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Journal of Cellular Biochemistry 117:382–389 (2016)

Journal of Cellular Biochemistry

CCL2 and CCR2 are Essential for the Formation of Osteoclasts and Foreign Body Giant Cells

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ABSTRACT

Osteoclasts are multinucleated cells responsible for bone resorption. They are derived from the fusion of cells in the monocyte/macrophage lineage. Monocytes and macrophages can also fuse to form foreign body giant cells (FBGC). Foreign body giant cells are observed at the interface between a host and a foreign body such as implants during a foreign body reaction. Macrophages are attracted to the site of bone resorption and foreign body reactions by different cytokines. Chemokine (C-C) ligand-2 (CCL2) is an important chemotactic factor and binds to a receptor CCR2. In this study we investigated the importance of CCL2 and the receptor CCR2 in the formation of osteoclasts and FBGC. CCL2 mRNA was more highly expressed in giant cell culture than macrophages, being 9-fold and 16-fold more abundant in osteoclasts and FBGC respectively. Significantly fewer osteoclasts and FBGC were cultured from the bone marrow of CCL2 and CCR2 knockout mice, when compared to wild type. Not only were the number of giant cells reduced but there was a significant reduction in the number of nuclei and the size of these cells in the cultures of CCL2 and CCR2 knockout mice. Formation of osteoclasts and FBGC were recovered in cultures by addition of exogenous CCL2 to the media containing marrow cells from CCL2-/- mice. We conclude that CCL2 and its receptor CCR2 are important for the formation of osteoclasts and FBGC and absence of these genes causes inhibition of osteoclast and FBGC formation. J. Cell. Biochem. 117: 382–389, 2016.

KEY WORDS: OSTEOCLASTS; CELL SIGNALLING; CELL DIFFERENTIATION; CHEMOKINE; MACROPHAGE; CCL2; CCR2

Osteoclasts are derived from the fusion of monocyte/macrophage lineage [Takami, 2011] and are responsible for bone homeostasis [Idris et al., 2008]. Since osteoclasts are the cells responsible for bone resorption, the control of osteoclast function and differentiation is critical in diseases such as rheumatoid arthritis [Schett, 2007], multiple myeloma and bone metastasis [Roodman, 2007; Zhu et al., 2007]. In the presence of a foreign body, macrophages may also undergo fusion and give rise to multinucleated cells, commonly known as foreign body giant cells (FBGC) [Anderson et al., 1999]. These cells are involved in secreting enzymes to degrade biomaterial [Luttikhuizen et al., 2006], cytokines for inflammation [Miller et al., 1989] and wound healing [Gretzer et al., 2006]. During foreign body reaction and osteolytic conditions, macrophages are attracted to the target site by different agents. One important chemotactic factor for macrophages is chemokine (C-C) ligand-2 (CCL2), also called monocyte chemotactic protein 1 (MCP1) [Uguccioni et al., 1995]. CCL2 belongs to the CC chemokine family and binds to its primary receptor CCR2 [Yoshimura and Leonard, 1991]. CCR2 is an important receptor for CCL2 [Boring et al., 1996] and blocking CCL2 and CCR2 disrupts macrophage function [Kurihara et al., 1997]. Expression of CCL2 stimulates osteoclastogenesis [Miyamoto et al., 2009] and FBGC formation, [Kyriakides et al., 2004] while absence of CCL2 leads to decreased bone resorption [Sul et al., 2012]. CCL2 binds to its receptor CCR2, which is expressed by many hematopoietic stem cells [Boring et al., 1997]. Some chemokines signal through more than one receptor. In the case of CCR2, MCP3 (or CCL7) also acts through CCR2 [Charo et al., 1994; Jia et al., 2008]. CCL2 and CCR2 interaction is evidently related to osseous inflammation [Rahimi et al., 1995]. CCL2 induction is also correlated with monocyte recruitment during normal bone growth, while CCR2 is associated with monocyte

Grant sponsor: National Health and Medical Research Council; Grant sponsor: Griffith University.

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Manuscript Received: 16 July 2015; Manuscript Accepted: 20 July 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 23 July 2015

DOI 10.1002/jcb.25282 • © 2015 Wiley Periodicals, Inc.

mobilization to the bone formation and resorption areas [Volejnikova et al., 1997].

This study aimed to identify the role of CCL2 during osteoclast and FBGC formation and the effect of removing CCL2 and its receptor CCR2 on the formation of osteoclasts and FBGC. We hypothesized that CCL2 and its receptor CCR2 are important for osteoclast and FBGC formation and removal of CCL2 and CCR2 will cause reduction in the formation of osteoclasts and FBGC.

MATERIALS AND METHODS

ANIMALS

Wild type, CCL2-/- and CCR2-/- mice, on the C57/BL6 genetic background, were obtained from Jackson Laboratories. All the mice were sex and age matched littermates. The mice were euthanized at the age of 6 to 8 weeks and dissected to obtain humerus and tibia. Approval for all our procedures was given by Griffith University Animal Ethic Committee.

