF-α and RANKL mRNA were increased in anti-GITR-treated mice 30 days pi (Fig. 5). These findings were confirmed by ELISA analyses, which demonstrate higher levels of TNF-α and RANKL expression in periodontal tissues 45 days pi.

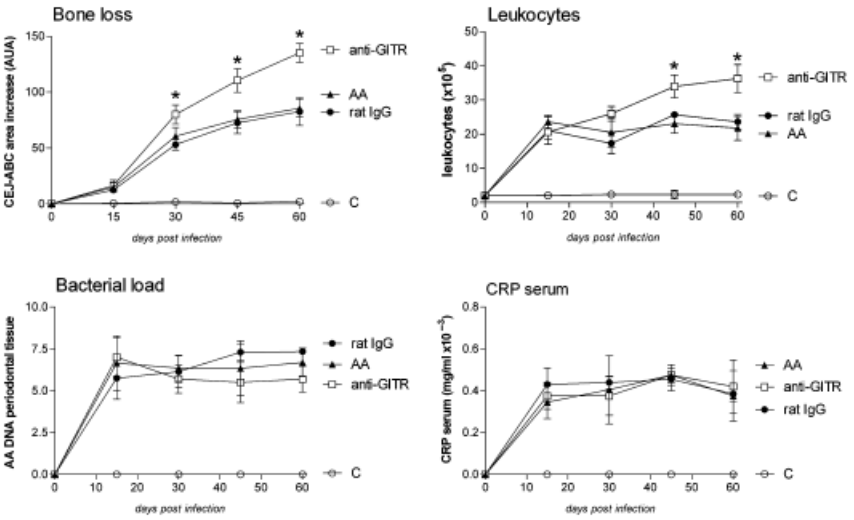

Fig. 3. Effects of glucocorticoid-inducible TNF receptor (GITR) treatment in the course of experimental periodontal disease. *Actinobacillus actinomycetemcomitans* (AA)-infected mice were treated with 500 μg/mice of purified mAb anti-GITR (or with control rat IgG) and evaluated after 15, 30, 45 and 60 days. Alveolar bone loss quantification was performed through the measurements of the cement–enamel junction (CEJ)–alveolar bone crest (ABC) area in the palatal face of maxillary molars, with ImageToll2.0 software (a). Values (mean+SD) obtained from five animals at each time point, from three independent experiments. Inflammatory cells were isolated and the cell viability was assessed by trypan blue exclusion and the total leucocytes were counted in a Neubauer chamber (b). Bacterial load was evaluated using the real-time polymerase chain reaction SYBR-Green System (c). The results are presented as the target mRNA level normalized to β-actin, using the cycle threshold (C_t) method. Each point represents the mean of triplicate measurements of each sample (d). Serum levels of C reactive protein, presented as mg/ml $\times 10^{-3}$. *$p<0.05$ *versus* AA-infected group, one-way ANOVA, Bonferroni post-test.

Discussion

Evidences from human PD demonstrate that Tregs (characterized by the expression of the phenotypic markers FOXp3, CTLA-4, IL-10, TGF-β, GITR, CD103 and CD45RO) are present in periodontal tissues (Nakajima et al. 2005, Ernst et al. 2007, Cardoso et al. 2008). In spite of a putative protective role for Tregs in the immunopathogenesis of PD, its exact role remains to be determined. In the present study, we demonstrated that there is an influx of Tregs ($CD4^+CD25^+$ $FOXp3^+$) into mice periodontal tissues after 30 days of experimental infection, a period characterized previously by a lower disease progression (Garlet et al. 2006b). Accordingly, Tregs were found to outnumber in chronic periodontitis *versus* human healthy (Cardoso et al. 2008, Dutzan et al. 2009) or gingivitis (Nakajima et al. 2005) tissues, in a possible attempt to restrain host response, important in the control of periodontal infection but also destructive (Garlet et al. 2007, 2008).

