UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

ANGÉLICA CRISTINA FONSECA

Evaluation of the role of CCR2 and CCR5 receptors in macrophage migration and alveolar bone repair outcome in mice

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Avaliação do papel dos receptores CCR2 e CCR5 na migração de macrófagos e no processo de reparo ósseo alveolar em camundongos

Dissertação apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Biologia Oral.

Orientador: Prof. Dr. Gustavo Pompermaier Garlet.

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LIST OF FIGURES

LIST OF ABREVIATIONS AND ACRONYMS

BMP	Bone morphogenic protein
c-fms	Colony-stimulating factor-1 receptor
CCL	C-C motif chemokine
CCR	C-C chemokine receptor type
COL1A2	Collagen type I alpha 2 chain
COL2A1	Collagen type II alpha 1 chain
CXCL	C-X-C motif chemokine
CX3CR	CX3C chemokine receptor
DLX5	Distal-less homeobox 5 gene
DMP	Dentin matrix protein
FGF-2	Fibroblast growth factors 2
IL	Interleukin
KO	*Knockouts*
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte Chemoattractant Protein-1
MIP–1 α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NFATc1	Nuclear factor of activated T-cells
NF-kB	Factor nuclear kappa β
OPG	Osteoprotegerin
OSX	Osteoblast-specific transcription factor Osterix
PDGF	Platelet-derived growth fator
RANK	Receptor activator of nuclear factor kappa β
RANKL	Receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt-related transcription factor 2
TGF- β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TRAF6	TNF receptor associated factor 6
VEGF	Vascular endothelial growth factor
WT	C57Bl/6 wild-type mice

SUMMARY

ABSTRACT

Macrophages play important roles in bone repair, including the control of immune response and inflammation, and regulating the transition between granulation tissue to osteogenesis and formation of new bone after an injury. In this context, chemokine receptors, such as CCR5 and CCR2, seems to be key players in the chemotaxis of monocytes/macrophages to sites of tissue injury. The objective of this project is investigate simultaneously the role of CCR2 and CCR5 receptors in the cell migration and its subsequent impact on the alveolar repair process in mice. Mice C57BL/6 WT and CCR5KO, eight weeks old, were submitted to extraction of the right upper incisor and distributed in groups (N=5) control and treated with the antagonist for CCR2 (RS504393, 2mg/kg/24h), in order to allow of the blockage analysis individually or simultaneously of the receptors. Samples were collected in the 0h, 7d, 14d and 21days post extraction periods, and analyzed by micro-computed tomography (μCT), histological analyzes (histomorphometry, immunohistochemistry and birefringence analysis), as well as molecular analysis by means of PCRArray for quantification of different markers involved in the repair process. Immunohistochemical analysis shows that CCR2 and CCR5 receptor blockade did not significantly influence the migration of monocytes/macrophages to alveolar bone repair. A similar pattern can be observed in MicroCT and in microscopic analyzes that do not demonstrate major changes in the parameters representatative of bone healing. On the other hand, molecular analyzes demonstrated that the simultaneous inhibition of CCR2 and CCR5 (CCR5KOantiCCR2 group) resulted in a a significantly higher mRNA expression of extracellular matrix markers (COL2A1 and MMP9), some bone markers (DMP1 and RANKL), FGF1 as well as IL-6 expression and decreased expression of growth factors (TGFb1 and VEGF), as well as RUNX-2 and cytokines (IL-10 and TNF-α) when compared to WT-C. Therefore, we conclude that, although CCR2 and CCR5 receptor blockade result in significant modulation of of growth factors, proinflammatory cytokines and osteoclastogenic factors expression, there are no significant differences in the control of macrophage migration as well as in the subsequent bone repair outcome.

Keywords: Dental socket. CCR2. CCR5. Macrophage. Osteoinmulogy. Bone repair

RESUMO

Sabe-se que macrófagos exercem um importante papel no processo de reparo tecidual, e estão presentes inclusive no processo de reparo ósseo, possivelmente atuando no controle e ativação da resposta imune/inflamatória, bem como na transição do tecido de granulação para um processo de osteogênese e a neo-formação de um tecido ósseo após a lesão. Neste sentido, os receptores de quimiocinas CCR2 e CCR5 parecem ser os principais envolvidos na quimiotaxia de monócitos/macrófagos para locais de injúria tecidual. Dessa forma, o objetivo deste projeto é investigar simultaneamente o papel dos receptores CCR2 e CCR5 na migração celular e seu impacto subsequente no processo de reparo alveolar. Camundongos C57Bl/6 WT e CCR5KO, com oito semanas de idade, foram submetidos à extração do incisivo superior direito e distribuídos em grupos (N=5) controle e tratados com o antagonista para CCR2 (RS504393, 2mg/Kg/24h), de modo a possibilitar a análise do bloqueio individual ou simultâneo dos receptores. As amostras foram coletadas nos períodos de 0h, 7d, 14d e 21dias pós-exodontia, e analisadas através de microtomografia computadorizada (MicroCT), análises histológicas (histomorfometria, imunoistoquímica e análise de birrefringência), bem como análise molecular por meio do PCRArray para quantificação de diferentes marcadores envolvidos no processo de reparo. A análise imuno-histoquímica nos mostra que o bloqueio dos receptores CCR2 e CCR5 não influenciou significativamente à migração de monócitos/macrófagos em direção ao reparo ósseo alveolar. Um padrão semelhante pode ser observado nas análises MicroCT e microscópicas que não demonstram mudanças importantes nos parâmetros representativos da cicatrização óssea . Já a análise molecular, demonstrou que essa mesma dupla inibição de CCR2 e CCR5 (grupo CCR5KOantiCCR2) resultou em uma expressão de mRNA significativamente maior de marcadores de matriz extracelular (COL2A1 e MMP9), alguns marcadores ósseos (DMP1 e RANKL), FGF1 e também na expressão de IL-6 e uma menor expressão dos fatores de crescimento (TGFb1 e VEGF), bem como RUNX-2 e e as citocinas (IL-10 e TNF-α) quando comparado ao WT-C. Assim concluímos que, embora, que o bloqueio dos receptores CCR2 e CCR5 resulte em uma modulação significativa de fatores de crescimento, citocinas pró-inflamátorias e expressão de fatores osteogênicos, não há diferença significativas no controle de migração de macrófagos, bem como no subsequente resultado do reparo ósseo alveolar.

Palavras-chave: Alvéolo dental. CCR2. CCR5. Macrófago. Osteoimunologia. Reparo ósseo.

1 INTRODUCTION

1 INTRODUCTION

The bone tissue is characterized as a connective and mineralized tissue, consisting of collagen, non-collagenous proteins, hydroxyapatite crystals, small organic molecules and water. While the collagenous constituent of the bone matrix determines mechanical resilience, the mineral constituents provide stiffness to this tissue (RIDDLE; CLEMENS, 2017). From the cellular viewpoint, bone presents a high complexity, being composed bydifferent cell types, such as osteoblasts, osteocytes and osteoclasts, which act in an integrated way to form and maintain bone tissue (ELSAYED; BHIMJI, 2017). Bone tissue maintenance involves a continuous remodeling process, which promotes tissue renewal and hemostasis, also provides a high repair capacity to bone (ZHAO; HUANG; ZHANG, 2016). Thus, upon bone injury (such as a fracture or a tooth extraction), new bone tissue is formed, and subsequently remodeled, in the injury site (GHIASI et al., 2017).

Osteoblasts are differentiated from mesenchymal cells under specific stimuli, such as from BMPs (Bone morphogenic protein), tthat trigger the activation of transcription factors such as RUNX2 (Runt-related transcription factor 2), DLX5 (Distal-less homeobox 5) and OSX (Osterix), whose activation characterizethe osteoprogenitory cells (GINALDI; MARTINIS, 2016). Subsequently, such cells receive the pre-osteoblast nomenclature, and become larger and cuboid, and positive for alkaline phosphatase activity. In the late maturation stages, the osteoblasts are presented in a single layer with basal and eccentric round nucleus, and a basophilic cytoplasm. At this stage, the characteristic function of osteoblasts is to secrete granules whose content in the extracellular medium is organized on the fibrils form, composing an osteoid matrix. The mineralization of this osteoid matrix, although it is not yet very clear, has the participation of the non-collagenous proteins that diffuse towards the front of mineralization where they control the deposition of mineral components, in addition, some cells present alkaline phosphatase, that are responsible for breaking the inorganic phosphate present in the tissue medium that also determines the onset and progression of the mineral crystals of the osseous tissue (NANCI, 2013; SAINT-PASTOU TERRIER; GASQUE, 2017). At the end of bone matrix synthesis, osteoblasts may undergo apoptosis or become bone-lining cells, assuming a flat shape overlying the bone surface, or differentiate into osteocytes (MATIC et al., 2016).

Osteocytes differentiate from osteoblasts after embedded in the bone matrix during mineralization. The osteocytes are flattened and characteristically present a decrease in organelles, as well a basophilic staining of the cytoplasm (NANCI, 2013). The osteocytes position themselves within lacunaes, connected by a canaliculi netwtork filled by the osteocytes prolongations, which helps in the intercommunication between the bone cells, allowing the passage of molecules, as well allow to osteocytes act as mechanical sensors contributing to the maintenance of bone function (PARK et al., 2017).

The third cell type found in bone tissue are the osteoclast, originated from hematopoietic monocyte/macrophage lineage, whose main function is bone matrix resorption (KANZAKI et al., 2016). The differentiation of these cells begins with the expression of cytokine M-CSF, secreted by osteoblasts, that when connected to the c-fms receptor, expressed in osteoclast precursors, will determine the upregulation of RANK (Receptor activator of nuclear factor kappa β). Upon RANK activation by RANKL, the osteoclast differentiation process proceedes under the influence of different factors such as TRAF6 (TNF receptor associated factor 6), NF-kB (factor nuclear kappa β), NFATc1 (Nuclear factor of activated T-cells), whichcharacterize the activate mature osteoclasts (KATSIMBRI, 2017).

Building on the above, it is clearer the understanding of the repair process after damage to the bone tissue, which starts with the formation of a clot to fill the bone defect (SIVARAJ; ADAMS, 2016). Sequentially, the clot is invaded by inflammatory cells, firstly by neutrophils that produce and release cytokines, such as IL-6 and CCL2, which in turn will recruit monocytes/macrophages. Macrophages, are thought to play several functions along tissue repair, being responsible for phagocytizing necrotic remains and also secrete inflammatory and chemotactic mediators, which contribute to the recruitment of mesenchymal stem cells (MSCs) and osteoprogenitor cells (TAKIZAWA; MANZ, 2017). Such cells, under the stimuli of proinflammatory cytokines and growth factors, such as members of the transforming growth factor (TGF-β) family, in particular TGF-β1, -β2, -β3 and BMPs, as well vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2), collectively promote neovascularization and formation of a provisional extracellular collagen matrix and proliferative activity of mesenchymal cells and its differentiation into osteoblasts (GARG et al., 2017). Subsequently, osteoid matrix will form and later mineralize, and a remodeling of this primary bone tissue will occur. Remodeling depends of the initial osteoclasts action, and the process of osteoclastogenesis occurs about direct control of the osteoblasts and osteocytes, which

involves the production M-CSF, RANKL, and Osteoprotegerin (OPG) (GINALDI; MARTINIS, 2016). Osteoclasts, when on the bone surface, alter their cytoskeleton in an important corrugated border conformation to generate a sealing area where they secrete hydrogen ions and lisasomal enzymes to degrade the organic component of this tissue. During resorption, growth factors are released from the bone matrix and will stimulate the migration and differentiation of osteoblasts, which will in turn will refill the resorption lacunae, depositing an osteoid tissue that will then undergo a mineralization process (MATSUMOTO et al., 2016; CHEUNG et al., 2016).

It is also important to consider that the control of the bone metabolism, both in physiological conditions and in pathological conditions, is influenced by several local and systemic factors that directly affect the behavior of the bone cells. Among these factors we have numerous polypeptides (systemic hormones, cytokines, differentiation factor and locally released growth factors), with emphasis on the association of the immune system with the skeletal system, which constitutes a recent research strand called osteoimmunology (ONO; TAKAYANAGI, 2017). Basically, the relationship between the immune and bone systems is characterized by the action of mediators produced by leukocytes, which directly affect the processes of bone formation and/or resorption (GRAVES; OATES; GARLET, 2011). This relationship can be exemplified by the action proinflammatory cytokines such as TNFα, which has an inhibitory role on bone formation by action under osteoblasts, while potentiating the resorptive activity of osteoclasts, by increasing expression of RANKL (TAKAYANAGI, 2017). In contrast, the anti-inflammatory cytokine IL-10 has an inhibitory action on osteoclastogenic activity, in part by the increase in OPG expression, thus affecting the RANK/RANKL/OPG system (GARLET et al., 2006).