CELL CULTURE

The femurs and tibias of adult mice were aseptically removed and dissected free of adhering tissues. The bone ends were cut off with scissors and the marrow cavity was flushed with α-MEM by slowly injecting at one end of the bone using a sterile needle. The bone marrow cells (BMCs) were collected, washed with α-MEM, and after washing cells were cultured in α-MEM containing 10% FCS and 1% penicillin for 2 days at 37°C in a humidified atmosphere of 5% CO_2 in air with M-CSF. On day 2, media was removed and cells were washed with PBS to remove non-adherent cells such as red blood cells, stromal cells and lymphoid cells [Khan et al., 2013]. Subsequently, cells were plated at a density of 1.5×10^4 cells per cm^2 in 96 and 6 well plates at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells destined for the macrophage lineage were treated only with M-CSF, for osteoclasts with M-CSF and RANKL and for FBGC with GM-CSF and IL-4. In the recovery experiments; cultures were grown as described above with the addition of exogenous CCL2 to these cultures on day zero, at the time when other cytokines were added, while there were still another set of cultures to which CCL2 was not added and were used as controls. MCSF was used at a concentration of 30 ng/ml, RANKL at 35 ng/ml, GM-CSF, IL-4, and CCL2 at 50 ng/ml. Cytokines were purchased from PeproTech Asia (Rehovot, Israel).

FIXING AND STAINING

Cell were fixed on day 1, 2, 3, and 4 with Acetone/Citrate fixative and subsequently stained for tartrate resistant acid phosphatase (TRAP) using a Acid phosphatase, Leukocyte (TRAP) kit Sigma–Aldrich (St. Louis, MO) according to the protocol provided. Cells that had more than three nuclei and were TRAP positive were counted.

QUANTITATIVE PCR

RNA was extracted from cultures of macrophages, osteoclasts and FBGC on each day from day 0 to day 4 with Nucleospin RNA II (Macherey-Nagel, PA) kit following manufacturer instructions. RNA was converted to cDNA using ImProm-II™ Reverse Transcriptase (RT, Promega) and oligo dt primer according to the manufacturer's protocol, and analysis done with Bio-Rad iCycler in the presence of Bio-rad SYBR green supermix according to the manufacturer's instructions. Day zero data was used as a baseline; primer sequence is given in Table I.

TISSUE SECTIONING AND STAINING

Ten week old mice from each genotype were euthanized and the back limbs dissected. After embedding in paraffin, tibiae and femora were collected and fixed in 4% paraformaldehyde at 4°C for 24 h. The samples were decalcified in 19% EDTA at 4°C for 10–14 days and then embedded in paraffin, sectioned at a thickness of 5 μm and allowed to air dry for several hours before deparaffinization as explained [Anders et al., 2001]. Tissue sections were then stained for TRAP (Sigma, USA).

STATISTICAL ANALYSIS

The cultures were done in triplicates and the whole experiment was repeated six times on different occasions, sacrificing six different donor mice of each genotype i.e. WT, CCL2-/- and CCR2-/-, with mRNA extracted from all the experiments. Cell counts were assessed from six non-overlapping images that were taken from each of the triplicate culture wells. In case of tissue sections three mice per genotype were utilised and 18 photomicrographs were taken 80–100 microns below the growth plate of femur and tibia in each section. In each image TRAP positive multinucleated giant cells (MNGC) were counted along with the number of nuclei per MNGC and finally the maximum diameter of each MNGC was recorded in pixels by the way of Feret's diameter of irregular bodies. Cell counts were compared with ANOVA and Bonferroni test was used for pair-wise comparisons. Data is presented as mean ± standard error.

RESULTS

OSTEOCLASTS AND FBGC EXPRESSED MORE CCL2 COMPARED TO MACROPHAGES

Realtime PCR expression analysis was used identify the expression of CCL2 in macrophages, osteoclasts and FBGCs derived from wild type mice BMCs. Our data showed that

TABLE I. Sequence of Primers Used for Gene Expression

Primer	Forward	Reverse	Accession number
18S	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA	X_00686
CCL2	TGAGTAGGCTGGAGAGCTACAAG	TGTATGTCTGGACCCATTCCTTC	NM_011333.3
CCR1	GAACGGTTCTGGAAGTACCT	TGGTTGACACCTATGGTCTG	NM_009912.4
CCR5	CTGGACTCCCTACAACATTG	ACACTGAGAGATAACTCCGG	NM_009917.5
CCR3	CAGTCACAATGACCTCTAGC	GAATTGTCAACTGGCCAGCA	NM_009914.4

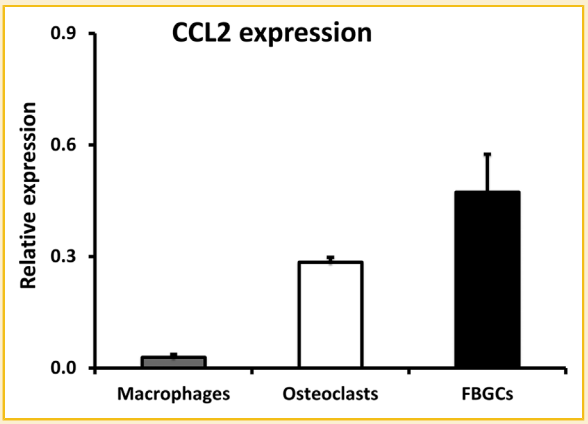

Fig. 1. Expression of CCL2 mRNA in bone marrow cultures from WT mice. CCL2 was expressed significantly ($P < 0.05$) more in osteoclasts and FBGC compared to macrophages. Osteoclasts expressed about 10-fold while FBGC expressed 16-fold more CCL2 compared to macrophages.

