CCR5 Mediates Pro-osteoclastic and Osteoclastogenic Leukocyte Chemoattraction

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ABSTRACT

Periodontal disease (PD) progression involves the selective leukocyte infiltration into periodontium, supposedly mediated by the chemokine/chemokine receptor system. In this study, we investigated the role of chemokine receptor CCR5 in the immunoregulation of experimental PD in C57BL/6 (WT) and CCR5KO mice. *Aggregatibacter actinomycetem comitans* infection triggered the chemoattraction of distinct CCR5+ leukocyte subpopulations (determined by flow cytometry): CCR5+F4/80+ leukocytes, which co-express CD14 , CCR2, TNF-α, and IL-1β, indicative of activated macrophages; and CCR5+CD4+ cells, which co-express CXCR3, IFN-γ, and RANKL, indicative of Th1 lymphocytes, therefore comprising pro-osteoclastic and osteoclastogenic cell subsets, respectively. CCR5KO mice presented a lower PD severity (lower inflammation and alveolar bone loss) when compared with the WT strain, since the migration of F4/80+, TNF-α+, CD4+, and RANKL+ cells specifically decreased due to the lack of CCR5. Also, ELISA analysis demonstrated that the production of TNF-α, IL-1β, IL-6, IFN-γ, and RANKL in periodontal tissues was significantly decreased in the CCR5KO strain. The periodontal bacterial load and antimicrobial patterns were unaltered in CCR5KO mice. Our results demonstrate that the chemokine receptor is involved in the migration of distinct leukocyte subpopulations throughout experimental PD, being a potential target for therapeutic intervention in PD.

KEY WORDS: CCR5, bone resorption, inflammation, chemotaxis, periodontal disease.

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INTRODUCTION

Periodontal diseases (PD) are chronic inflammatory diseases characterized by the inflammatory bone resorption of the tooth-supporting structures, being the most prevalent form of bone pathology in humans and a modifying factor of the systemic health of patients (Bartold *et al*., 2010). After the recognition of bacteria harboring the subgingival biofilm that triggers host response, the succeeding selective leukocyte subset infiltration into periodontal tissues determines the nature and the extent of inflammatory immune response and, consequently, contributes to determine PD outcome (Silva *et al*., 2007; Garlet, 2010).

Among the inflammatory mediators potentially involved in selective leukocyte migration to the periodontium, chemokines have been implicated in PD pathogenesis (Graves, 1999; Silva *et al*., 2007). Chemokines are a superfamily of small chemotactic cytokines recognized as regulators of inflammatory processes and cell trafficking, which orchestrate the migration of specific cells to organs or tissues through the binding to specific receptors (*i.e*., chemokine receptors) selectively expressed by leukocyte subpopulations (Luther and Cyster, 2001; Bonecchi *et al*., 2009). The chemokine receptor CCR5, and its ligands CCL3, CCL4, and CCL5 are highly expressed in diseased periodontium, where they are thought to contribute to inflammatory cell migration and PD development (Gamonal *et al*., 2001; Kabashima *et al*., 2002; Garlet *et al*., 2003; Silva *et al*., 2007; Repeke *et al*., 2010). Accordingly, in different inflammatory models, CCR5 and its ligands are described to be involved in the migration of T-lymphocytes polarized into the Th1 phenotype, and also in the chemoattraction of dendritic cells and monocytes/macrophages (Luther and Cyster, 2001; Silva *et al*., 2007; Bonecchi *et al*., 2009). Interestingly, while Th1 cells are thought to be the major source of RANKL in diseased periodontium (Kotake *et al*., 2005), dendritic cells and macrophages are potential osteoclast precursors (Takeshita *et al*., 2000; Yu *et al*., 2004; Maitra *et al*., 2010), reinforcing its potential role in PD development. While a recognized redundancy in the chemokine system suggests an overlapping role for CCL3, CCL4, and CCL5 in experimental PD, the absence of CCR5 results in decreased leukocyte infiltration and bone loss (Repeke *et al*., 2010).