The understanding of the interaction between bone tissue and the imune system is necessary because the inflammatory process can be a critical determinant in the reestablishment of tissue hemostasis after bone injury or the outcome of pathological situations. Generally, the inflammation process induces an increase of pro- inflammatory factors such as TNFα and IL-6, which can determine an imbalance of bone metabolism, where bone resorption overlaps the formation process, generating a loss of bone mass, as has been demonstrated in periodontal and periapical lesions (REPEKE et al., 2010; GRAVES; OATES; GARLET, 2011). On the other hand, under physiological conditions of repair, the immune/inflammatory response occurs in the initial periods after the occurrence of the lesion, presenting a "constructive" and self-limited profile with the theoretical predominance of anti-

inflammatory mediators and growth factor, and in this distinct environment leukocytes and their different mediators appear to contribute positively to tissue formation, remodeling and repair (THOMAS; PULEO, 2011).

Macrophages compose a group of leukocytes regarded as keymodulators of the process of bone repair, although there are aspects related to its activation, profile and action that are not yet fully understood. Initially, it was believed that its function was closely related to its phagocytic role in the immune/inflammatory response, however recent studies have shown that the role of these cells also involves maintaining homeostasis and repairing tissues (SINDER et al., 2016). Currently the macrophages are subdivided into M1 and M2 subsets. The M2 cells are activated in response to IL-4, IL-12 and IL-10 anti-inflammatory cytokines with the purpose of promoting the resolution of the inflammatory process, while M1 subsets is activated in response to cytokines such as IL-1 and IL-6 acting throughout the pro-inflammatory response. As regards the macrophage activity in the bone tissue, we can find it on the form of macrophages originated from the bone marrow, osteoclasts and a population of macrophages recently defined as "osteomacs" macrophages (MIRON; BOSSHARDT, 2016). It should be noted that this study seeks to identify the acting of macrophages throughout all phases of bone repair, interfering in the initial inflammatory process until bone remodeling, and thus accelerate or even delay such remodeling process (MICHALSKI; MCCAULEY, 2017). Recruitment of monocytes/macrophages to injury sites occurs through the binding of chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CXCL9 and CXCL10 and CCL2, CCL3, CCL4, CCL5, CCL11, CCL17 and CCL22, with specific chemokine receptors expressed in the cell surface (GILCHRIST; STERN, 2015).

Among the chemokine receptors that can mediate macrophage migration, CCR2 and CCR5 are considered as key elements of macrophage mobilization during inflammatory responses. CCR2 and CCR5 respond to certain subtypes of chemokines of the CC family (characterized by having the two cysteines adjacent and close to the N-terminal region), such as CCL2 (MCP-1), CCL1 (MIP-1α) and CCL5 (RANTES) (RIGAMONTI et al., 2008). Studies cite that chemokines can be classified into inflammatory and hemostatic. Several hemostatic chemokines may interact with a single receptor, unlike the inflammatory chemokines ones that present a lower specificity (BERNARDINI et al., 2017). Constituting a basis on possible cooperative effects of CCR2 and CCR5 receptors (KOTHANDAN; GADHE; CHO, 2012), present mainly in monocytes/macrophages with an inflammatory

profile, which influences the migration of most F4/80+ cells (specific surface marker to mouse macrophages) to the injured tissue, affecting the course of various pathological processes (XIAO et al., 2017) or even repair/regeneration of different tissues (LU et al., 2011; KHAN et al., 2013). In contrast, the CX3CR1 receptor, also commonly expressed at injury sites, is related to another subpopulation of macrophages, called resident macrophages, because they are found physiologically in the tissue, do not appear to significantly interfere in tissue repair (KHAN et al., 2013).

We can thus verify the role of these receptors in different inflammatory conditions associated with bone tissue, being the activity of CCR2 previously investigated in orthodontic movement (TADDEI et al., 2012a), rheumatoid arthritis (IWAMOTO et al., 2008), osteoporosis (ERALTAN et al., 2012,) in the repair of fracture of endochondral bones (ISHIKAWA et al., 2014) and the alveolar bone repair (VIEIRA et al., 2015). In the case of the CCR5, our group previously demonstrated its influence on the migration of leukocytes and pre-osteoclasts to the bone tissue in bone pathologies such as periodontal disease (REPEKE et al., 2011), as well as during bone remodeling during orthodontic movement in mice (ANDRADE et al., 2009), but its role in the process of alveolar bone repair remains unknown.

In this context, previous studies show the cooperative role of both receptors in the migration of inflammatory profile macrophages. Among them, a study previously developed by our research group, focused in the analysis of the alveolar bone repair process in CCR2KO mice (BIGUETTI, 2014). Such study desmonstrated by immunohistochemical analysis a statistically significant decrease in both F4/80+ and CCR5+ cell recruitment in CCR2KO animals compared to WT animals, indicating that, even reduced, the remaining macrophage migration could be related to the presence of the CCR5 receptor in CCR2KO animals. The results of the immunohistochemistry in addition to those of PCRarray, from the same study, show some statistical relevance in the higher expression of CCR5 in WT in relation to CCR2KO animals (BIGUETTI, 2014). Therefore, we can suppose that there is a possibility that the receptors CCR2 and CCR5 have a cooperative role on the recruitment of inflammatory cells to the repair site during the immune/inflammatory response that occours along bone repair. Considering the evidence previously discussed it would be reasonable to assume that the absence of the CCR5 receptor could affect alveolar bone repair in a manner similar to that occurring in CCR2KO animals, but that only the blockade and/or absence of both receptors (CCR2 and CCR5) could interfere with the repair. Thus, the present work aims

to analyze the simultaneous influence of CCR2 and CCR5 receptors throughout the process of alveolar bone repair in mice, as well as to investigate the molecular mechanisms by which CCR2 + and CCR5 + cells contribute to this process.

2 ARTICLE

2 ARTICLE

Bone

Microtomographical, histological and molecular analysis of the role of the CCR5 receptor, and possible cooperative role of CCR2 receptor, in macrophage migration and its impact on the alveolar repair process post extraction in mice

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ABSTRACT

Macrophages play important roles in bone repair, including the control of immune response and inflammation, and regulating the transition between granulation tissue to osteogenesis and formation of new bone after an injury. In this context, chemokine receptors, such as CCR5 and CCR2, seems to be key players in the chemotaxis of monocytes/macrophages to sites of tissue injury. The objective of this project is investigate simultaneously the role of CCR2 and CCR5 receptors in the cell migration and its subsequent impact on the alveolar repair process in mice. Mice C57BL/6 WT and CCR5KO, eight weeks old, were submitted to extraction of the right upper incisor and distributed in groups (N=5) control and treated with the antagonist for CCR2 (RS504393, 2mg/kg/24h), in order to allow of the blockage analysis individually or simultaneously of the receptors. Samples were collected in the 0h, 7d, 14d and 21days post extraction periods, and analyzed by micro-computed tomography (μCT), histological analyzes (histomorphometry, immunohistochemistry and birefringence analysis), as well as molecular analysis by means of PCRArray for quantification of different markers involved in the repair process. Immunohistochemical analysis shows that CCR2 and CCR5 receptor blockade did not significantly influence the migration of monocytes/macrophages to alveolar bone repair. A similar pattern can be observed in MicroCT and in microscopic analyzes that do not demonstrate major changes in the parameters representatative of bone healing. On the other hand, molecular analyzes demonstrated that the simultaneous inhibition of CCR2 and CCR5 (CCR5KOantiCCR2 group) resulted in a a significantly higher mRNA expression of extracellular matrix markers (COL2A1 and MMP9), some bone markers (DMP1 and RANKL), FGF1 as well as IL-6 expression and decreased expression of growth factors (TGFb1 and VEGF), as well as RUNX-2 and cytokines (IL-10 and TNF-α) when compared to WT-C. Therefore, we conclude that, although CCR2 and CCR5 receptor blockade result in significant modulation of of growth factors, proinflammatory cytokines and osteoclastogenic factors expression, there are no significant differences in the control of macrophage migration as well as in the subsequent bone repair outcome.

Keywords: Dental socket. CCR2. CCR5. Macrophage. Osteoinmulogy. Bone repair

1. Introduction

Bone repair depends on the integration of bone tissue with other systems, such as the immunological system (1,2). Thus, when a fracture occurs or even the production of bone defects a new tissue is formed and remodeled at the injury site under the influence of several local and systemic factors that directly affect the behavior of bone cells site (3) process macrophages compose a group of leukocytes regarded as keymodulators of the process of bone repair, although there are aspects related to its activation, profile and action that are not yet fully understood. Initially, it was believed that its function was closely related to its phagocytic role in the immune/inflammatory response, however recent studies have shown that the role of these cells also involves maintaining homeostasis and repairing tissues (4).

Recruitment of monocytes/macrophages towards injury occurs through the binding of chemokines to surface receptors (5,6), such as the CCR2 and CCR5 receptors, which respond to certain CC chemokine subtypes (7). We can thus verify the role of these receptors in different inflammatory conditions associated with bone tissue, being the activity of CCR2 previously investigated in orthodontic movement (8), rheumatoid arthritis (9), osteoporosis (10) in the repair of fracture of endochondral bones (11) and the alveolar bone repair (12). In the case of the CCR5, our group previously demonstrated its influence on the migration of leukocytes and pre-osteoclasts to the bone tissue in bone pathologies such as periodontal disease (13), as well as during bone remodeling during orthodontic movement in mice (14), but its role in the process of alveolar bone repair remains unknown.

In this context, previous studies show the cooperative role of both receptors in the migration of inflammatory profile macrophages. Among them, a study previously developed by our research group, focused in the analysis of the alveolar bone repair process in CCR2KO mice (15). Such study desmonstrated by immunohistochemical analysis a statistically significant decrease in both F4/80+ and CCR5+ cell recruitment in CCR2KO animals compared to WT animals, indicating that, even reduced, the remaining macrophage migration could be related to the presence of the CCR5 receptor in CCR2KO animals. The results of the immunohistochemistry in addition to those of PCRarray, from the same study, show some statistical relevance in the higher expression of CCR5 in WT in relation to CCR2KO animals (15). Therefore, we can suppose that there is a possibility that the receptors CCR2 and CCR5 have a cooperative role on the recruitment of inflammatory cells to the repair site during the immune/inflammatory response that occours along bone repair. These studies are based on the understanding that chemokines can be classified as inflammatory and hemostatic, where one

or two hemostatic chemokines may interact with a single receptor, other than the inflammatory ones that have a lower specificity (16). It thus provides a basis for possible cooperative effects of CCR2 and CCR5 (7,17,18).

Thus, by investigating the cooperative effect between CCR2 and CCR5 receptors, we promoted a genetic blockade by using CCR5KO animals and a pharmacological blockade by administration of the CCR2 antagonist. The choice of this antagonist was made on the basis of studies demonstrating a similar effect to the use of CCR2KO animals (19-23). For a better understanding of the mechanism of action of this antagonist, it is necessary to understand the signaling pathway that occurs between the CCR2 receptor and its main ligand CCL2 (MCP-1). The different chemokines have a variable sequence of amino acids, but all are characterized by being arranged under a three-dimensional fold consisting of a short unstructured N-terminal region and an enlarged N-loop region followed by three β-chains stabilized by disulfide bonds between two cysteines (covalently bond) and a C-terminal helice. Thus, for intracellular signaling to occur, determining monocyte/macrophage migration, a more stable binding occurs between the N-loop of CCL2 and the N-terminal of CCR2 (G protein-coupled receptor containing seven transmembrane domains, presenting a region C-terminus and another N-terminus) and a more unstable binding between the short N-terminal region of CCL2 binds to the intermembrane region of CCR2 termed the binding pocket in the transmembrane domain. Structurally there is a division in large binding pocket (transmembrane helice 3, 4, 5, 6 and 7) and a smaller binding pocket (transmembrane helice 1, 2, 3 and 7) (20).

In the case of the CCR2 antagonist, RS504393 (Biotechnology® Santa Cruz), is classified in the Spiropiperidine antagonist group, whose structure is formed by the basic Nitrogen present in the Piperidine ring, where the positively charged Nitrogen compound occupies the chemokine binding site CCL2, called Glutamate 291(Glu291) which has negatively charged, an amino acid residue present in the transmembrane region seven between the major and minor binding pocket (17,20). In addition, other structural compounds of this antagonist bind to other regions of the CCR2 receptor, where a second Nitrogen and Oxygen are bound by Hydrogen bridges there are Tyr49 (residue present in the transmembrane region one) and Tyr120 (residue present in the transmembrane region three), respectively (17,24). Determining that this antagonist has a specific inhibition pathway of CCL2/CCR2, not demonstrating effectiveness in the blockade of the other receptors.