CCL2 expression was significantly different in macrophages, osteoclasts and FBGC ($P = 4 \times 10^{-4}$). Furthermore, osteoclasts expressed ninefold more CCL2 compared to macrophages, while FBGC expressed 16-fold more than macrophages (Fig. 1).

CCL2 AND CCR2 KNOCKOUT RESULTS IN DECREASE OSTEOCLAST AND FBGC FORMATION IN IN-VITRO CULTURE

There was a significant reduction in the number of osteoclasts produced from the marrow of CCL2-/- and CCR2-/- mice compared to WT controls ($P = 2.9 \times 10^{-9}$) (Fig. 2A and B). In addition, the number of nuclei ($P = 2.5 \times 10^{-10}$) and cell size ($P = 1.4 \times 10^{-10}$) of these osteoclasts were significantly reduced in CCL2-/- and CCR2-/- compared to WT mice, confirming that the reduction in osteoclast number did not result from increased fusion (Fig. 2C). Similarly, the number of FBGC were significantly reduced (5.9×10^{-9}) in the two knockouts compared to wild type, and the number of nuclei ($P = 4.4 \times 10^{-14}$) and cell size ($P = 2.5 \times 10^{-12}$) were reduced (Fig. 2D).

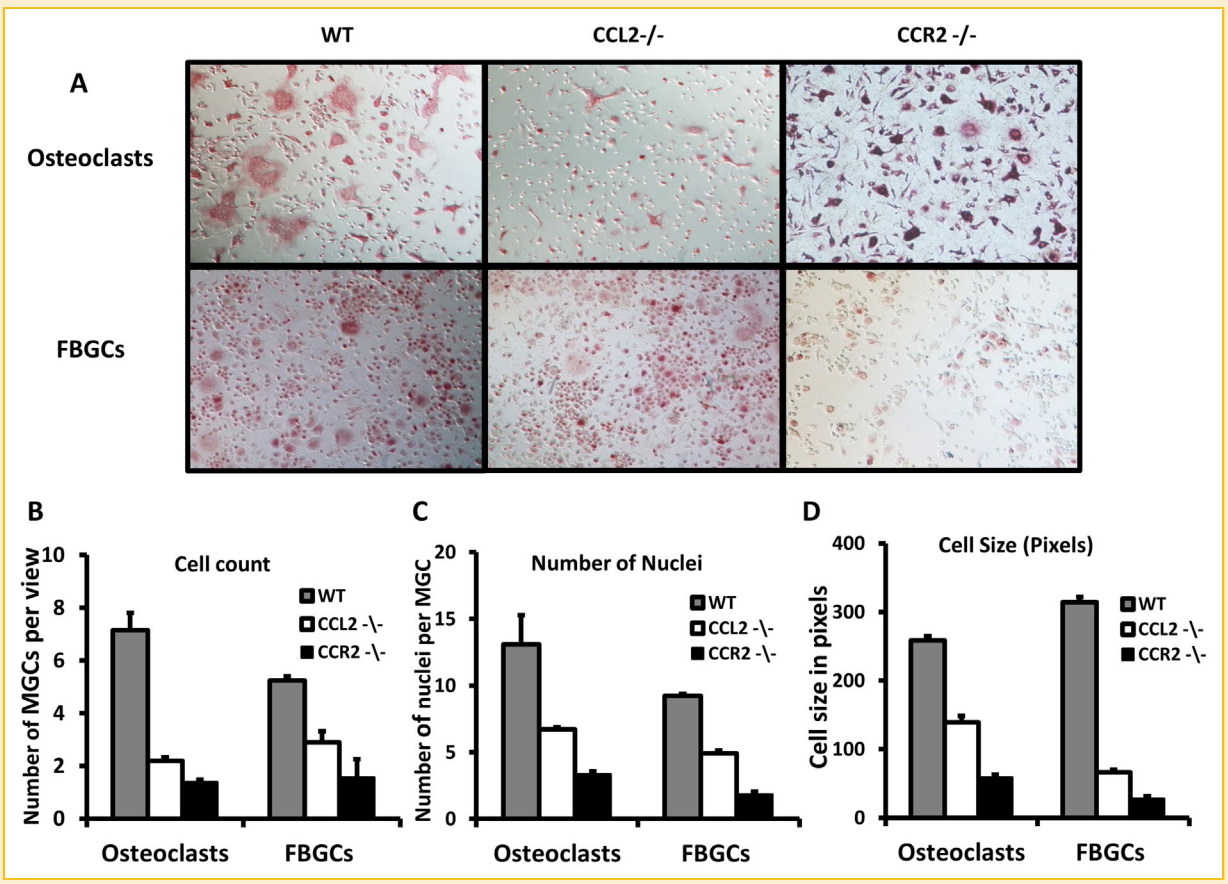

Fig. 2. Osteoclasts and FBGC generation was reduced in BMCs from CCL2-/- and CCR2-/- compared to wild type mice (WT). A: Cultures were TRAP stained and number of cells with more than three nuclei was counted, number of nuclei per multinucleated giant cells (MNGCs) was also recorded along with the mean size of MNGCs. B: There was a significant reduction ($P = 2.9 \times 10^{-9}$) in the number of TRAP positive MNGCs derived from CCL2-/- and CCR2-/- compared to WT in the cultures of osteoclasts and FBGC. C: Number of nuclei per MNGCs also showed significant inhibition in osteoclasts ($P = 2.5 \times 10^{-10}$) and FBGC cultures ($P = 4.4 \times 10^{-14}$). D: Size of osteoclasts and FBGC observed in cultures taken from CCL2-/- and CCR2-/- mice were markedly reduced compared to wild type.