However, the exact nature of CCR5+ cells in the periodontal environment remains unknown, as do the mechanisms by which CCR5 modulates inflammatory cell migration and alveolar bone loss throughout experimental PD. To

clarify this question, we infected wild-type and CCR5 genetically deficient C57Bl/6 mice with the periodontopathogen *Aggregatibacter actinomycetemcomitans* and comparatively evaluated the patterns of leukocyte migration and cytokine production to determine the role of CCR5 in PD pathogenesis.

MATERIALS & METHODS

Experimental Groups

Experimental groups were comprised of 8-week-old male wild-type (WT) C57BL/6 mice, and mice with targeted disruption of the CCR5 (CCR5KO, breeding pairs obtained from Jackson Laboratory [Bar Harbor, ME, USA], bred and maintained in the animal facilities of FOB/USP). Throughout the period of the study, the mice were fed with sterile standard solid mouse chow (Nuvital, Curitiba, PR, Brazil) and sterile water, and were periodically weighed. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use.

Experimental Periodontitis

Periodontitis induction was performed as described previously (Repeke *et al*., 2010). In brief, the animals received an oral delivery of 1 x 10^9 *A. actinomycetemcomitans* JP2 (grown anaerobically in supplemented agar medium, TSBV) CFU diluted in 100 µL 2% of carboxymethylcellulose PBS solution, at 0, 48, and 96 hrs. Negative controls included sham-infected mice, which received PBS with carboxymethylcellulose in solution without *A. actinomycetemcomitans*, and non-infected animals. Experimental periodontitis severity was determined by the analysis of alveolar bone loss degree (please see the Appendix for details) and the quantitative analysis of inflammatory cell infiltration (please see the Appendix for details). Flow cytometry was used for the phenotypic analysis of leukocytes isolated from periodontal tissues (please see the Appendix for details).

Protein Extraction and ELISA

Measurements of cytokines (TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and RANKL) in periodontal tissues were performed by ELISA as previously described (Repeke *et al*., 2010). For protein extraction, buccal and palatal periodontal tissue from upper molars was homogenized in phosphate-buffered saline (PBS), pH 7.4, by Ultra Turrax T18 (IKA, Staufen, Germany), centrifuged at 100 *g* at 4°C, and the concentrations of cytokine in periodontal extracts were determined by ELISA with commercially available kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, and expressed as picograms of cytokine (± SD) *per* milligram of periodontal tissue.

Real-time PCR Reactions

Real-time PCR was performed with SybrGreen PCR MasterMix (Applied Biosystems, Foster City, CA, USA), in a MiniOpticon System (BioRad, Hercules, CA, USA), as previously described (Repeke *et al*., 2010; Trombone *et al*., 2010). The extraction of total RNA from periodontal tissues was performed with Trizol (Life Technologies, Rockville, MD, USA), and the complementary DNA was synthesized with 3 µg of RNA. For mRNA analysis, the relative level of gene expression was calculated in reference to β-actin by means of the delta-delta CT method. For *A. actinomycetemcomitans* and 16S bacterial DNA quantification, DNA extraction from periodontal tissue samples was performed with the DNA PurificationSystem (Promega Biosciences, San Luis Obispo, CA, USA). For DNA analysis, expression levels were normalized by the tissue weight.

Quantification of Antimicrobial Mediators

The levels of serum C-reactive protein (CRP) were determined in serum samples by means of a commercially available agglutination kit (Labtest Diagnóstica, São Paulo, Brazil), as previously described (Garlet *et al*., 2007, 2008). Myeloperoxidase (MPO) activity in homogenized periodontal tissue was measured as by enzymatic reaction, measured through the absorbance at 450 nm, and presented as OD; the serum titer of total and *Aa*-specific IgG was measured by ELISA, both as previously described (Repeke *et al*., 2010).