Indeed, the expression of the transcription factor FOXp3 (essential for Tregs development and function, and an exclusive and definitive marker of Tregs lineage in mice) (Allan et al. 2008) in leucocytes extracted from periodontal tissues of infected mice clearly defines these cells as Tregs. Accordingly, the pattern of FOXp3 mRNA expression perfectly matches with $CD4^+FOXp3^+$ cell influx into periodontal tissues (data not shown), as described in human PD (Cardoso et al. 2008). A possible explanation for the control of kinetics of Tregs' influx throughout periodontitis is the pattern of chemokines expressed in periodontal tissues. In human PD, 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). Accordingly, CCR4 and its ligands' expression were reported to be detectable in the latter stage of experimental PD (Garlet et al. 2005), in parallel with Tregs influx pattern demonstrated in this study. Similarly, TGF-β, IL-10 and CTLA-4 are expressed in significant levels in the early and late disease stages, but the noteworthy increase in its levels parallels with Tregs' influx into tissues (Cools et al. 2007, Yong et al. 2007). Tregs can act by direct cell–cell contact mediated by CTLA-4 and cell-surface TGF-β and also independent of cell contact through the production of soluble TGF-β and IL-10 (Shevach 2009). The positive correlation between IL-10 and CTLA-4 levels with the Tregs counts suggests that Tregs are important sources of these molecules in the periodontal environment. While previous studies demonstrated its expression

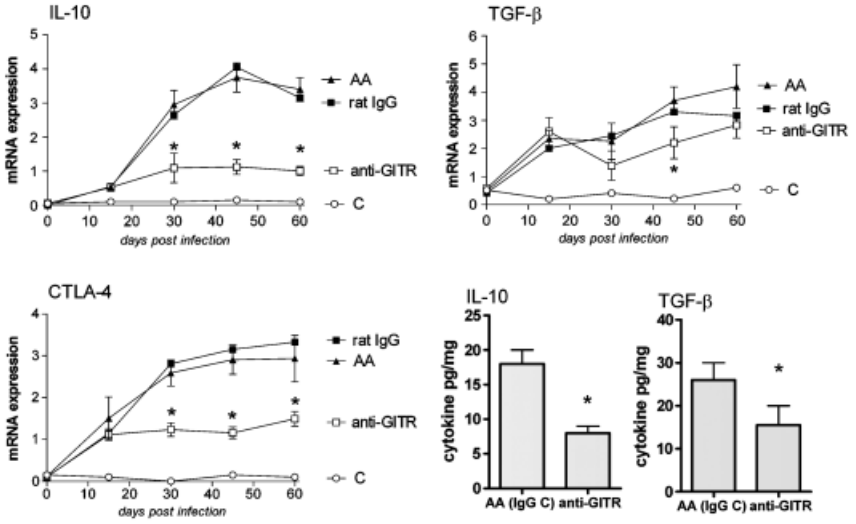

Fig. 4. Quantitative expression of interleukin-10 (IL-10), cytotoxic T-lymphocyte antigen-4 (CTLA-4) and transforming growth factor-β (TGF-β) in anti-glucocorticoid-inducible TNF receptor (GITR)-treated mice. Periodontal tissues of C57BL/6 mice inoculated orally with *Actinobacillus actinomycetemcomitans* (AA) were harvested from 0 (before infection) until 60 days of infection. AA-infected mice were treated with 500 μg/mice of purified mAb anti-GITR (or with control rat IgG) and evaluated after 15, 30, 45 and 60 days. (a–c) The RNA was extracted at each time point, and the levels of IL-10 (a), CTLA-4 (b) and TGF-β (c) mRNA were measured quantitatively using the real-time polymerase chain reaction SYBR-Green System. The results are presented as the target mRNA level normalized to β-actin, using the cycle threshold (C_t) method. Each point represents the mean of triplicate measurements of each sample. The concentrations of cytokines in periodontal extracts were determined by ELISA using commercially available kits (R&D Systems) (d). The results were expressed as picograms of cytokine ($\pm$ SD) per milligram of periodontal tissue, for one experiment representative of three. $^*p<0.05$ *versus* AA-infected group, one-way ANOVA, Bonferroni post-test (unpaired *t*-test for ELISA data).

in periodontal tissues (Orima et al. 1999, Aoyagi et al. 2000, Lappin et al. 2001, Cardoso et al. 2008), this is the first indication of Tregs as a potential major cellular source of both IL-10 and CTLA-4 in inflamed periodontal tissues. However, a positive correlation between TGF-β and Tregs counts could be expected, but the kinetics of TGF-β expression does not perfectly match with the Tregs migration pattern, which was not confirmed by the linear regression analysis. This apparent inconsistency is probably due to the pleiotropic TGF-β nature and the existence of several potential cellular sources of this cytokine in periodontal lesions (Steinsvoll et al. 1999, Cornelini et al. 2003, Ye et al. 2003, Chen et al. 2005).