ADDITION OF CCL2 TO THE CULTURES OF CCL2 KNOCKOUT MICE RESULTED IN THE RESCUING OF OSTEOCLAST NUMBERS AND FBGC FORMATION

Addition of exogenous CCL2 to cultures of BMCs resulted in an increase number of giant cells (Fig. 3). In the case of WT mice, there was a significant increase in the number of osteoclasts and FBGC compared to the control cultures ($P = 1.3 \times 10^{-10}$), as well as the number of nuclei ($P = 2.2 \times 10^{-6}$) and an increase in cell size ($P = 1.4 \times 10^{-6}$) (Fig. 4).

After adding exogenous CCL2 to the media containing cells from CCL2-/- mice, there was a significant increase ($P = 6.2 \times 10^{-6}$) in the number of osteoclasts and FBGC compared to cultures in which CCL2 was not added (Fig. 4A and B). The number of nuclei ($P = 1.6 \times 10^{-10}$) and size of these cells recovered as well ($P = 2 \times 10^{-9}$) (Fig. 4C–F). In cultures of cells from CCR2-/- mice, there was also a recovery ($P = 2.7 \times 10^{-4}$) in the number of osteoclasts and FBGC after adding exogenous CCL2 to the media. We also observed a significant increase in the number of nuclei (1.1×10^{-4}) and cells (3.4×10^{-4}) in cultures with exogenous CCL2 compared to controls (Fig. 4).

IN VIVO OSTEOCLAST FORMATION IS ALSO INHIBITED IN THE CCL2 AND CCR2 KNOCKOUT MICE

Trap staining was used on sections from wild type, CCL2 and CCR2 knockout mice to identify the formation and morphology of giant cells (Fig. 5). The number of cells ($P = 4.9 \times 10^{-8}$) (Fig. 5A and B), cell size ($P = 1.1 \times 10^{-6}$) (Fig. 5C) and number of nuclei ($P = 4.5 \times 10^{-9}$) (Fig. 5D) were significantly less in the metaphysis of tibiae and femora of CCL2 and CCR2 knockout mice.

CCR2-/- MICE EXPRESSED HIGHER CCR5 AND CCR1 MRNA COMPARED TO WT

Osteoclasts from CCR2-/- expressed more than 46-fold CCR5 compared to WT ($P = 4.7 \times 10^{-8}$). There was also a significant difference in CCR5 expression in the FBGC taken from WT, CCL2-/- and CCR2-/- ($P = 2.6 \times 10^{-12}$). FBGC taken from CCR2-/- expressed 32-fold more CCR5 compared to WT (Fig. 6A).

Osteoclasts cultures taken from CCR2-/- mice expressed more CCR1 mRNA compare to WT and CCL2-/- ($P = 7.5 \times 10^{-7}$). Osteoclasts taken from CCR2-/- expressed CCR1 24-fold more than WT. FBGC taken from CCR2 -/- mice also expressed CCR1 significantly more than WT and CCL2-/- ($P = 3.7 \times 10^{-9}$). FBGC taken form CCR2-/- expressed 24-fold more CCR1 compared to WT (Fig. 6C).

OSTEOCLASTS FROM CCL2-/- EXPRESSED SIGNIFICANTLY HIGHER CCR3 MRNA COMPARED TO CCR2-/- MICE

Gene expression of CCR3 was significantly greater in osteoclasts taken from CCL2-/- compared to WT and CCR2-/- mice ($p = 7.4 \times 10^{-5}$). Osteoclast from CCL2-/- expressed CCR3 34-fold more than WT. The difference between WT and CCR2-/- osteoclasts was not significant. FBGC from CCL2-/- were significantly different from WT and CCR2-/- in terms of CCR3 expression ($p = 0.002$). FBGC taken from CCR2-/- expressed CCR3 136-fold more than WT (Fig. 6B).