Statistical Analysis

Data presented as means ± SD were analyzed by ANOVA/Bonferroni or unpaired *t* test, performed with Prism5.0 (GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

CCR5+ Cell Migration Pattern, Cellular Phenotype, and Its Impact in Experimental PD Outcome

We initially characterized the migration kinetic of total leukocytes and CCR5+ cells throughout experimental PD (Fig. 1). While total leukocytes were found in a rising number until a day 30 peak and a subsequent decrease, CCR5+ cells peaked at day 15 and then decreased. When the phenotype of CCR5+ was investigated by FlowCytometry, our results demonstrated a substantial number of cells co-stained for F4/80, CD14, CD3, and CD4. When CCR5KO mice were investigated concerning experimental PD outcome (Fig. 2), our results demonstrated a significant reduction in disease severity phenotype, represented by the lower bone loss and decreased total leukocyte counts and alveolar bone loss area when compared with those of the WT strain. The *A. actinomycetemcomitans* and 16S bacterial DNA loads were similar in WT and CCR5 strains, as were the CRP serum levels and the weights of the mice at 60 days of infection (Fig. 2). The antimicrobial mediators MPO, iNOS (mRNA), and *Aa*-specific IgG levels were not affected by CCR5 lack (Appendix Fig.).

CCR5+ Cells Comprise an Inflammatory Cytokine-producing F4/80+ Subpopulation

In view of the potential presence of subpopulations within CCR5+ leukocytes, we next performed an additional phenotypic analysis of CCR5+F4/80+ cells (Fig. 3), and verified that these

cells were also largely positive for TNF-α, IL-1β, and CCR2. Accordingly, the relative numbers of F4/80+- and TNF-α-positive cells were significantly reduced in CCR5KO mice at 30 days post-infection. When macrophage-associated cytokines were measured in periodontal tissues, our results demonstrated that TNF-α, IL-1β, and IL-6 levels were significantly decreased in the CCR5KO strain when compared with their WT counterparts at day 30 post-infection.

CCR5+ Cells Comprise a RANKL+ CD4 Th1 Subpopulation

Our next step was the phenotypic analysis of CCR5+CD4+ cells (Fig. 4), which demonstrated that these cells were also largely positive for IFN-γ, RANKL, and CXCR3. Accordingly, the relative numbers of CD4+- and RANKL+-positive cells were significantly reduced in CCR5KO mice at 30 days post-infection. CD4+ and F4/80+ were independently co-stained for distinct cytokines, reinforcing the existence of 2 distinct CCR5+ subpopulations in periodontal tissue infiltrate. When Th-associated cytokines were measured in periodontal tissues, our results demonstrated that while IFN-γ levels were significantly decreased in CCR5KO strain, IL-4 and IL-17A levels were unaltered. In addition, the CCR5KO strain also presented a significant decrease in RANKL levels when compared with WT mice.