Interestingly, the expression of Tregs-associated factors IL-10, TGF-β and CTLA-4, as well the influx of Tregs into the periodontal environment, parallel the latter stage of PD, characterized by a lower disease progression rate and by reduced MMPs/tissue inhibitors of metalloproteinases (TIMPs) and RANKL/OPG ratios (Garlet et al. 2006b). Therefore, the overall balance between pro- and immunoregulatory mediators (in this specific case, products of Tregs) seems to determine (or at least strongly influence) the outcome of PD.

However, only based on such an association, strong statements regarding the role of Tregs in the immunopathogenesis of PD, as well regarding the putative mechanisms involved in its function, are impossible. Therefore, we next inhibited Tregs function with anti-GITR antibodies, which are known to inhibit Treg functions (Shimizu et al. 2002, Mariano et al. 2008), in order to determine Tregs' role in the experimental PD outcome. Our results demonstrated that Tregs inhibition resulted in a significant increase of alveolar bone loss and inflammatory cell influx in the latter disease stage, coinciding with the period where a significant Tregs influx into periodontal tissues was found. As could be expected, anti-GITR treatment results in a significant decrease in the levels of Tregs markers IL-10 and CTLA-4, and in a slight decrease of TGF-β levels. These results suggest that Tregs possibly act via IL-10 and CTLA-4, while the minor decrease of TGF-β levels is possibly due to its production by other cell types in periodontal tissues (Ye et al. 2003).

Interestingly, the Tregs-associated cytokines IL-10 and TGF-β have been considered as putative protective factors against periodontal tissue destruction influx in view of its anti-inflammatory and anabolic properties (Cardoso et al. 2008, Claudino et al. 2008). IL-10 is widely expressed in diseased periodontal tissues and is associated with lower disease severity (Lappin et al. 2001, Garlet et al. 2004). Indeed, IL-10 plays a protective role against tissue destruction, inhibiting both MMPs and RANK systems (Garlet et al. 2004, Zhang & Teng 2006). In addition, IL-10 characteristically induces the upregulation of a group of endogenous proteins named TIMPs, which are capable of inhibiting almost every member of the MMP family in a non-specific way (Baker et al. 2002, Silva et al. 2007). IL-10 also stimulates the production of OPG, a decoy receptor of RANKL, which strongly inhibits bone resorption by preventing RANK–RANKL engagement (Baker et al. 2002, Silva et al. 2007). In agreement, a positive correlation was demonstrated between the levels of IL-10, TIMPs and OPG in both human and experimental PDs (Garlet et al. 2004, 2006b, Claudino et al. 2008). Similar to IL-10, TGF-β can also play important roles in the attenuation of inflammatory damage to periodontal tissues. TGF-β is a pleiotropic cytokine that can regulate cell growth, differentiation and matrix production, and is therefore (as a general rule) anabolic in nature (Okada & Murakami 1998). Indeed, in active periodontal lesions, higher levels of TGF-β have been negatively correlated with RANKL levels (Dutzan et al. 2009). Also in accordance with our findings, Tregs marker FOXp3 was inversely correlated with RANKL+ lymphocytes and RANKL expression in diseased periodontal tissues (Ernst et al. 2007, Dutzan et al. 2009).

Besides a direct effect of Tregs-derived cytokines in MMPs/TIMPs and RANKL/OPG expression, the modulation of the cytokine milieu in the periodontal environment possibly contributes to attenuate experimental PD progression. Indeed, with the lack of Tregs activity, downregulation of the pro-inflammatory and Th1-type response associated with a low disease progression period 30 days pi (Garlet et al.