DISCUSSION

CCL2 has been detected at the sites of Rheumatoid arthritis [Koch et al., 1992], tooth eruption [Wise et al., 2002], other lytic conditions of bone [Aggarwal et al., 2006; Lu et al., 2007] as well

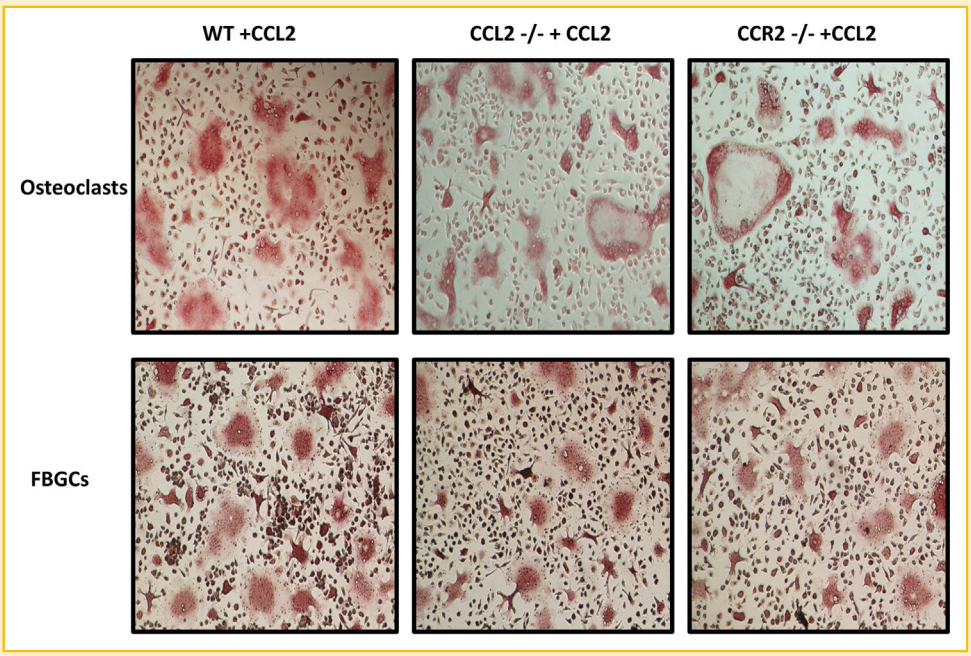

Fig. 3. Exogenous CCL2 caused increase number of MNGCs formation in cultures taken from CCL2-/- and CCR2-/- mice. CCL2 was added to the cultures of osteoclasts and FBGC taken from wild type, CCL2-/- and CCR2-/-. Osteoclasts cultures were grown for 4 days while FBGC cultures were grown for 8 day, the media was changed every third day. Cultures were fixed then TRAP stained and finally images were taken to count the number of MNGCs, number of nuclei per MNGC and size of these cells.