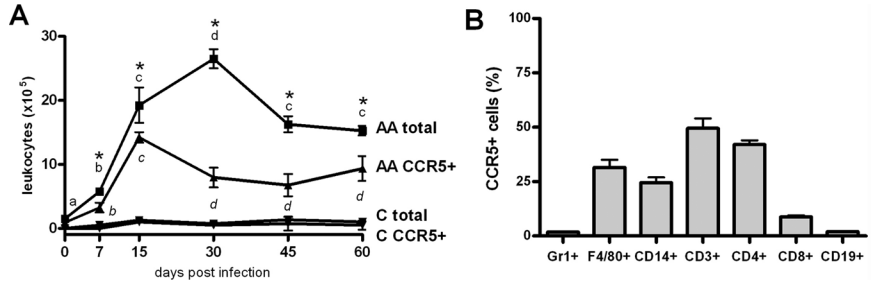

Figure 1. CCR5+ cells in experimental periodontal disease in WT mice: migration pattern and phenotypic analysis and role in disease development. C57Bl/6 (WT) mice infected orally with *A. actinomycetemcomitans* (AA) and non-infected controls (C) and evaluated for: **(A)** total leukocyte (performed in a Neubauer chamber) and CCR5+ cells (determined by flow cytometry subsequent to Neubauer chamber count) migration in WT mice; and **(B)** CCR5+ cell phenotype analyzed by flow cytometry (double staining with anti-CCR5 and anti-leukocyte subset markers). Values (mean ± SD) obtained from 5 animals at each point, from one experiment representative of 3; different letters represent statistically significant differences in the kinetic analysis within each group ($P < 0.05$; one-way ANOVA with Bonferroni's post-test); *$P < 0.05$ (unpaired *t* test) represents statistically significant differences between WT and CCR5KO strains at each time-point. All values in AA-infected strains were significantly different from those in non-infected controls.

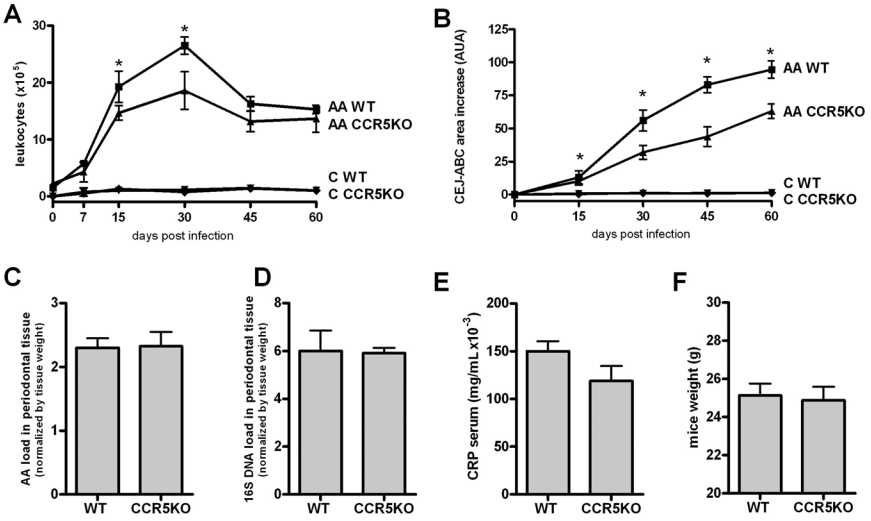

Figure 2. Experimental periodontitis phenotype in CCR5KO mouse strain. C57Bl/6 (WT) and CCR5KO mice were infected orally with *A. actinomycetemcomitans* (AA) and non-infected controls (C) of both strains and evaluated for: **(A)** total leukocyte count in periodontal tissues (performed in a Neubauer chamber); **(B)** alveolar bone loss quantification, represented by the cement-enamel junction-alveolar bone crest (CEJ-ABC; measured in the palatal face of maxillary molars) area increase in AA groups normalized by the respective controls; **(C)** *A. actinomycetemcomitans* (AA DNA) and **(D)** total bacterial (16S DNA) load periodontal tissues, quantified by Real-time PCR, with the SybrGreen System and normalized by tissue weight; **(E)** serum levels of C-reactive protein, presented as mg/mL x 10^{-3}; and **(F)** weight of mice in grams. Values (mean ± SD) obtained from 5 animals at each point, from one experiment representative of 3; *$P < 0.05$ (unpaired *t* test) represents statistically significant differences between AA-infected WT and CCR5KO strains at each time-point. All values in AA-infected strains were significantly different from those in non-infected controls.

DISCUSSION

Periodontal diseases (PD) are chronic inflammatory diseases triggered by the subgingival biofilm and highly influenced by host inflammatory immune response (Graves, 2008; Garlet, 2010). Among inflammatory mediators responsible for leukocyte migration, chemokines have been implicated in PD pathogenesis (Silva *et al*., 2007). The chemokine receptor CCR5 was described as a higher PD severity mediator in humans and mice (Gamonal *et al*., 2001; Kabashima *et al*., 2002; Garlet *et al*., 2003; Silva *et al*., 2007; Repeke *et al*., 2010), but the mechanisms by which CCR5 determines PD outcome remain unknown.