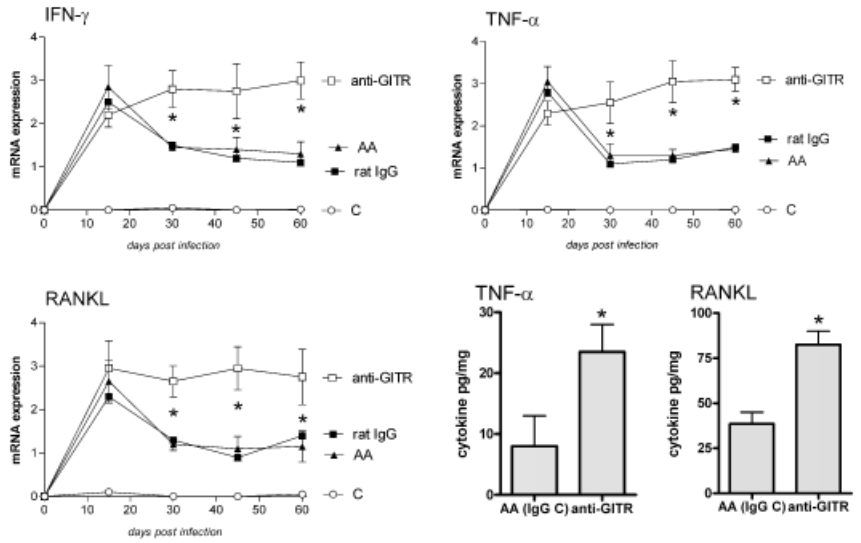

Fig. 5. Quantitative expression of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and receptor activator for nuclear factor κB ligand (RANKL) in anti-glucocorticoid-inducible TNF receptor (GITR)-treated mice. Periodontal tissues of C57BL/6 mice inoculated orally with *Actinobacillus actinomycetemcomitans* (AA) were harvested from 0 (before infection) until 60 days of infection. AA-infected mice were treated with 500 μg/mice of purified mAb anti-GITR and evaluated after 15, 30, 45 and 60 days. The RNA was extracted at each time point, and the levels of IFN-γ (a), TNF-α (b) and RANKL (c) mRNA were measured quantitatively using the real-time polymerase chain reaction SYBR-Green System. The results are presented as the target mRNA level normalized to β-actin, using the cycle threshold (C_t) method. Each point represents the mean of triplicate measurements of each sample. The concentrations of cytokines in periodontal extracts were determined by ELISA using commercially available kits (R&D Systems) (d). The results were expressed as picograms of cytokine ($\pm$ SD) per milligram of periodontal tissue, for one experiment representative of three. $^*p < 0.05$ *versus* AA-infected group, one-way ANOVA, Bonferroni post-test (unpaired *t*-test for ELISA data).

2006b) was not observed in anti-GITR-treated mice. Conversely, our results demonstrated a sustained high TNF-α and IFN-γ expression, associated with high RANKL levels. In accordance, both TNF-α and IFN-γ cytokines were described to upregulate RANKL expression in the periodontal environment and to consequently increase alveolar bone loss (Garlet et al. 2004, Cochran 2008, Graves 2008). Previous studies suggest that the protective role of IL-10 may be mediated by the direct downregulation of inflammatory cytokines and their signalling, an event mediated by suppressors of cytokine signalling family members (Garlet et al. 2006a). Similar to IL-10, TGF-β can downregulate the transcription of pro-inflammatory factors such as IL-1β and TNF-α (Page 1992, Birkedal-Hansen 1993, Steinsvoll et al. 1999). Furthermore, these inflammatory cytokines have been linked to inflammatory cell migration in the periodontal environment (Garlet et al. 2004, 2007), and consequently, Tregs-derived TGF-β could have a direct effect in restraining leucocyte influx throughout experimental PD. Besides TGF-β and IL-10, the classic Tregs marker CTLA-4 is expressed by leucocytes in diseased periodontium (Gemmell et al. 2001), and was found to be increased in $CD4^+$ cells of periodontitis patients when compared with healthy subjects (Orima et al. 1999, Aoyagi et al. 2000). In addition, CTLA-4 suppresses the proliferation of T cells in response to periodontopathogens (Aoyagi et al. 2000), and the co-stimulation mediated by B7/CD28 signalling (characteristically inhibited by CLTA4) is required for inflammatory cell migration and bone resorption in experimental PD (Kawai et al. 2000), reinforcing its protective role in PD immunopathogenesis.