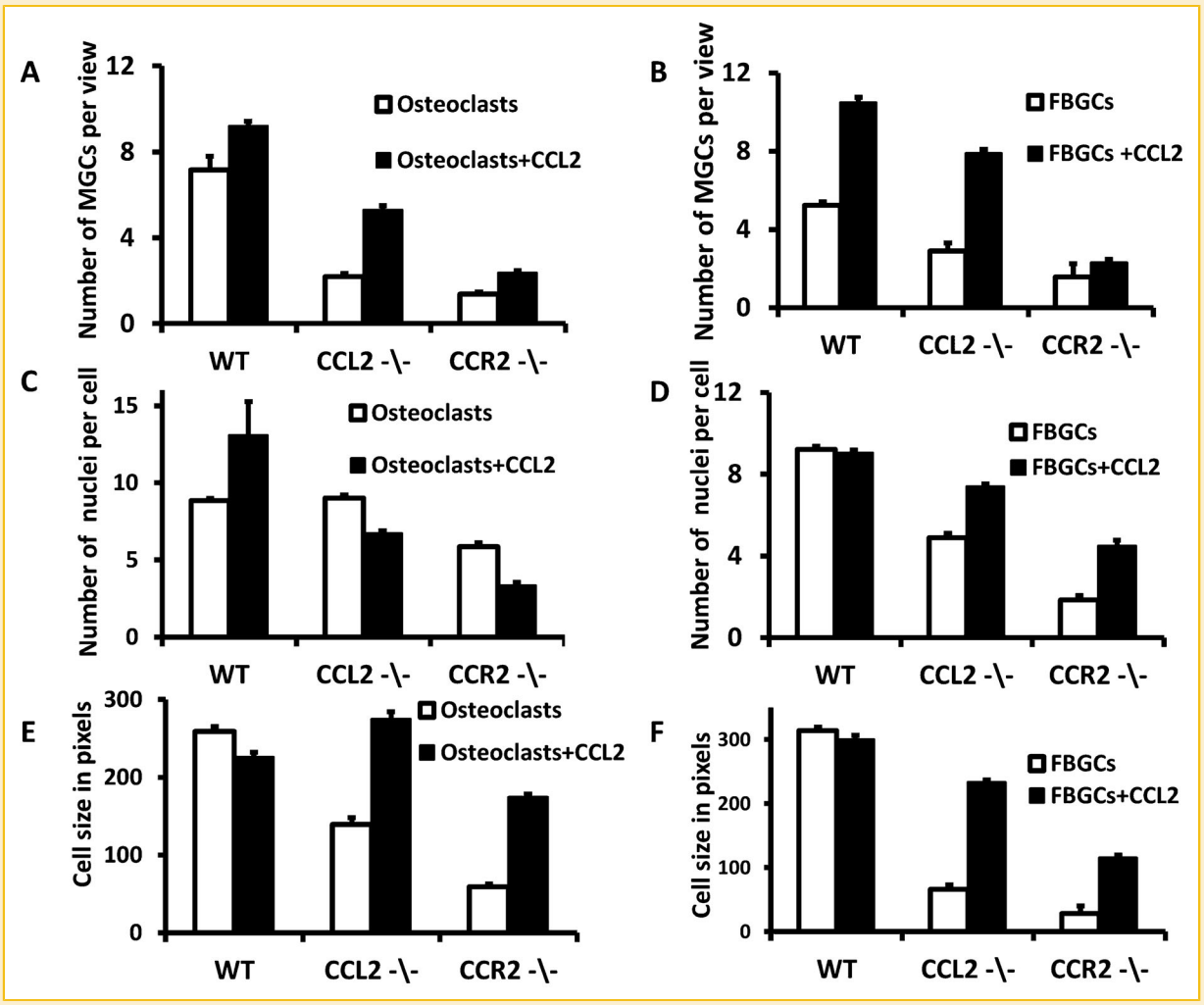

Fig. 4. Recovery of MNGCs formation in cultures of osteoclasts and FBGC taken from CCL2-/- and CCR2-/- mice. A: Addition of CCL2 to the cultures caused increase in osteoclasts numbers in wild type compared to cultures in which CCL2 was not added. In CCL2-/- mice addition of CCL2 resulted in rescuing of the inhibition of osteoclasts formation. In CCR2-/- mice however we recorded very little recovery of osteoclast formation. B: FBGC formation was enhanced by the addition of exogenous CCL2 to the cultures taken from wild type mice. We observed recovery of inhibition of FBGC formation in CCL2-/- mice when exogenous CCL2 was added to the cultures. C: Exogenous CCL2 however did not cause a significant change in the number of nuclei per MNGCs in osteoclasts cultures taken from WT, CCL2-/- and CCR2-/- mice. D: Number of nuclei per MNGCs in FBGC cultures taken from WT mice were not different, however in CCL2-/- and CCR2-/- mice exogenous CCL2 increased the number of nuclei per MNGCs in FBGC cultures. E: There was no significant effect on cell size of the osteoclasts by adding exogenous CCL2 to the cultures taken from WT mice. We observed a significant increase in the size of osteoclasts and FBGC after adding exogenous CCL2 to the cultures taken from CCL2 and CCR2-/- mice. F: Size of FBGC did not change by adding exogenous CCL2 to the cultures taken from WT mice. In contract, we saw a significant increase in the mean size of FBGC in cultures taken from CCL2-/- and CCR 2-/- mice.

as foreign body reactions [Kyriakides et al., 2004]. As evidenced by our results, CCL2 is induced during osteoclast formation (Fig. 1), our lab and Miyamoto et al. found that CCL2 produced acts in an autocrine/paracrine manner to regulate osteoclast formation [Kim et al., 2005; Miyamoto et al., 2009], and hence CCL2 has an important role in the formation and regulation of osteoclasts. We observed that osteoclast formation is inhibited in mice lacking CCL2, which is also consistent with previous reports [Sul et al., 2012]. To further support our hypothesis, we also demonstrated that inhibition of osteoclast and FBGC formation in CCL2 deficient mice can be rescued by the addition of recombinant CCL2 (Fig. 3 and 4), consistent with Miyamoto et al. [Miyamoto et al., 2009]. Sul et al. found that mice lacking CCL2 exhibited significantly reduced serum markers of osteoclastogenesis, while the osteoblastic markers remain unchanged [Sul et al., 2012], suggesting that osteoclast formation/function has been compromised, and is consistent with our observation of reduced osteoclast number recorded in the metaphysis of femora and tibiae (Fig. 5).

To evaluate the importance of CCL2 during formation of FBGC, we added exogenous CCL2 to the cultures of FBGC taken from wild type mice and we observed a twofold increase in FBGC number