Incorporating the aforementioned concepts about osteoimmunology and directing them to the field of dentistry, studies aimed at understanding the molecular mechanisms that

regulate this bone repair process could promote clinical benefits related to the installation of osseointegrable implants or even a better understanding of the progression and an adequate treatment of Periodontal disease (25-27). Following this line of investigation of the immune/inflammatory response in the process of bone repair within the dental area, our research group developed a research model that aims to analyze aspects related to the influence of the immune system on alveolar bone repair post-exodontia in C57Bl/6- WT mice (12). Such an animal model has been a viable alternative for comparing alveolar repair in WT mice with genetically deficient mice (Knockouts-KO) to potential immune/inflammatory targets, allowing the establishment of direct cause and effect relationships between such factors and their influence on the biological events that succeed the repair, as well as the clarification of the mechanisms involved in the regulation of the metabolism of the bone tissue (28-29).

Thus, the present work aims to analyze the simultaneous influence of CCR2 and CCR5 receptors throughout the process of alveolar bone repair in mice, as well as to investigate the molecular mechanisms by which CCR2+ and CCR5+ cells contribute to this process. Para isto, we promoted a genetic blockade when using CCR5KO animals and a pharmacological blockade through the administration of the CCR2 antagonist (RS504393) to identify macrophages performance throughout all phases of bone repair, interfering in the initial inflammatory process until bone remodeling, which may accelerate or delay such remodeling process (30).

2.Matherial and methods

2.1 Animals and Ethics Statement

For this study we used 8-week-old male C57BL/6 mice wild type (WT) and CCR5 knockouts provided by the Center for Special Animal Breeding from FMRP/USP. All animals were treated according to the approval of the Committee on Animal Research and Ethics [Comissão de Ética no Ensino e Pesquisa em Animais] CEEPA-FOB/USP, processes #012/2015. WT mice treated with RS504393 (WTantiCCR2) or not (WT-C "control") compared to Knockout mice for CCR5 that received RS504393 (CCR5KOantiCCR2) or not (CCR5KO), each comprised of nine mice (five for both the microtomographic, histological, histochemical and immunohistochemistry analysis, and four for the RealTimePCRarray analysis).

2.2 Treatment with CCR2 Antagonists

CCR2 antagonist RS504393 (Biotechnology® Santa Cruz), is included in the group of Spiropiperidine antagonists, whose structure is formed by the basic Nitrogen present in the Piperidine ring, where the positively charged Nitrogen compound binds to the chemokine site CCL2, negatively charged (17,20). Animals from the treated groups was receive the CCR2 antagonist drug, diluted in DMSO and administered orally, at a dosage of 2mg/kg diluted in 20µl of solution. Administration started one day prior to the surgical procedure, being the daily dose until the end of the respective experimental period (0h, 7,14 and 21 days) (19).

2.3 Experimental protocol and mice tooth extraction model

The animals were anaesthetized by intramuscular administration of 80mg/kg of ketamine chloride (Dopalen, Agribrans Brasil LTDA) and 160mg/kg of xylazine chloride (Anasedan, Agribrands Brasil LTDA) in the proportion 1:1, determined according to the animal body mass. The extraction of the upper right incisor was performed with the aid of a stereomicroscope (DF Vasconcellos S.A., Brasil) under 25x magnification. A dental exploratory probe was used to promote the dental element luxation by a smooth movement to avoid root fracture, followed by the use of clinical tweezers to seize and remove the tooth. After extraction, the removed tooth was checked for integrity. Animals presenting fractured teeth during the extraction were excluded from further analysis. At the end of the experimental periods (0h, 7, 14 and 21 days post tooth extraction), the animals were killed with an excessive dose of anesthetic, and the maxillae were collected. Samples for the µCT and histological analyses were fixed in PBS-buffered formalin (10%) solution (pH 7.4) for 48h at room temperature, subsequently washed over-night in running water and maintained temporarily in alcohol fixative (70% hydrous ethanol) until the conclusion of the µCT analysis, and them decalcified in 4.13% EDTA (pH 7,2) and submitted to histological processing. Samples for molecular analysis were stored in RNAlater (Ambion, Austin, TX) solutions (12).

2.4 Micro-computed tomography (µCT) assessment

The maxillae samples were scanned by the Skyscan 1174 System (Skyscan, Kontich, Belgium) at 50 kV, 800 µA, with a 0.5 mm aluminium filter and 15% beam hardening correction, ring artifacts reduction, 180 degrees of rotation and exposure range of 1 degree. Images were captured with 1304x1024 pixels and a resolution of 14µm pixel size. Projection images were reconstructed using the NRecon software and three-dimensional images obtained

by the CT-Vox software. Morphological parameters of trabecular bone microarchitecture were assessed using the CTAn software in accordance with the recommended guidelines (31). A cylindrical region of interest (ROI) with an axis length of 2,5 a 2,6mm and diameter of 1mm was determined by segmenting the trabecular bone located from the coronal to apical thirds. Trabecular measurements analyzed included the tissue volume (TV), bone volume (BV) bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm), and trabecular separation (Tb.Sp) (12,32).

2.5 Histology *sample preparation and* histomorphometric analysis

Serial sections (8 semi-serial sections of each maxilla, with a 5 μm thickness for each section) were obtained using a microtome (Leica RM2255, Germany) and stained with H.E. (hematoxylin and eosin). Morphometric measurements were performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100x immersion objective and a Zeiss kpl 8X eyepiece containing a Zeiss II integration grid (Carl Zeiss Jena GmbH, Jena, Germany) with 10 parallel lines and 100 points in a quadrangular area. The grid image was successively superimposed on approximately 13 histological fields per histological section, comprised of all tooth sockets from the coronal limit adjacent to the gingival epithelium until the lower apical limit. For each animal/socket, sections from the coronal, medial and apical thirds were evaluated. In the morphometric analysis, points were counted coinciding with the images of the following components of the alveolar socket: clot, inflammatory cells, blood vessels, fibroblasts, collagen fibers, bone matrix, osteoblasts, osteoclasts and other componentes (empty space left by the inflammatory exudate or intercellular liquid and bone marrow) (12,33). The results are presented as the volume density (mean) for each evaluated structure.

2.6 Birefringent fibers

The Picrosirius-polarization method and quantification of birefringent fibers were performed to assess the structural changes in the newly formed bone trabeculae matrix based on the birefringence of the collagen fiber bundles, as previously described (12,34,35). Serial sections (8 semiserial sections of each maxilla) with 5 μm thickness were cut and stained with Picrosirius Red Stain; all sections were stained simultaneously to avoid variations due to possible differences in the staining process. Picrosirius Red-stained sections were analyzed through a polarizing lens coupled to a binocular inverted microscope (Leica DM IRB/E), and

all images were captured with the same parameters (the same light intensity and angle of the polarizing lens 90° to the light source). AdobePhotoshopCS6 software was used to delimit the region of interest (alveolar area comprised of new tissue with the external limit comprised of the alveolar wall), totalizing 1447680 pixels^2. The quantification of the intensity of birefringence brightness was performed using the AxioVision 4.8 software (CarlZeiss). For quantification, the images were binarized for definition of the green, yellow and red color spectra, and the quantity of each color pixels^2 corresponding to the total area enclosed in the alveoli were measured. Mean values of 4 sections from each animal were calculated in pixels^2.

2.7 Immunohistochemistry analysis

We performed immunoexpression so that we could locate and quantify macrophages present in the alveolar bone repair (36-41). Histological sections from 7, 14 and 21 days were deparaffinised following standard procedures. The material was pre-incubated with 3% Hydrogen Peroxidase Block (Spring Bioscience Corporation, CA, USA) and subsequently incubated with 7% NFDM to block serum proteins. The histological sections from of all groups were incubated with anti-CCR2 polyclonal primary antibody (sc-31564) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CCR5 polyclonal (sc-6129) and anti-F4/80 (a pan macrophage marker for mice) polyclonal primary antibodies (sc-26642) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at 1:100 concentrations for 1h at room temperature. The identification of antigen–antibody reaction was performed using 3-3'-diaminobenzidine (DAB) and counter-staining with Mayer's hematoxylin. Positive controls were performed in the mouse spleen for positive leucocytes to CCR2, CCR5 and F4/80 receptors. The analysis of immunolabeled cells was performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100x immersion objective. As the histomorphometric analysis, 13 histological regions were successively analyzed. The total number of immunolabeled cells was obtained to calculate the mean for each section.

2.8 RealTimePCR array reactions

RealTimePCR array reactions were performed as previously described (12). The extraction of total RNA from the remaining alveolus was performed with the RNeasyFFPE kit (Qiagen Inc, Valencia, CA) according to the manufacturers' instructions. The integrity of the RNA samples was verified by analyzing 1 mg of total RNA in a 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions, and the

complementary DNA was synthesized using 3 μg of RNA through a reverse transcription reaction (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA). RealTimePCR array was performed in a Viia7 instrument (LifeTechnologies, Carlsbad, CA) using a custom panel containing targets "Wound Healing" (PAMM-121), "Inflammatory cytokines and receptors"(PAMM-011) and "Osteogenesis" (PAMM-026) (SABiosciences, Frederick, MD) for gene expression profiling. RealTimePCR array data was analyzed by the RT2 profiler PCR Array Data Analysis online software (SABiosciences, Frederick, MD) for normalizing the initial geometric mean of three constitutive genes (GAPDH, ACTB, Hprt1) and subsequently normalized by the control group, as previously described (42). RealTimePCR array analysis was conducted with a pool of samples of all time points (N=4/time pont), and the data is presented as the relative expression/fold change relative to the normalizing control (regular maxillar alveolar bone); as previously described (42).

2.9 Statistical analysis

Differences among data sets were statistically analyzed by One-Way analysis of variance (ANOVA) followed by the Tukey multiple comparison post test or the student's t-test where applicable; for data that did not fit in the distribution of normality, the Mann-Whitney and Kruskal-Wallis (followed by the Dunn's test) tests were used. The statistical significance of the experiment involving the PCR Array was evaluated by the Mann-Whitney test, and the values tested for correction by the Benjamini—Hochberg Procedure (43). Values of $p<0.05$ were considered statistically significant. All statistical tests were performed with the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 μCT analysis

Three-dimensional images obteined from the μCT of maxillae demonstrate the evolution of the alveolar bone healing process post-extraction (Fig.1A), being the microtomographic characteristics observed compatible with the the respective experimental periods of 0h, 7, 14 and 21 days, as previously described (12). In agreement with the original description, it is possible to observe the evolution of bone healing over time, with some variation between them the microarquiteture bone characteristics in the different groups (Fig.1B). The total volume (TV) did not present statistical differences between the groups during the different periods. However, in the bone volume (BV) and bone volume fraction (BV/TV) it was observed a progressive increase over experimental times in the non-treated

groups (WT-C; CCR5KO) whereas in the treated groups (WTantiCCR2; CCR5KOantiCCR2) there is a decrease in the 21 days, with statistical difference between these and 7 days ($p<0.05$WTantiCCR2vsCCR5KOantiCCR2). Also, in the WTantiCCR2 group there was a increase in the number of trabecular (Th.N), decreasing on day 21 with difference between the groups treated at 7 and 14 days. Regarding the trabecular thickness parameter (Tb.Th), the WT groups treated or not (WTantiCCR2; WT-C) showed an increase between the periods while in the CCR5KO groups treated or not (CCR5KOantiCCR2; CCR5KO) a decrease in the 21 days, resulting in the difference statistic between WTantiCCR2 and CCR5KOantiCCR2 at 21 days. These results formed similar but inversely proportional in the trabecular separation parameter (Th.Sp), with difference between the groups treated at 7days, apart from the difference at 14 days when comparing CCR5KOantiCCR2 with WT-C and WTantiCCR2.

3.2 Histological and histomorphometric analyses

The histological and histomorphometric analysis of the alveolar repair in WT mice is in agreement with previous description, demonstrating that the bone repair progresses following the classic events sequence. When blocking CCR2 and CCR5 receptors (individually or simultaneously), some morphometric and quantitative differences were observed (Fig.2A).