However, the exact target (or targets) of Tregs and their products in periodontal tissues remain unclear. The current scientific literature demonstrates that B cells produce RANKL in response to periodontopathogen challenge (Han et al. 2009), and also that the majority of B cells in periodontal lesions are positive for RANKL staining (Kawai et al. 2006). Interestingly, B-cell deletion was demonstrated to prevent *Porphyromonas gingivalis*-induced bone loss in mice (Baker et al. 2009), but B cells are not essential for inflammatory bone loss process because T cells are able to mediate bone resorption in the absence of B cells (Yamaguchi et al. 2008). Indeed, T cells are also a potential source of RANKL in the periodontal environment, and consequently, are potentially involved in periodontitis outcome determination (Kawai et al. 2000, Kawai et al. 2006). In any case, Tregs are able to specifically regulate the activation, proliferation and effector function of both B and T cells (Lim et al. 2005, Appay et al. 2008, Belkaid & Tarbell 2009, Sallusto & Lanzavecchia 2009, Shevach 2009). Therefore, further studies are required to determine the exact nature of the leucocyte subsets under the suppressive influence of Tregs throughout experimental periodontitis.

Taken together, the results described until this point a protective role for Tregs, this T-cell subset being involved in the attenuation of the inflammatory reaction and bone loss after periodontal infection. However, it is also very important to consider that in a series of infectious diseases, Tregs inconveniently impair the immune response against the pathogens (Belkaid et al. 2002, Joosten & Ottenhoff 2008). Nevertheless, our results demonstrate that Tregs migration does not compromise the control of periodontal infection; also Tregs inhibition does not result in an improved infection control. However, the ability of *A. actinomycetemcomitans* to attach and colonize the subgingival biofilm, an environment that may confer efficient protection to the periodontopathogens (Jenkinson & Lamont 2005, Fine et al. 2006), possibly impairs its clearance by the immune system, even with the development of robust responses. Accordingly, mice strains genetically selected to maximal (AIRmax) or minimal (AIRmin) inflammatory responsiveness present similar *A. actinomycetemcomitans* loads after experimental infection (Trombone et al. 2009). Interestingly, the C57BL/6 strain usually used in our studies develops an intermediary response between AIRmin and AIRmax strains, and also presents a bacterial load similar to that seen in the AIR strains (Garlet et al. 2006b), reinforcing the finding that the development of a robust host response

against the periodontal biofilm is ineffective to clear the infection, and that a less intense reaction (i.e. as host response under Tregs control) is equally effective in controlling (at least partially) periodontal infection, and also results in a lower degree of tissue damage (Trombone et al. 2009).

Taken together, our results demonstrate that Tregs influx naturally occurs throughout experimental PD, being associated with an attenuation of disease progression rate. Furthermore, Tregs are associated with IL-10, TGF-β and CTLA-4 production and downregulation of pro-inflammatory and osteoclastogenic factors. Very importantly, in contrast to a series of infectious diseases, Tregs do not seem to impair the control of experimental periodontal infection. Therefore, Tregs may be considered potential therapeutic targets aimed to modulate the host immune response in order to minimize the tissue destruction associated with periodontitis development. However, it is very important to remember 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 in the outcome of different forms of human periodontitis.

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Clinical Relevance

Scientific rationale for the study: While the mechanisms involved in periodontal destruction by inflammatory cytokines are well known, regulatory mechanisms involved in the control of periodontitis remain unknown. In this scenario, Tregs appear as a T-helper subset potentially involved in disease outcome determination.

Principal findings: Tregs migration to periodontal tissues is associated with higher levels of IL-10, CTLA-4 and TGF-β. The inhibition of Tregs function resulted in increased alveolar bone loss and inflammatory cell migration, associated with decreased anti-inflammatory and increased inflammatory cytokine and RANKL production. Interestingly, the control of experimental infection was not affected by the inhibition of Tregs.

Practical implications: Tregs can be an interesting therapeutic target in the management of PDs.