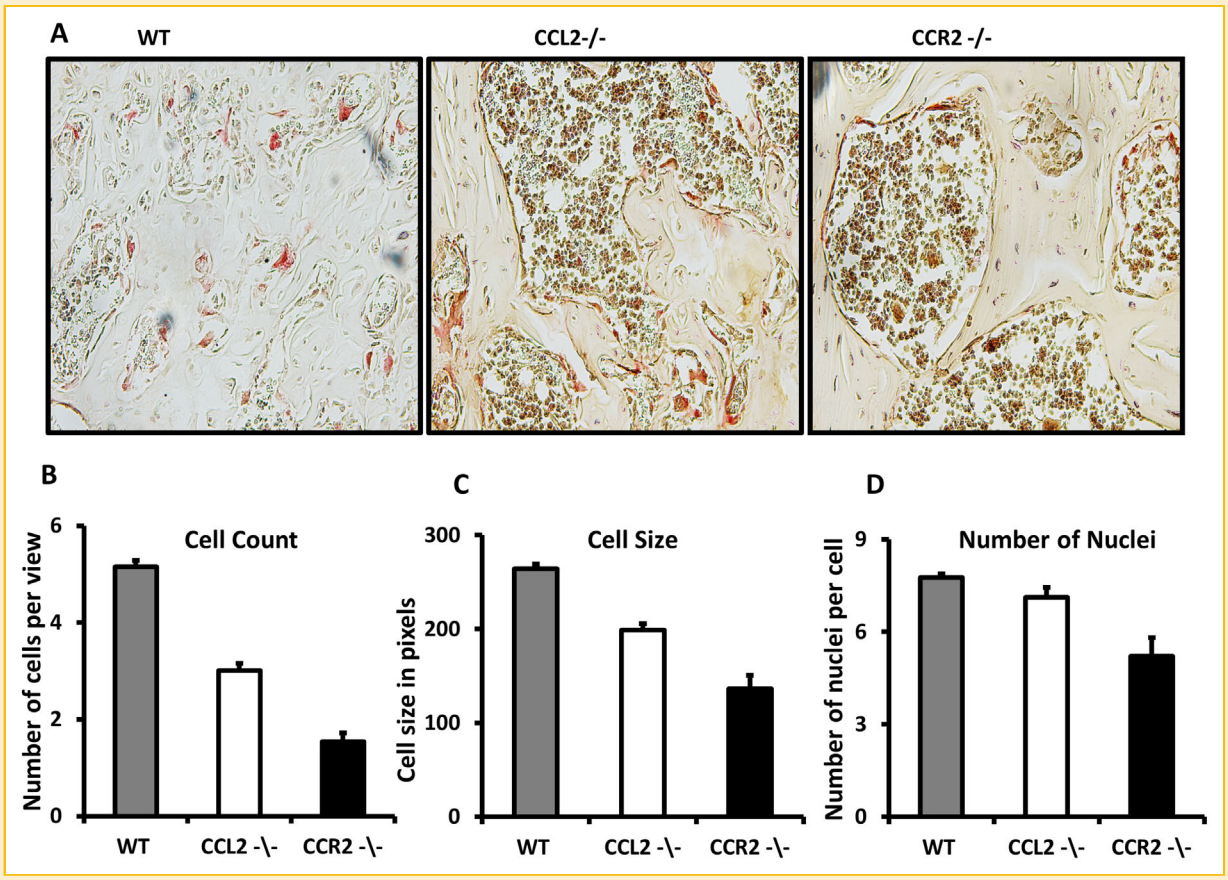

Fig. 5. In vivo inhibition of osteoclasts in CCL2-/- and CCR2-/- KO mice compared to WT mice. A: Fewer TRAP positive multinucleated giant cells attached to bone at the growth plate of tibiae and femora were observed in CCL2-/- and CCR2-/- KO mice compared to WT mice. B: There was a significant reduction in the number of TRAP positive multinucleated cells in CCL2-/- mice and to CCR2-/- compared to WT. The average cell size (C) as well as the number of nuclei per multinucleated giant cells (D) were also inhibited in the CCL2-/- and CCR2-/- compared to WT mice.

compared to control. Kyriakides et al showed that FBGC formation was inhibited in CCL2 deficient mice, our results also showed twofold inhibition of FBGC formation in CCL2 deficient mice compared to wild type. In addition we also showed that this inhibition of cultures taken from CCL2-/- mice can be rescued profoundly by the addition of exogenous CCL2 to the cultures of FBGC.

Binder et al. showed that CCR2-/- mice are resistant to ovariectomy-induced bone loss because of the decreased formation and function of osteoclasts [Binder et al., 2009]. Likewise, we have shown that the number of osteoclasts was significantly reduced in the metaphysis of CCR2-/- mice (Fig. 5), we also observed more than fivefold reduction of osteoclasts number in cultures of CCR2-/- mice compared to wild type. In contrast to Binder et al, we observed almost twofold recovery in osteoclasts number when exogenous CCL2 was added to the cultures taken from CCR2-/- mice, however it was still less than wild type cultures, the reason for this might be that CCL2 can bind to CCR4 as well as CCR2 [Craig and Loberg, 2006]. Furthermore, we also report that osteoclast number in cultures taken from CCL2 -/- mice were significantly more than CCR2-/- ($P = 1.2 \times 10^{-9}$), the reason for this might be that in CCL2-/- mice the receptor CCR2 was present and CCR2 can bind to other ligands, such as CCL7 [Jia et al., 2008].

Kuziel et al. reported more than three times inhibition of macrophage recruitment in response to foreign body in CCR2-/- mice compared to wild type [Kuziel et al., 1997]. Here we report that CCR2 is also important for macrophage fusion during foreign body reaction as we have observed threefold inhibition of FBGC formation in cultures taken from CCR2-/- compared to wild type mice while number of FBGC were twofold more in CCL2-/- mice compared to CCR2-/- mice. Exogenous CCL2 did not result in increased number of FBGC, however the number of nuclei and cell size were significantly increased implying that the same number of FBGC are present but with more nuclei per cell and greater size.

There was a significant inhibition in the number of osteoclasts in CCL2-/- and CCR2-/- both in vivo and in vitro, which might be as a result of increased expression of CCR3 by the CCL2-/- mice compared to CCR2-/- mice. This may be consistent with previous observations that osteoclasts express significantly higher amounts of CCR3 during their formation [Yu et al., 2004; Khan et al., 2014].