Histomorphometric analysis (quantitative) (Fig.2B) of the mice of the different groups/periods allowed the systematic quantification by means of area density calculation (%), so that we could carry out a comparative analyses of the events occurred. Immediately after tooth extraction (0h) the alveolus is filled by blood clot that was smaller in WT-C mice ($p<0.05$) when compaedwith WTantiCCR2 and CCR5KO. Similar results, however inversely proportional, were observed in the other structures parameters due to the voids that interposes to the clot and inflammatory cells present. At 7days, it was observed the presence of granulation tissue with characteristic presence of angiogenesis, an intense inflammatory cell infiltrate and immature connective tissue. CCR5KO and CCR5KOantiCCR2 mice showed a significant increase in fiber volume density compared to WT-C and WTantiCCR2 mice ($p<0.05$). Emphasizing a greater neo-formation of bone tissue in the animals of the WTantiCCR2 group ($p<0.05$WTantiCCR2vsCCR5KO in 7days) and an inverse result of fiber density present in the socket. Also at 7 days, we can observe that the density of osteoblasts decreases, over the respective periods, in the WTantiCCR2, WT-C, CCR5KOantiCCR2 and CCR5KO groups, with the difference ($p<0.05$) between WTvsCCR5KO, WTantiCCR2vsCCR5KO and WTantiCCR2vsCCR5KOantiCCR2. At 14 days a sequential

decrease in the density of the fibers and fibroblasts, parallel to the greater density of bone matrix occurs and a lower amount of osteoblasts. With emphasis on the fibroblast density difference WTantiCCR2 vs CCR5KOantiCCR2 ($p<0.05$) and between the CCR5KOvsWTantiCCR2 groups in relation to osteoblast density. Still at 14 days, it was observed a significant number of osteoclasts in all experimental groups, specially in CCR5KOantiCCR2 group. Density of inflammatory cells presented a statistically significant difference between CCR5KOantiCCR2 with WT-C and CCR5KO ($p <0.05$ at 14 days). In the last period to be analyzed, at 21 days we can observe a lower density of fibroblast in relation to the period of 14 days ($p<0.05$ in WT-C), except in the group CCR5KOantiCCR2, resulting in a statistical difference ($p<0.05$) when comparing this group with CCR5KO and WT-C. In relation to the blood vessels parameter WTantiCCR2 presented higher vessel density ($p<0,05$WTantiCCR2vsCCR5KO and WTantiCCR2vsCCR5KOantiCCR2). The osteoblast density is statistically different between CCR5KO vs CCR5KOantiCCR2 and WTantiCCR2vsCCR5KOantiCCR2 ($p<0.05$), in the case of osteoclasts, the difference is between CCR5KOvsCCR5KOantiCCR2 ($p<0.05$).

In the birefringence analysis (Fig.3), in the 0h period it was observed a remnant of periodontal ligament fibers, whose birefringence is probably greenish due to the predominant thinner type III collagen constitution. The replacement of immature fibers progressively occurs over time in parallel with bone tissue formation. At 7 days, despite the presence of red and yellow fibers (type I collagen) there is a greater predominance of greenish fibers, the main component of immature connective tissues, and progressively these greenish fibers decreased and were replaced by red fibers, which increased at 14 and 21 days, composing the mature bone matrix. Although there is no statistically significant difference when comparing the different groups with each other during the same experimental period, we can observe than in 7 days there is a greater amount of greenish fibers, with less organization and thickness, in the WT groups treated or not. In the subsequent periods in which there is a greater maturation and organization of the fibers occurs in the CCR5KO group while the inverse we can be noticed in CC5antiCCR2 (Fig.3B).

3.3 Immunohistochemistry of F4/80+, CCR2+ and CCR5+ cells throughout alveolar bone healing in mice

In the view of the of the potential involvement of CCR2 and CCR5 receptors in the migration of monocytes/macrophages into sites of inflammation, we used immunohistochemistry to confirm the presence CCR5+ cells on the site of alveolar bone

healing at different time points (7,14 and 21 days) post tooth extraction, and the presence of CCR2+ and F4/80+ cells in WT and CCR5KO mice (Fig.4D-F). During the early inflammatory phase (7days), CCR5+ and F4/80+ cells, except for CCR5KOantiCCR2, presented a peak in the granulation tissue and inflammatory infiltrate in the socket of C57BL/6 mice. At day 14, F4/80+, CCR2+ and CCR5+ cells were found in permeating the connective tissue surrounding bone formation areas, while at day 21 these cells were found predominantly in the bone marrow and surrounding blood vessels, with an emphasis on the peak of CCR2+ cells in 21 days in all groups.

3.4 Molecular analysis using PCRArray

Molecular analysis of the gene expression patterns in the alveolar bone healing of WT and CCR5KO mice, treated with or not with the CCR2 antagonist, was performed by RealTimePCR array with a pool of samples from all experimental time periods. The resulst demonstrate a differential gene expression of several molecules, such growth factors (BMPs, TGFβ, VEGF and FGF1), extracellular matrix markers (COL1a1, COL1a2 and MMPs), bone markers (RUNX2, DMP1, RANKL and OPG) and cytokines (IL-1B, IL-6, TNF and IL-10) within the different groups, when compared with the normalizing control (normal alveloar maxillary bone) (Fig.5).When the relative expression of the experimental groups, it was observed that in the growth factors group, there is a higher expression of of TGFβ mRNA in WT-C and lower expression in CCR5KOantiCCR2 ($p<0.05$). BMPs are more expressed in the CCR5KOantiCCR2 group, both BMP2 and BMP4 being equally expressed in CCR5KOantiCCR2 and WT-C with difference of these groups when compared to CCR5KO ($p<0.05$ in CCR5CASvsCCR5KO and WT-CvsCCR5KO). The angiogenic factor VEGF has a higher expression in WTantiCCR2 followed by WT-C, with no difference between CCR5KO and CCR5KOantiCCR2. In contrast, the growth factor FGF1 was found to be upregulated in the CCR5KOantiCCR2 group. Among the extracellular matrix and bone markers evaluated, the early expression of the bone formation marker RUNX2 mRNA was higher expression in WT-C ($p<0.05$), while the expression of COL1A2 is smaller in WTantiCCR2 determining statistical difference between WTantiCCR2 and the other groups ($p<0.05$). Regarding the MMPs expression, MMP1 and MMP2 are less expressed in WT-C, and a higher expression of MMP9 mRNA was observed in the treated groups. The late bone formation maker DMP1 presented a similar pattern of expression, and RANKL is also expressed in higher levels in treated and non-treated CCR5KO groups ($p<0.05$). Considering the mRNA expression of cytokines, the expresion of the proinflammatory cytokines IL-1b do not show any difference

between the groups, TNF levels are higher in WT-C when compared with the experimental groups, whereas IL-6 mRNA levels are higher in the WTantiCCR2 and CCR5KOantiCCR2 ($p<0.05$). IL-10, an anti-inflammatory cytokine, is more expressed in CCR5KO and less expressed in CCR5KOantiCCR2 group ($p<0.05$).

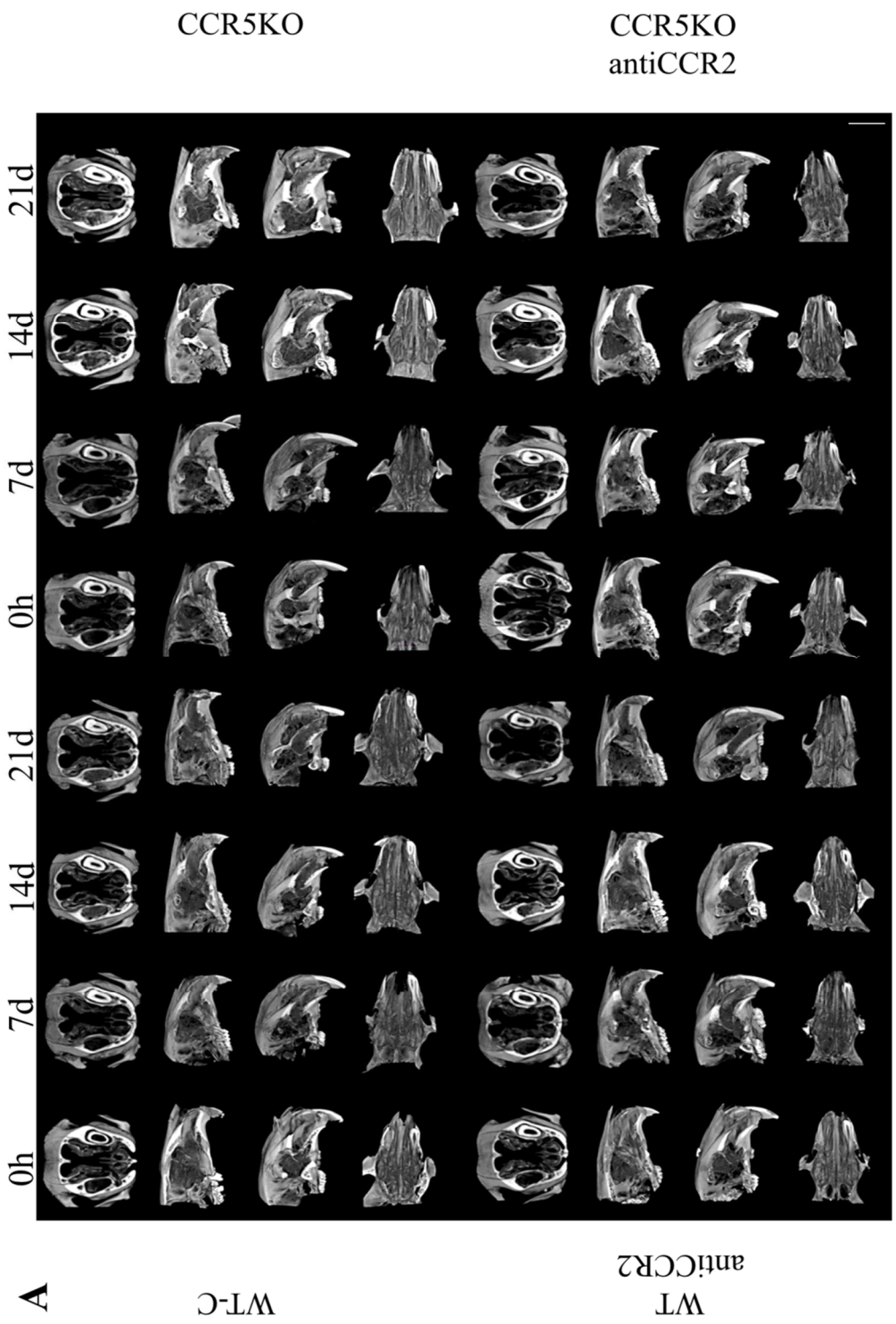

Fig.1 Micro-computed tomography (μCT) analysis of bone healing process among WT-C, CCR5KO, WTantiCCR2 and CCR5KOantiCCR2, at 0,7,14 and 21 days post tooth extraction. (A) At 0 hours and 7 days the socket is occupied by clot and the presence of a granulation tissue, respectively, determining a hypodense image delimited by hyperdense regions are corresponding to the medial wall of the socket, in all groups. At 14 and 21 days, the bone mineralization is evidenced by hyperdense images inside the socket. From top to bottom, the sectioned maxilla are represented at the transverse (horizontal); sagittal; sagittal with inclination and transaxial planes.