Our results demonstrates that, in the absence of CCR5, both inflammatory cell migration and alveolar bone loss throughout experimental PD were significantly reduced, while the periodontal bacterial load was not affected, demonstrating the role of CCR5 in determining an increased PD severity phenotype. Similarly, CCR5 was previously associated with the severity of rheumatoid arthritis, which shares with PD the chronic inflammatory bone resorption activity (Qin *et al*., 1998; Trombone *et al*.,

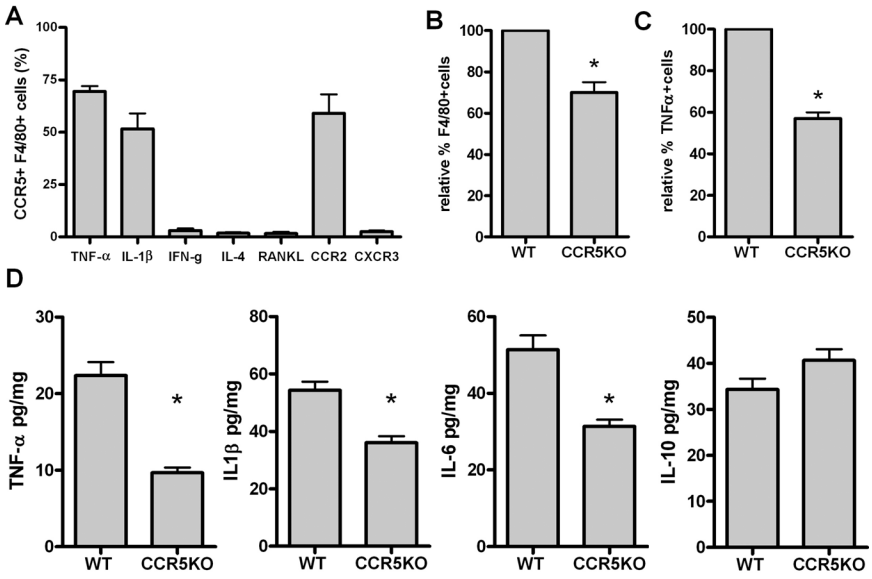

Figure 3. The CCR5+F4/80+ leukocyte subset: phenotypic analysis, migration pattern, and association with pro- and anti-inflammatory cytokine production throughout experimental periodontitis. C57Bl/6 (WT) and CCR5KO mice were infected orally with *A. actinomycetemcomitans* (AA) and evaluated for: **(A)** phenotype of CCR5+F4/80+ cells extracted from periodontal tissues of WT mice, analyzed by flow cytometry; relative numbers of **(B)** F4/80+ and **(C)** TNF-α+ cells in periodontal tissues of WT *vs.* CCR5KO strains, analyzed by flow cytometry; and **(D)** the levels of TNF-α, IL-1β, IL-6, and IL-10 protein in periodontal tissues, determined by ELISA. Values (mean ± SD) obtained from 5 animals at each point, from one experiment representative of 3; *P < 0.05 (unpaired *t* test) represents statistically significant differences between WT and CCR5KO strains.