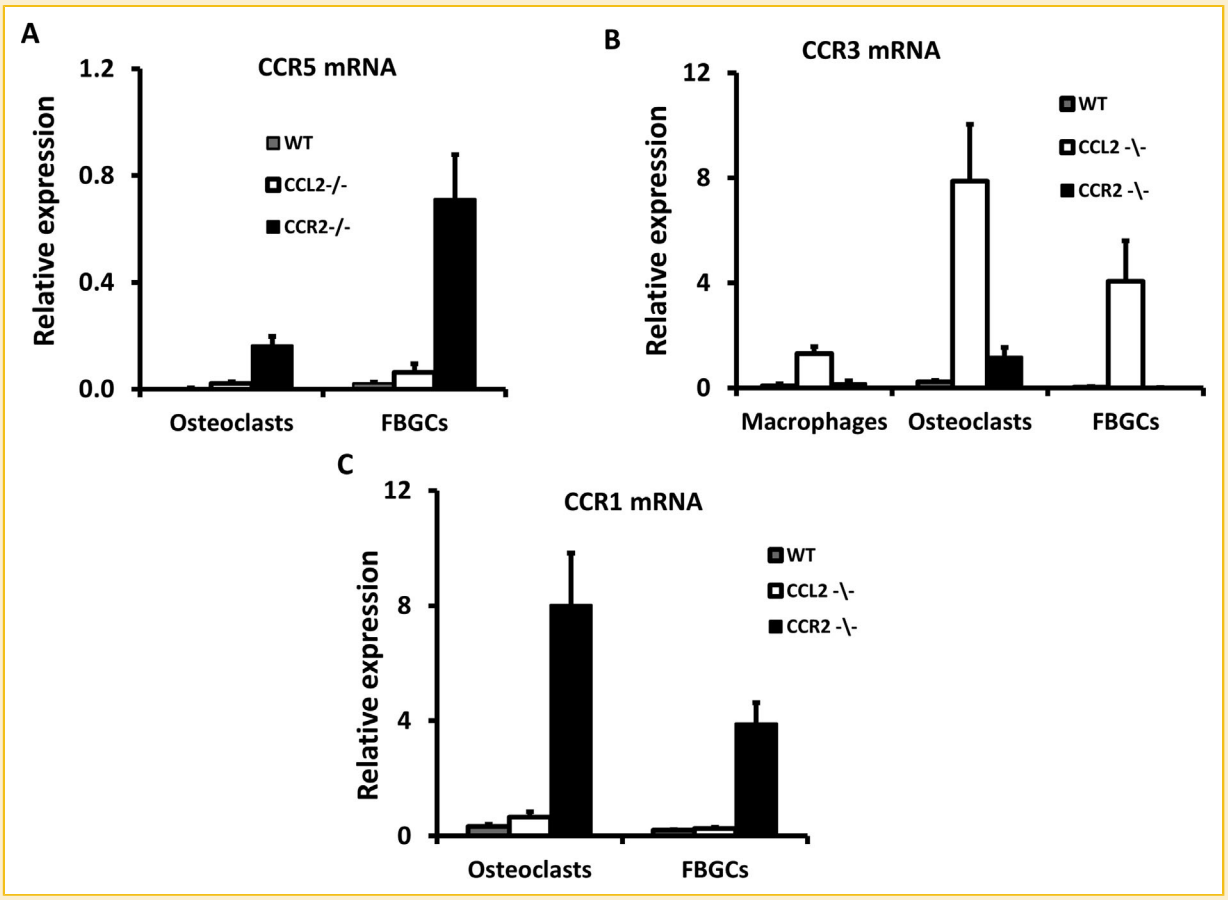

Fig. 6. Expression of chemokine receptors by osteoclasts and FBGC taken from WT, CCL2, and CCR2 KO mice. A: There was a significant $P < 0.05$) difference in CCR5 expression between WT, CCL2-/-, and CCR2-/- mice. Osteoclasts from CCR2-/- expressed 46-fold more CCR5 compared to WT while FBGC from CCR2-/- expressed 32-fold more CCR5 compared to WT. B: There is a significant $P < 0.05$) difference in CCR3 expression between CCL2-/-, WT, and CCR2-/-. Osteoclasts from CCL2-/- expressed 34-fold more CCR3 compared to WT while FBGC form CCL2-/- mice expressed 136-fold more CCR3 compared to WT mice. C: Cells from CCR2-/- expressed CCR1 significantly more than WT and CCL2-/-. Osteoclasts derived from CCR2-/- expressed 24-fold more expression of CCR1 compared to osteoclasts derived from WT mice. FBGC derived from CCR2-/- mice expressed 18-fold more CCR1 compared to WT.

We were able to observe osteoclasts in CCR2-/- mice, possibly because CCR2-/- mice expressed more CCR1 mRNA than WT and CCL2-/- mice. Increase expression of CCR1 may be a compensatory mechanism in response to the reduced CCL2, and CCR1 was reported by another study to have a significant effect on osteoclast formation and function [Hoshino et al., 2010].

In conclusion our study has found that both CCL2 and CCR2 are required for the formation of osteoclasts and FBGC. Removing CCL2 and CCR2 resulted in significant reduction of osteoclasts and FBGC formation. Furthermore, the addition of exogenous CCL2 not only increased the number of osteoclasts in WT mice, but also recovered the inhibition of osteoclast formation in CCL2-/- mice, when tested in vitro.

ACKNOWLEDGMENTS

The above project was supported by National Health and Medical Research Council grant awarded to N.A.M. Dr Khan was supported by a scholarship from Griffith University.

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