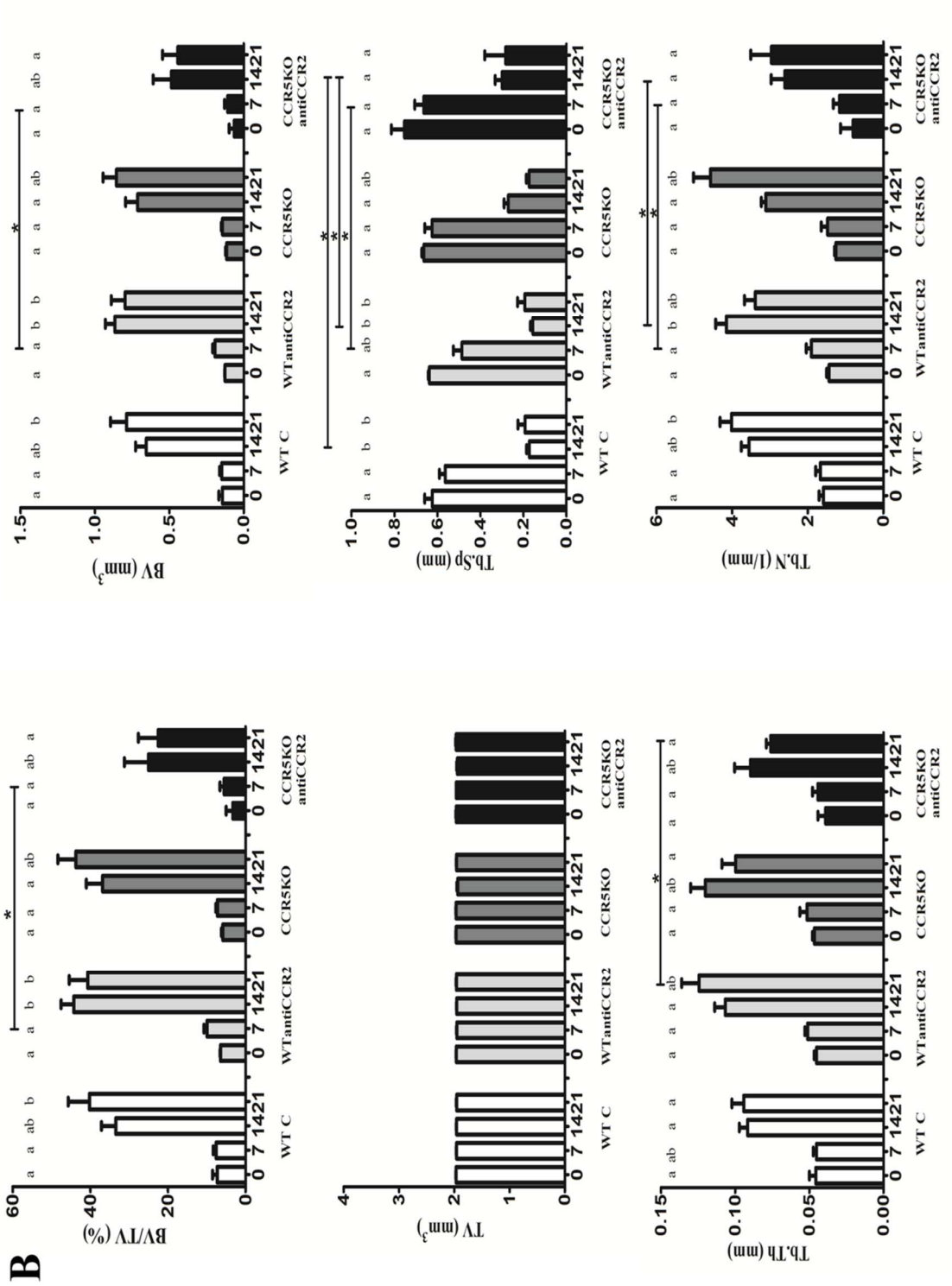

Fig.1 Micro-computed tomography (μCT) analysis of bone healing process among WT-C, CCR5KO, WTantiCCR2 and CCR5KOantiCCR2, at 0,7,14 and 21 days post tooth extraction. (B) The bone analysis is corresponding: Percent bone volume (%), Bone volume (mm3), Tissue volume (mm3), Trabecular thickness (mm), Trabecular number (1/mm), Trabecular separation (mm). The results represent the average values and standard deviation in each of the periods analyzed. * ($P < 0.05$) indicates a statistically significant difference between the different experimental groups within the same period and lowercase letters represent statistically significant difference ($p < 0.05$) between different periods within the same group.

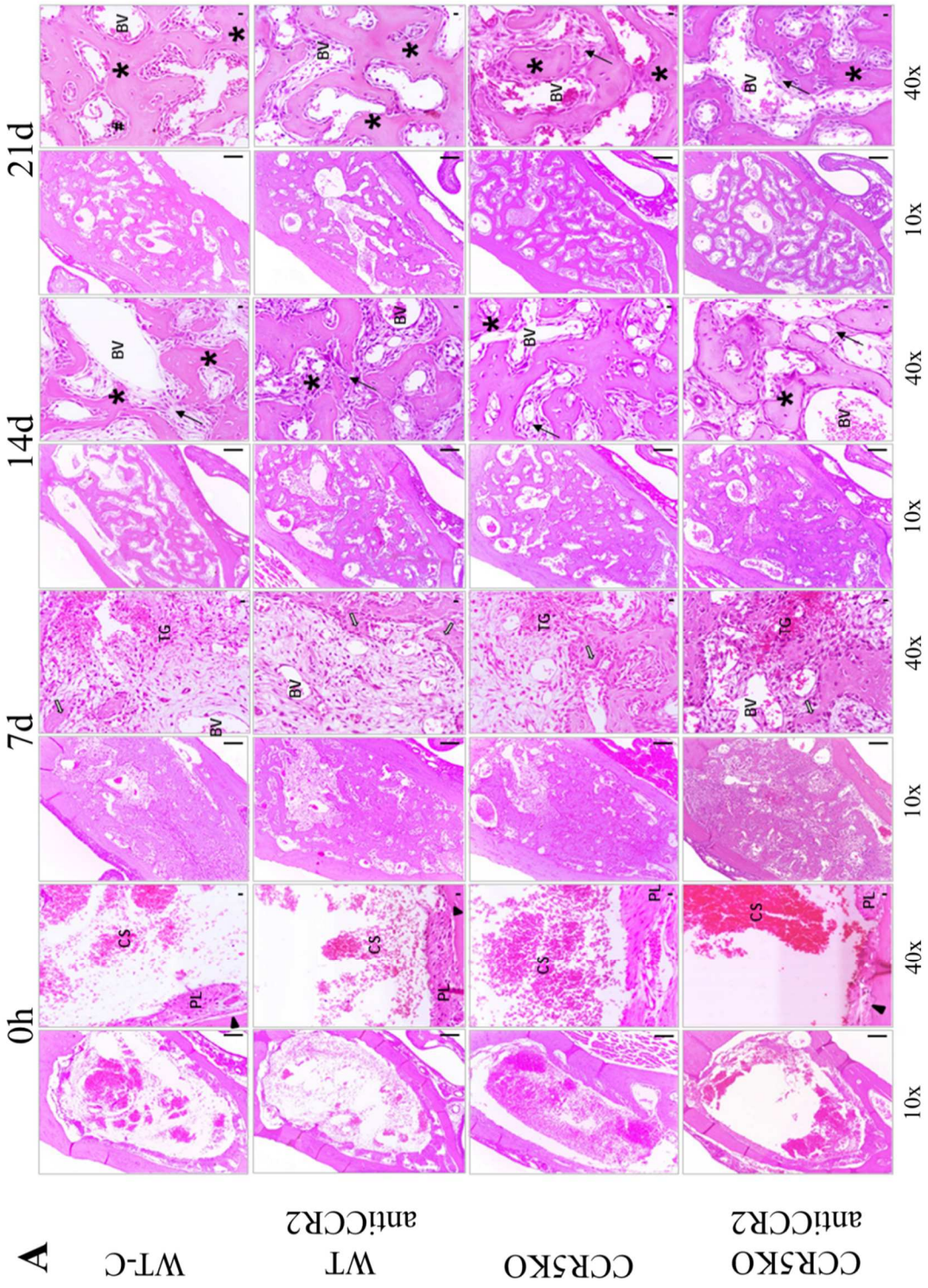

Fig.2 Histological description of the bone repair process among WT-C, WTantiCCR2, CCR5KO and CCR5KOantiCCR2 at 0,7,14 and 21 days post tooth extraction.
(A) At 0 hour, the socket is occupied by blood clot (BC), it is surrounded by residual cortical bone (closed arrowheads) and with presence of the periodontal ligament (PL). At 7 days, there are granulation tissues (GT) and early new bone formation (open arrow). At 14 days, the persistence of connective tissue to 14days (thin arrow) and the presence of newly formed trabecular bone (*) at 14 and 21 days. Bone marrow (#) in 21 days. HE staining; objective of 10x and 40x.

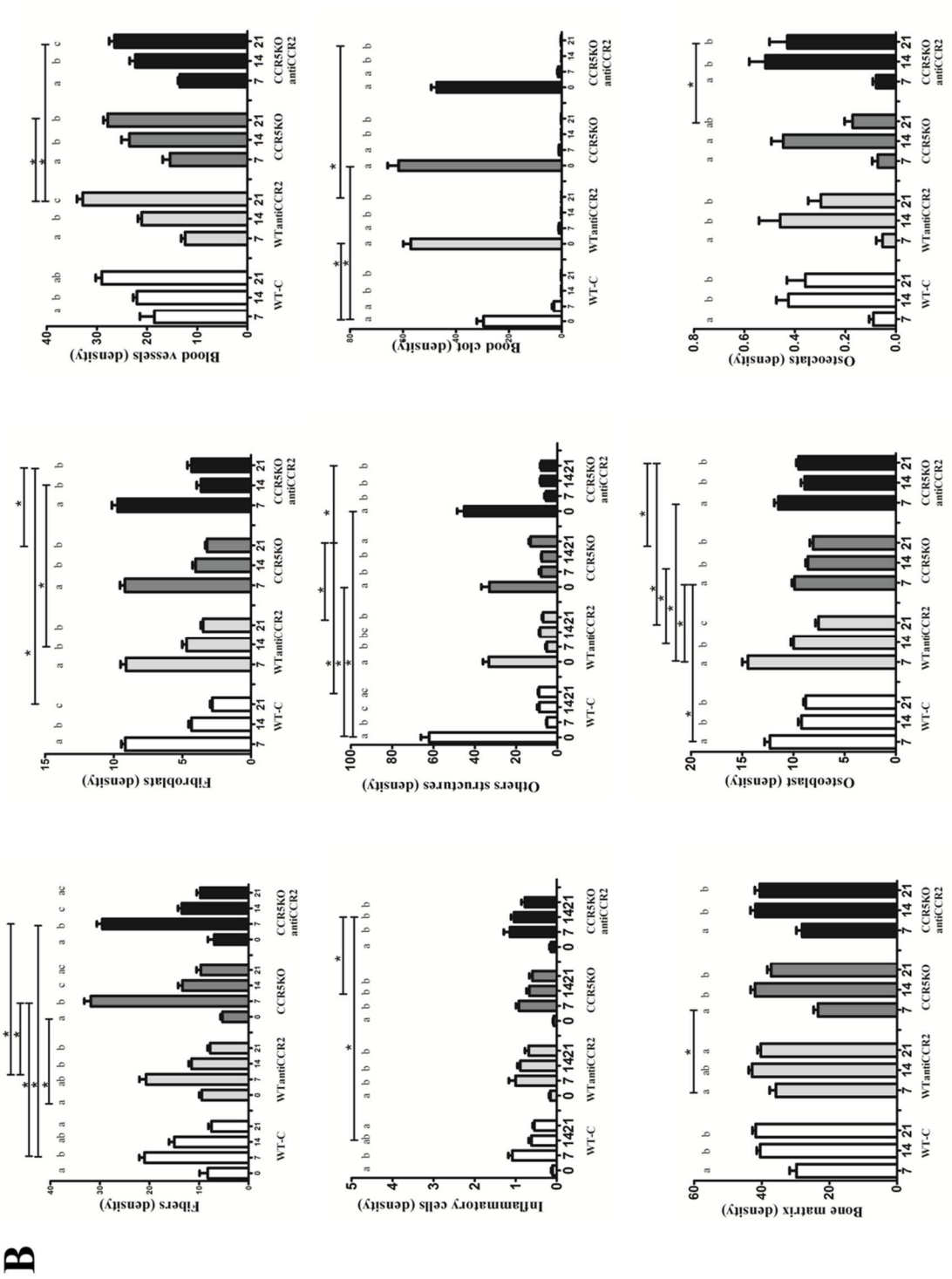

Fig.2 Histomorphometric analysis of the bone repair process among WT-C, WTantiCCR2, CCR5KO and CCR5KOantiCCR2 at 0,7,14 and 21 days post tooth extraction.
(B) Histomorphometric analysis of the total areal density (%) corresponding: Collagen fibers, Fibroblasts, Blood vessels, Inflammatory cells, Other structures, Bone matrix, Osteoblasts, Osteoclasts and Blood clot present in the dental socket. The results represent the values of the mean and standard deviation of the reporting period. * ($P < 0.05$) indicates a statistically significant difference between the different experimental groups within the same period and lowercase letters represent statistically significant difference ($p < 0.05$) between different periods within the same group.