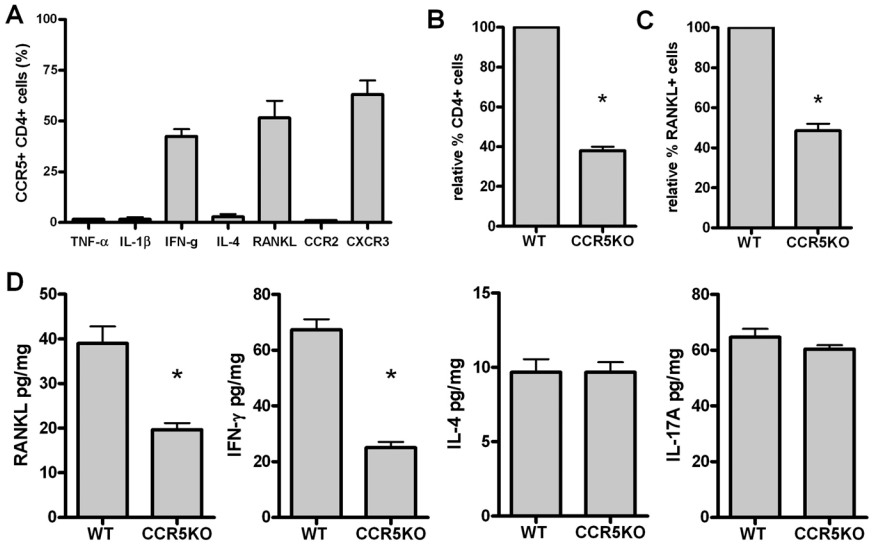

Figure 4. The CCR5+CD4+ leukocyte subset: phenotypic analysis, migration pattern, and association with RANKL and prototypical T-helper cytokine production throughout experimental periodontitis. C57Bl/6 (WT) and CCR5KO mice were infected orally with *A. actinomycetemcomitans* (AA) and evaluated for: **(A)** phenotype of CCR5+CD4+ cells extracted from the periodontal tissues of WT mice, analyzed by flow cytometry; relative numbers of **(B)** CD4+ and **(C)** RANKL+ cells in periodontal tissues of WT *vs.* CCR5KO strains, analyzed by flow cytometry; and **(D)** the levels of RANKL, IFN-γ, IL-4, and IL-17A protein in periodontal tissues determined by ELISA. Values (mean ± SD) obtained from 5 animals at each point, from one experiment representative of 3; *P < 0.05 (unpaired *t* test) represents statistically significant differences between WT and CCR5KO strains.

2010). Interestingly, the bone loss rate attenuation is more prominent than the leukocyte number reduction, suggesting that CCR5+ cells may play active roles in the bone resorption process. Our results also demonstrated that CCR5+ cells comprise a significant fraction of total leukocytes that infiltrate periodontal tissues, and that these cells largely co-express F4/80, CD14, CD3, and CD4. These markers are characteristically expressed by distinct leukocyte subpopulations, namely, dendritic cells and monocytes/macrophages (F4/80 and CD14), and CD4 (CD3 and CD4) lymphocytes, these being the cell types implicated in the pathogenesis of PD (Takeshita *et al*., 2000; Berglundh and Donati, 2005; Garlet *et al*., 2005; Garlet, 2010).

In view of the existence of distinct leukocyte subpopulations among CCR5+ cells infiltrating periodontal tissues, a detailed phenotypic characterization of these cells was performed. CCR5+ F4/80+ cells co-express CD14 and the chemokine receptor CCR2, but not CD3 and CD4. Such a phenotype suggests a myeloid lineage that can be composed of dendritic cells and monocytes/macrophages (Takeshita *et al*., 2000; Tacke and Randolph, 2006; Crane *et al*., 2009), putative antigen-presenting cells, and inflammatory cytokine sources in the periodontium (Silva *et al*., 2007; Takayanagi, 2009; Ford *et al*., 2010). Accordingly, our results also demonstrate that CCR5+F4/80+ are positive for TNF-α and IL-1β intracellular staining, which also suggests an activated phenotype (Graves, 2008; Garlet, 2010). In fact, TNF-α and IL-1β have been associated with the development of PD, and mononuclear leukocytes are classically described as a source of inflammatory cytokines (Graves and Cochran, 2003; Graves, 2008). Accordingly, our results demonstrated that the levels of TNF-α, IL-1β, and IL-6 (also destructive in the context of PD) (Graves, 2008) were significantly lower in the periodontal tissues of CCR5KO, reinforcing the potential tissue-destructive role of CCR5+ cells. It is also important to consider that dendritic cells and macrophages are potential osteoclast precursors (Nicholson *et al*., 2000; Takeshita *et al*., 2000; Maitra *et al*., 2010). Accordingly,