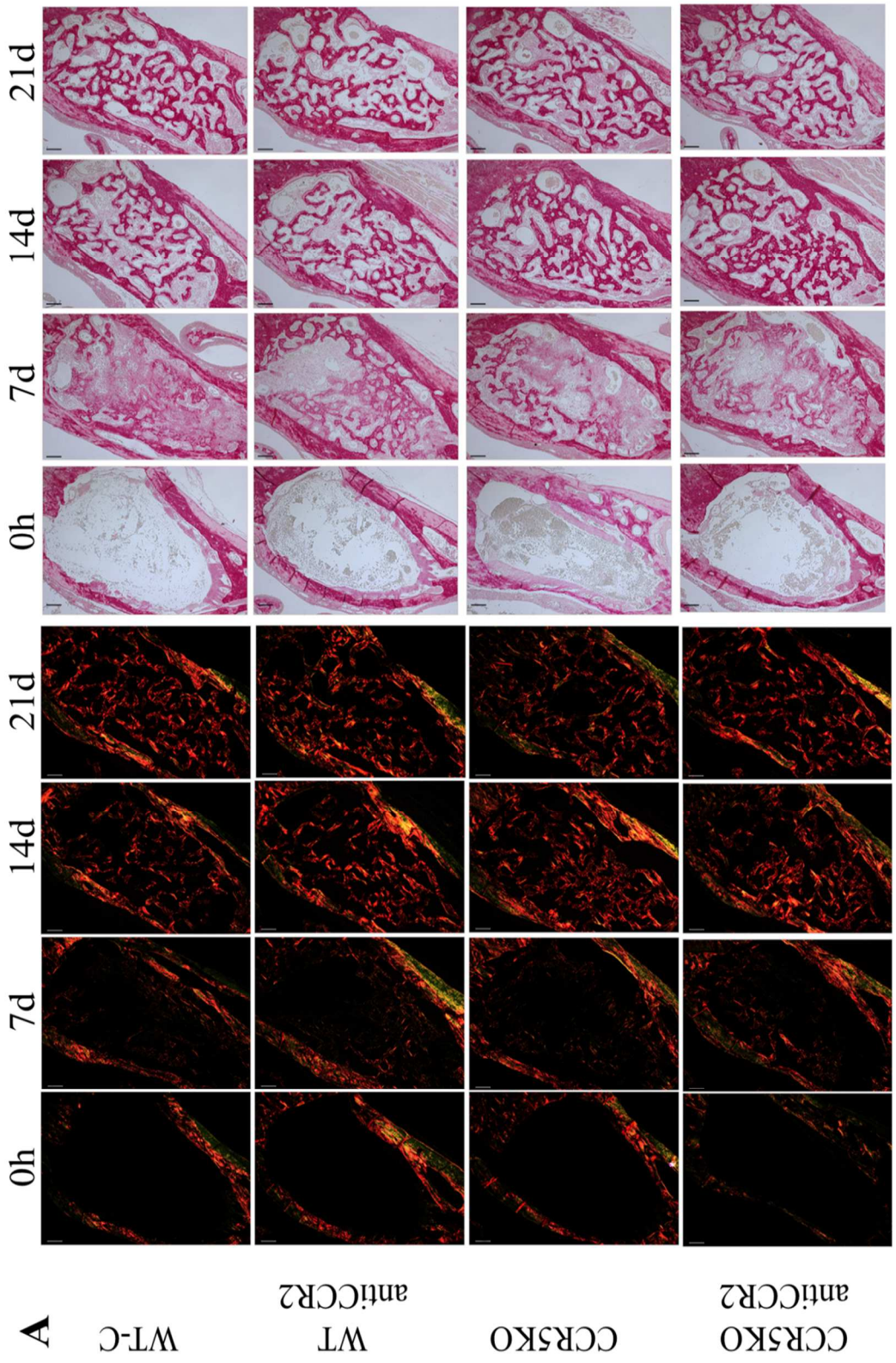

Fig.3 Birefringence analysis of collagen fibers in the alveolus of WT-C, WTantiCCR2, CCR5KO and CCR5KOantiCCR2 animals in the 0h, 7, 14 and 21 days post-exodontia periods.
(A) Photomicrographs are representative of the middle region of dental alveolus, captured under conventional light and polarized light. Picrosirius red coloration; objective of 10x.

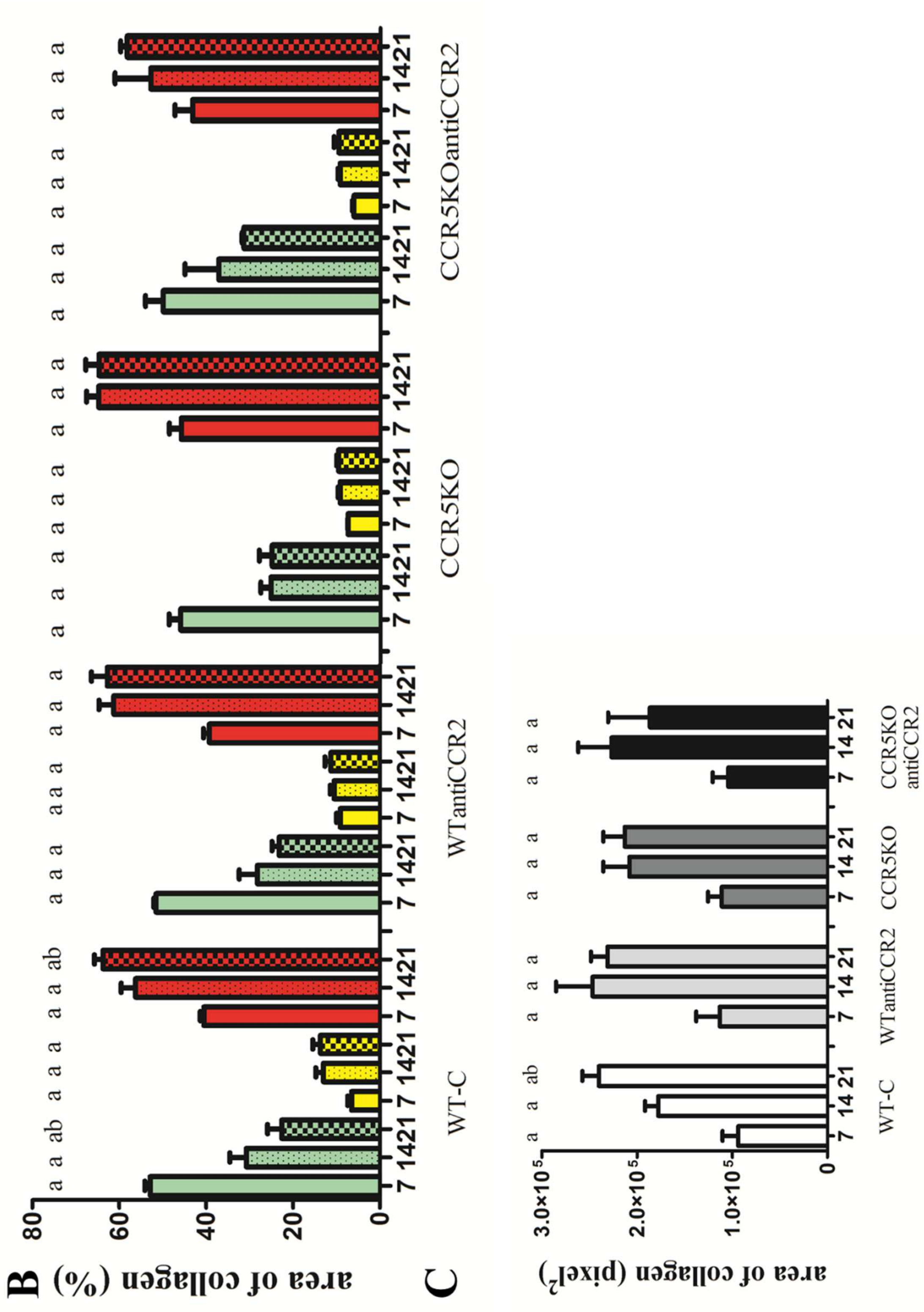

Fig.3 Birefringence analysis of collagen fibers in the alveolus of WT-C, WTantiCCR2, CCR5KO and CCR5KOantiCCR2 animals in the 0h, 7, 14 and 21 days post-exodontia periods.

(B) Area of collagen from each birefringence color (%) and (C) total area of collagen fibers (pixel^2) and Results are presented as the mean (±SEM) of percentage or pixels^2 for each color in the birefringence. Lowercase letters represent statistically significant difference ($p < 0.05$) between different periods within the same group.

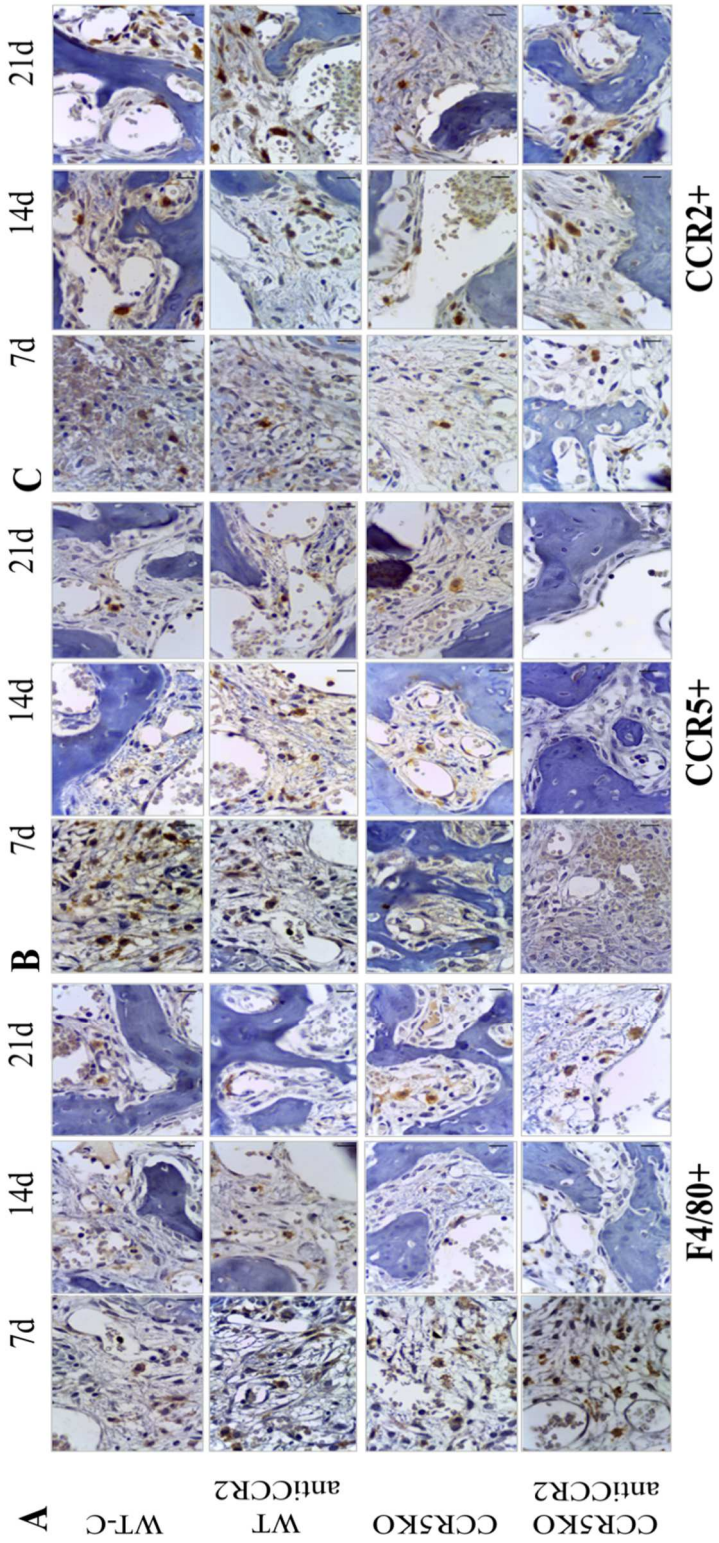

Fig.4 Immunohistochemistry description for (A) F4/80+, (B) CCR5+ and (C) CCR2+ cells present in the bone repair process in the WT-C, WTantiCCR2, CCR5KO and CCR5KOantiCCR2 groups at 7,14 and 21 days after dental extraction. At 7 days, we observed a predominance of marked cells with decrease in the subsequent periods of 14 and 21 days, with the exception of the immunolabeling performed with CCR2 where we can observe a considerable amount of positive cells in 21 days. Indirect staining MACH4+DAB, anti-staining Mayer hematoxylin; objective of 100x.

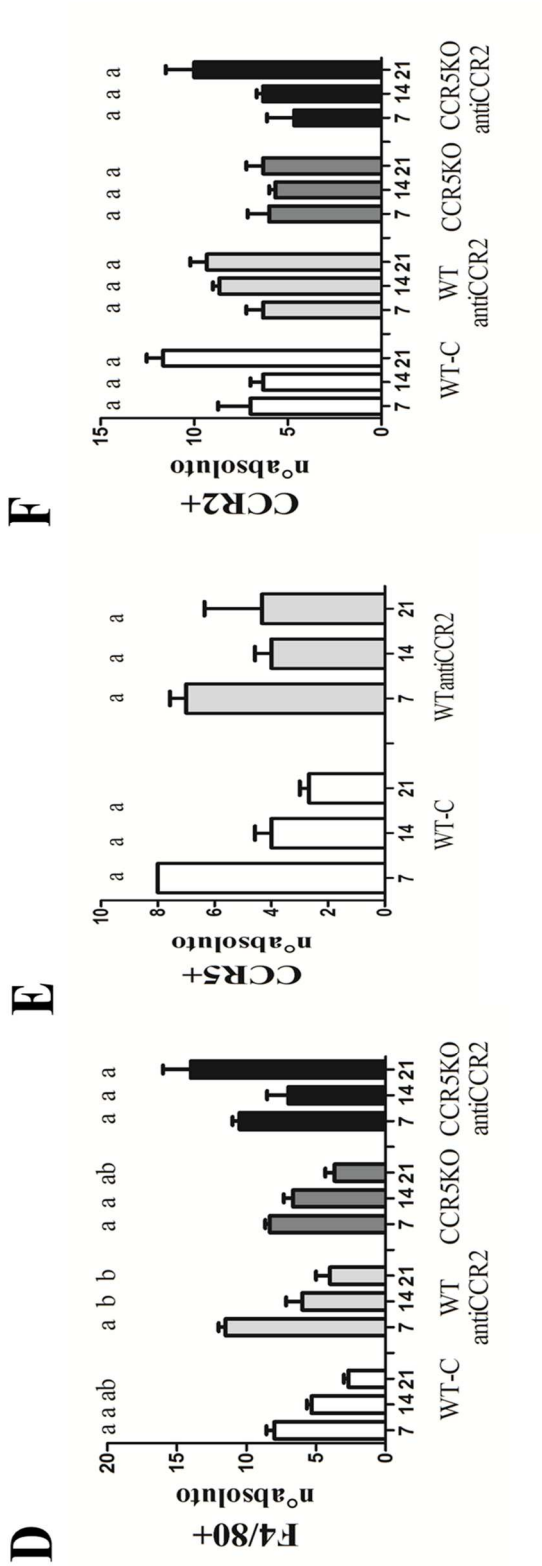

Fig.4 Immunohistochemistry quantification of the absolute number is corresponding:
(D) F4/80+ immunolabelled with anti-F4/80 (E) CCR5+ immunolabelled with anti-CCR5 (F) CCR2+ immunolabelled with anti-CCR2. * ($P < 0.05$) indicates a statistically significant difference between the different experimental groups within the same period and lowercase letters represent statistically significant difference ($p < 0.05$) between different periods within the same group.

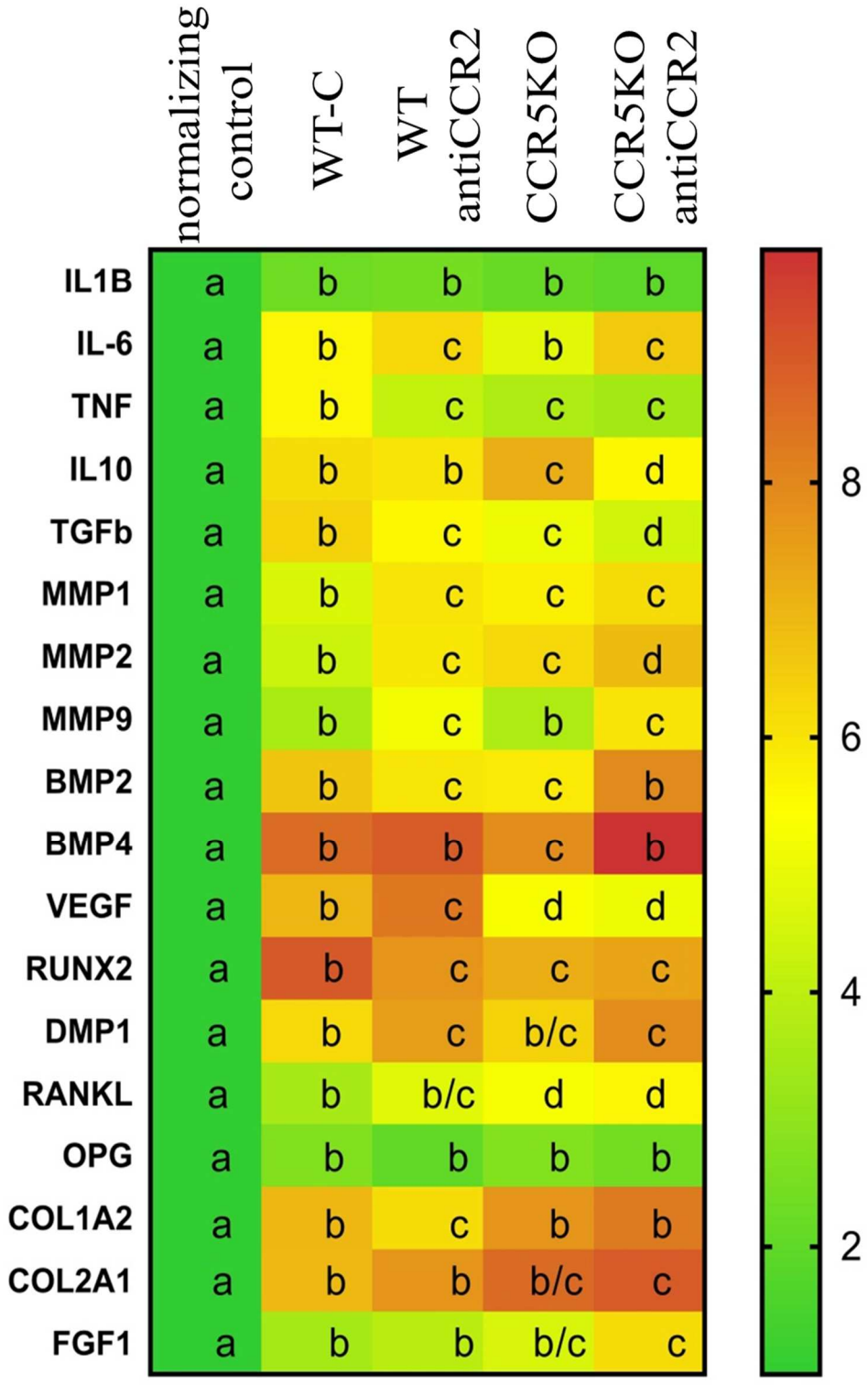

Fig.5 Molecular analysis (PCRArray) using Heat map to quantify the expression of the growth factors (BMPs, TGFβ, VEGF and FGF1), extracellular matrix markers (COL1a1, COL1a2 and MMPs), bone markers (RUNX2, DMP1, RANKL and OPG) and cytokines markers (IL-1B, IL-6, IL-10 and TNF) in bone healing process among WT-C, CCR5KO, WTantiCCR2 and CCR5KOantiCCR2, post tooth extraction. Results were obtained when comparing the relative expression of the different groups to the normalizing control, thus, lowercase letters represent statistically significant difference ($p<0.05$) between indicated groups.

4. Discussion

Macrophages are among the first immune cells required to trigger and modulate the inflammatory response, and their initial recruitment from circulation into injured tissues is an essential initial event for a proper tissue healing. Macrophage chemmoatraction to different tissues in inflammatory conditions can be mediated by distinct chemokine receptors, being inflammatory macrophages characterized by CCR2 and CCR5 expression (7,17,18,44). In this study, we performed a comparative characterization of the intramembranous alveolar bone healing post tooth extraction in CCR5KO and C57Bl/6-WT mice, treated or not with a CCR2 antagonist (RS504393), in order to investigate the role of CCR2 and CCR5, individually or simultaneously.

Our results initially demonstrate the presence of CCR5+ and F4/80+ (a common marker for murine macrophages) cells in bone repair sites of WT-C mice, with a peak at 7 days time point. Regarding CCR2+ cells, a different pattern was observed, since the immunolabeling was greater at 21 days time pointe, being the immunolabeling evident in mesenchymal stem cells in the bone marrow region (45) (Fig.4F) When WT-C group was compared with the experimental groups, during the the inflammatory stage (7 days) it is possible to observe that the influx of macrophages CCR2+ and CCR5+ was higher in WT control animals in comparison to the other groups (24,46-50) (Fig.4E,F). However, inverse results were observed when quantifying F4/80+ macrophages, since WT-C group presented the the smallest number of F4/80+ cells, being this data divergent from previous studies results, where both CCR2 and CCR5 receptor blockade resulted in a decrease of F4/80+ cell (19,51) (Fig.4D). Therefore, we assume that, due to the increased F4/80+ cells counts, there may be a distinct subpopulation of macrophages that migrates in a CCR2 and CCR5 independent way. Indeed, studies demonstrate the presence of other chemokine receptors in monocytes/macrophages, which could be responsible for this migration (44).

Subsequently the analysis of the presence of macrophages in the alveolar bone repair, we investigated if CCR2 and/or CCR5 deficiency, individually or simultaneously, resulted into modifications of the subsequent bone healing stages. Initially, it is important to emphasize that the analyzes (uCT, histomorphometric and birefringence) performed on the WT-C animals resulted in data similar to that previously described (12,52,53). When WT-C group was compared with the experimental groups, in the uCT analysis, analysis, the CCR5KO group presented an increase in bone volume in 14 and 21 days, but without statistical difference (54). While, WTantiCCR2 group, with an increase in 7, 14 days (22) (Fig.1B).

The histomorphometric analysis, focused specificallyin the density of the area occupied by collagenous fibers (Fig.2B), demonstrate that the WTantiCCR2 group collagen fibers quantification was lower in comparison to the other groups in the periods of 7 and 14 days. This finding may be a result of the CCR2 blockade promoted by the use of RS504393, since this same antagonist was descrived to promote an improvement in kidney fibrosis, evidenced by extensive areas of type I collagen in WT mice after the unilateral ureteral obstruction model, but fibrosis was lower in CCR2KO mice. This finding is also in agreement with the lowest percentage of red birefringence fibers (type I collagen) at 7 days in WTantiCCR2 animals (Fig.3B) (19).

Regarding the bone cell analysis, osteoblasts may express the CCR5 receptor (55,56),coinciding with the lower density of osteoblasts in CCR5KO mice at 7 and 14 days (Fig.2B). Regarding osteoclast density, there are also possible correlations with the presence of both the CCR2 receptor and CCR5 (8,57,58), so we assume that blocking the receptors individually or simultaneously would reduce the density of osteoclasts, which was only observed in the 7 day period, with the WT-C group presenting a greater amount of osteoclasts, but at 14 days, the period of greater resorption activity, WT-C had the lowest amount of osteoclasts compared to the other groups, specially in the CCR5KOantiCCR2 group where the two receptors were simultaneously disabled (Fig.2B). In regards to the bone matrix density, it was observed the same pattern in relation to the bone volume (BV) in WT animals that received CCR2 antagonist (22), and also in the CCR5KO group, that again did not present significant differences with WT–C (54), although CCR5KO presented in the histomorphometric analysis lower density of bone matrix (Fig.2B). Thus, bone matrix density in the CCR5KOantiCCR2 mice is a compensation for the lower bone formation in the CCR5KO animals with administration of the CCR2 antagonist.

Finally, it is worth emphasizing that the bone healing is a complex and coordinated process that involves numerous mediators and cell types that perform functions along each healing step, this process has also been previously described in C57B1/6 WT animals (12). Thus, when focusing on the simultaneous inhibition of CCR2 and CCR5, in the CCR5KOantiCCR2 group, there is a significantly higher mRNA expression of extracellular matrix markers (COL1A2 and MMP9), bone markers (DMP1 and RANKL) and IL-6 when compared to WT-C (Fig.5). In contrast, the expression of growth factors (TGFb1 and VEGF), as the key osteogenic differentiation transcription facgtor RUNX-2 and and the cytokines of opposing classes, the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine TNF-α were downregulated when compared to the WT-C group. Although it is possible to

observe multiple differences at molecular level derived from the simultaneous inhibition of CCR2 and CCR5, in the view of our uCT and histological/histomorphometric data, it is reasonable to consider that such variations were not sufficient to promote significant changes in the healing phenotype. However, it is possible to correlate some significant variation in the mRNA expression with histological/histomorphometric data, in order to dissect possible influences of CCR2 and CCR5 inhibition in specific events of the bone healing cascade. Higher expression of MMP9 mRNA that was higher in the treated WT and CCR5KO groups, we can correlate with the greater immunolabeling of F4/80+ cells in the same groups as well as the higher density of inflammatory cells quantified in the histomorphometry of the CCR5KOantiCCR2 group in 7 days, thus indicating the presence of macrophages or other inflammatory cells compensating for the blockade of CCR2 and CCR5. Since the expression of these matrix remodeling enzymes is relevant in the initial stages of the healing process (12), and has relevance in the migration of inflammatory cells, such as macrophages (59). These data can also be justified by the greater expression of IL-6 in the WT and CCR5 groups treated with the CCR2 antagonist (60-62).