pre-osteoclasts express CCR5 (Oba *et al*., 2005), and CCL3-CCR5 binding triggers its differentiation into osteoclasts (Oba *et al*., 2005). Therefore, CCR5 can also contribute to PD development as a pro-osteoclastic leukocyte subpopulation, and preliminary results demonstrate that CCR5+F4/80+ cells can develop an osteoclastic phenotype *in vitro* (data not shown).

While CCR5+F4/80+ leukocytes constitute a pro-osteoclastic inflammatory cytokine-producing subset, CCR5+CD4+ cells present a potential osteoclastogenic phenotype. Our results demonstrated that CCR5+CD4+ cells co-express CXCR3 and IFN-γ, characterizing a Th1 polarized lymphocyte subset (Qin *et al*., 1998; Luther and Cyster, 2001; Bonecchi *et al*., 2009). Th1 cells are regarded as the major RANKL source in diseased periodontium (Kotake *et al*., 2005), and, accordingly, CCR5+CD4+ cells are positive for RANKL staining. Also, the levels of RANKL in periodontal tissues of CCR5KO strain were significantly reduced. Therefore, CCR5+CD4+ cells can potentially induce the pro-osteoclastic CCR5+F4/80+ subset differentiation, generating a complementary osteoclastogenic network. In addition, confirming the alleged Th1 phenotype, the lack of CCR5 resulted in decreased IFN-γ (the prototypical Th1 cytokine) levels in periodontal tissues. While described as anti-osteoclastogenic *in vitro*, the remarkable inflammatory properties of IFN-γ *in vivo* overcome the inhibitory effect and lead to an osteoclastogenic outcome (Garlet *et al*., 2008; Takayanagi, 2009). In fact, IFN-γKO presents decreased inflammatory cell migration and alveolar bone loss (Garlet *et al*., 2008). Interestingly, while the lack of CCR5 significantly restrains Th1-cell migration, T-cells polarized into Th2 and Th17 phenotypes (whose migration is mediated by CCR4 and CCR6, respectively) are still able to migrate to periodontal tissues and probably correspond to the remaining CD4 T-cells found in the CCR5KO strain. Accordingly, the levels of IL-4 (the prototypical Th2 cytokine) and IL-17A (Th17-related) were unaltered by the lack of CCR5, reinforcing the specificity of this chemokine receptor in Th1-cell chemoattraction. While analysis of our data demonstrates the osteoclastogenic role of CCR5+ cells in experimental PD, CCR5 down-regulates osteoclast function in orthodontic tooth movement (Andrade *et al*., 2009). These opposing roles may rely on differences in the triggering factors (*i.e*., microbial factors *vs*. mechanical loading), and in the nature of inflammatory processes (*i.e*., chronic *vs*. transitory inflammation) and leukocyte populations involved.

While limiting PD progression, the lack of CCR5 could impair the control of periodontal infection mediated by pro-inflammatory and Th1 cytokines (Garlet *et al*., 2007, 2008). However, our results demonstrated that the bacterial load, as well as the levels of iNOS, MPO, and *Aa*-specific IgG, was similar in CCR5KO and WT strains. Previous studies have demonstrated that host inflammatory-immune hyper-responsiveness is not required for proper control of the periodontal infection (Trombone *et al*., 2009).

In summary, CCR5 mediates the migration of distinct leukocyte populations, which include pro-osteoclastic and osteoclastogenic leukocytes, depicting an interesting complementary network involved in experimental PD progression. Furthermore, since the lack of CCR5 does not impair the control of periodontal infection, this chemokine receptor is a potential target for therapeutic intervention in PD.

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