In later periods of bone healing IL-6 and RANKL act to induce osteoclastogenesis, thus it is possible to correlate the greater IL-6 expression in the treated groups as well as the higher expression of RANKL in the CCR5KO and CCR5KOantiCCR2 groups, with the highest osteoclast density in the CCR5KOantiCCR2 group (63,64). In counterpoint to the effect of the simultaneous inhibition of CCR2 and CCR5, now specifically aimed at the performance of molecules expressed in osteogenesis, we can not observe a very well established pattern of expression of BMPs, TGF-b or even RUNX-2 with the density of osteoblasts or bone matrix density, although such a relationship is proven in previous studies (65,66). Another factor of the molecular analysis that deserves to be highlighted is the lower expression ($p<0.05$) of COL1A2 mRNA, gene responsible for the formation of type I collagen, in WTantiCCR2, thus confirming the established correlation between the histomorphometric and birefringence analysis mentioned above.

5.Conclusions

Therefore, we conclude that, although CCR2 and CCR5 receptor blockade result in significant modulation of of growth factors, proinflammatory cytokines and osteoclastogenic factors expression, there are no significant differences in the control of macrophage migration as well as in the subsequent bone repair outcome.

6. Acknowledgements

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3 DISCUSSION

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The function of the bone tissue is not restricted only to the function of the osteoblasts and osteoclasts, cells that make up this tissue, having the influence of a great variety of cells of other systems and the liberation of their products (BORGIANI; DUDA; CHECA, 2017). Macrophages are among the first immune cells required to trigger and modulate the inflammatory response, and their initial recruitment from circulation into injured tissues is an essential initial event for a proper tissue healing. Macrophage chemmoatraction to different tissues in inflammatory conditions can be mediated by distinct chemokine receptors, being inflammatory macrophages characterized by CCR2 and CCR5 expression (MACK et al., 2001; PUENGEL et al., 2017) . In this study, we performed a comparative characterization of the intramembranous alveolar bone healing post tooth extraction in CCR5KO and C57Bl/6-WT mice, treated or not with a CCR2 antagonist (RS504393), in order to investigate the role of CCR2 and CCR5, individually or simultaneously.

Our results initially demonstrate the presence of CCR5+ and F4/80+ (a common marker for murine macrophages) cells in bone repair sites of WT-C mice, with a peak at 7 days time point. Regarding CCR2+ cells, a different pattern was observed, since the immunolabeling was greater at 21 days time pointe, being the immunolabeling evident in mesenchymal stem cells in the bone marrow region (RINGE et al., 2007) (Fig.4F). When WT-C group was compared with the experimental groups, during the the inflammatory stage (7 days) it is possible to observe that the influx of macrophages CCR2+ and CCR5+ was higher in WT control animals in comparison to the other groups (KAWANO et al., 2016; MOSSANEN et al., 2016) (Fig.4E,F). However, inverse results were observed when quantifying F4/80+ macrophages, since WT-C group presented the the smallest number of F4/80+ cells, being this data divergent from previous studies results, where both CCR2 and CCR5 receptor blockade resulted in a decrease of F4/80+ cell (GLASS et al., 2001; KITAGAWA et al., 2004) (Fig.4D). Therefore, we assume that, due to the increased F4/80+ cells counts, there may be a distinct subpopulation of macrophages that migrates in a CCR2 and CCR5 independent way. Indeed, studies demonstrate the presence of other chemokine receptors in monocytes/macrophages, which could be responsible for this migration (MANTOVANI et al., 2004).

Subsequently the analysis of the presence of macrophages in the alveolar bone repair, we investigated if CCR2 and/or CCR5 deficiency, individually or simultaneously, resulted into modifications of the subsequent bone healing stages. Initially, it is important to emphasize that the analyzes (uCT, histomorphometric and birefringence) performed on the WT-C animals resulted in data similar to that previously described (VIEIRA et al., 2015; UMOH et al., 2009; OSORIO et al., 2016). Due to the common lineage between macrophages and osteoclasts, we assume that possible inhibitions on monocyte/macrophages chemotactic would promote changes in the bone tissue (LIU et al., 2014; XUAN et al., 2017). Thus, when comparing the WT-C group, we noticed a greater bone volume of the CCR5KO group, although without statistical difference, indicating that the absence of this receptor did not lead to significant bone changes (TAKEBE et al., 2015) (Fig.1B). In the WTantiCCR2 group, if on the one hand, there is a study that shows a decrease in bone resorption, however, there being no demonstration of what this condition would specifically cause in bone volume (TADDEI et al., 2012b), another study demonstrates by means of a model of osteoarthritis induction with early administration of CCR2 antagonist (RS504393), that there is a decrease in cartilage degradation as well as a protective effect of bone tissue, resulting in the decrease of the size of osteophytes formed, determinants for disease progression (LONGOBARDI et al., 2017). Thus, the insignificant but greater volume of osseous tissue can be correlated according to a model of induction of ovariectomy in mouse CCR2KO, which shows that the inhibition of this receptor affects the maturation and function of osteoclasts, decreasing its function of resorption causing greater resistance to bone loss in these animals (BINDER et al.,2009) (Fig.1B). Although the bone volume parameter demonstrates greater importance on an overview of alveolar bone repair, it is worth noting that other parameters may indicate important data about the quality of this bone, such as Tb.Th (trabeculae thickness) and Tb.N (number of trabecualar) as discritos in the results (Fig.1B).

The histomorphometric analysis, focused specificallyin the density of the area occupied by collagenous fibers (Fig.2B), demonstrate that the WTantiCCR2 group collagen fibers quantification was lower in comparison to the other groups in the periods of 7 and 14 days. This finding may be a result of the CCR2 blockade promoted by the use of RS504393, since this same antagonist was descrived to promote an improvement in kidney fibrosis, evidenced by extensive areas of type I collagen in WT mice after the unilateral ureteral obstruction model, but fibrosis was lower in CCR2KO mice. This finding is also in agreement

with the lowest percentage of red birefringence fibers (type I collagen) at 7 days in WTantiCCR2 animals (Fig.3B) (KITAGAWA et al., 2004).

Regarding the bone cell analysis, osteoblasts may express the CCR5 receptor (DE BOER, 2005; GARLET et al., 2008; BARMANIA; PEPPER, 2013),coinciding with the lower density of osteoblasts in CCR5KO mice at 7 and 14 days (Fig.2B). Regarding osteoclast density, there are also possible correlations with the presence of both the CCR2 receptor and CCR5 (TADDEI et al., 2012b; LIU et al., 2014; XUAN et al., 2017), so we assume that blocking the receptors individually or simultaneously would reduce the density of osteoclasts, which was only observed in the 7 day period, with the WT-C group presenting a greater amount of osteoclasts, but at 14 days, the period of greater resorption activity, WT-C had the lowest amount of osteoclasts compared to the other groups, specially in the CCR5KOantiCCR2 group where the two receptors were simultaneously disabled (Fig.2B). In regards to the bone matrix density, it was observed the same pattern in relation to the bone volume (BV) in WT animals that received CCR2 antagonist (BINDER et al.,2009), and also in the CCR5KO group ,that again did not present significant differences with WT–C (TAKEBE et al., 2015), although CCR5KO presented in the histomorphometric analysis lower density of bone matrix (Fig.2B). Thus, bone matrix density in the CCR5KOantiCCR2 mice is a compensation for the lower bone formation in the CCR5KO animals with administration of the CCR2 antagonist.

Finally, it is worth emphasizing that the bone healing is a complex and coordinated process that involves numerous mediators and cell types that perform functions along each healing step, this process has also been previously described in C57B1/6 WT animals (VIEIRA et al., 2015). Thus, when focusing on the simultaneous inhibition of CCR2 and CCR5, in the CCR5KOantiCCR2 group, there is a significantly higher mRNA expression of extracellular matrix markers (COL2A1 and MMP9), bone markers (DMP1 and RANKL), IL-6 and FGF1 when compared to WT-C (Fig.5). In contrast, the expression of growth factors (TGFb1 and VEGF), as the key osteogenic differentiation transcription facgtor RUNX-2 and and the cytokines of opposing classes, the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine TNF-α were downregulated when compared to the WT-C group. Although it is possible to observe multiple differences at molecular level derived from the simultaneous inhibition of CCR2 and CCR5, in the view of our uCT and histological/histomorphometric data, it is reasonable to consider that such variations were not sufficient to promote significant changes in the healing phenotype. However, it is possible to

correlate some significant variation in the mRNA expression with histological/histomorphometric data, in order to dissect possible influences of CCR2 and CCR5 inhibition in specific events of the bone healing cascade. Higher expression of MMP9 mRNA that was higher in the treated WT and CCR5KO groups, we can correlate with the greater immunolabeling of F4/80+ cells in the same groups as well as the higher density of inflammatory cells quantified in the histomorphometry of the CCR5KOantiCCR2 group in 7 days, thus indicating the presence of macrophages or other inflammatory cells compensating for the blockade of CCR2 and CCR5. Since the expression of these matrix remodeling enzymes is relevant in the initial stages of the healing process (VIEIRA et al., 2015), and has relevance in the migration of inflammatory cells, such as macrophages (WANG et al., 2013). These data can also be justified by the greater expression of IL-6 in the WT and CCR5 groups treated with the CCR2 antagonist (RECKNAGEL et al., 2013; TAKIZAWA; MANZ, 2017; PARK, 2017).

In later periods of bone healing IL-6 and RANKL act to induce osteoclastogenesis, thus it is possible to correlate the greater IL-6 expression in the treated groups as well as the higher expression of RANKL in the CCR5KO and CCR5KOantiCCR2 groups, with the highest osteoclast density in the CCR5KOantiCCR2 group (DE BENEDETTI et al., 2006; GINALDI; MARTINIS, 2016). In counterpoint to the effect of the simultaneous inhibition of CCR2 and CCR5, now specifically aimed at the performance of molecules expressed in osteogenesis, we can not observe a very well established pattern of expression of BMPs, TGF-b or even RUNX-2 with the density of osteoblasts or bone matrix density, although such a relationship is proven in previous studies (NUNTANARANONT; SUTTAPREYASRI; VONGVATCHARANON, 2014; GRUBER et al., 2006). Another factor of the molecular analysis that deserves to be highlighted is the lower expression ($p<0.05$) of COL1A2 mRNA, gene responsible for the formation of type I collagen, in WTantiCCR2, thus confirming the established correlation between the histomorphometric and birefringence analysis mentioned above.

4 CONCLUSIONS

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Therefore, we conclude that, although CCR2 and CCR5 receptor blockade result in significant modulation of of growth factors, proinflammatory cytokines and osteoclastogenic factors expression, there are no significant differences in the control of macrophage migration as well as in the subsequent bone repair outcome.

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ANNEX

ANNEXS

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 012/2015

Bauru, 12 de agosto de 2015.

Senhor Professor,

Informamos que o projeto de pesquisa intitulado "***Análise microtomográfica, histológica e molecular do papel do receptor CCR5, e do possível papel cooperativo do receptor CCR2, na migração de macrófagos e seu impacto no processo de reparo alveolar pós-exodontia em camundongos",*** que envolve a utilização de animais (roedores), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA), em reunião realizada no dia **31 de julho de 2015**.

Esta comissão solicita que ao final da pesquisa seja enviado relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Vigência do projeto:	*Agosto/2015 a Julho/2017*
Espécie/Linhagem:	*Camundongo isogênico C57Bl/6 / CCR5KO*
Nº de animais:	*n = 144 (72 para cada espécie/linhagem)*
Peso/Idade	*25 g/8 semanas*
Sexo:	*Macho*
Origem:	*Biotério de criação da FOB-USP (WT) e FMRP-USP (Knockout)*

Atenciosamente,

Profª Drª Ana Paula Campanelli

Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Prof. Dr. Gustavo Pompermaier Garlet
Docente do Departamento de Ciências Biológicas

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