Chemokine signals are crucial for enhanced homing and differentiation of circulating osteoclast progenitor cells

Alan Sucur1,2, Zrinka Jajic3, Marinko Artukovic4, Marina Ikic Matijasevic4, Branimir Anic5, Darja Flegar1,2, Antonio Markotic1,2, Tomislav Kelava1,2, Sanja Ivcevic1,2, Natasa Kovacic1,6, Vedran Katavic1,6 and Danka Grcevic1,2*

Abstract

Background: The peripheral blood (PB) monocyte pool contains osteoclast progenitors (OCPs), which contribute to osteoresorption in inflammatory arthritides and are influenced by the cytokine and chemokine milieu. We aimed to define the importance of chemokine signals for migration and activation of OCPs in rheumatoid arthritis (RA) and psoriatic arthritis (PsA).

Methods: PB and, when applicable, synovial fluid (SF) samples were collected from 129 patients with RA, 53 patients with PsA, and 110 control patients in parallel to clinical parameters of disease activity, autoantibody levels, and applied therapy. Receptors for osteoclastogenic factors (CD115 and receptor activator of nuclear factor-κB [RANK]) and selected chemokines (CC chemokine receptor 1 [CCR1], CCR2, CCR4, XCC chemokine receptor 3 [CXCR3], CXCR4) were determined in an OCP-rich subpopulation (CD3−CD19−CD56−CD11b+CD14+) by flow cytometry. In parallel, levels of CC chemokine ligand 2 (CCL2), CCL3, CCL4, CCL5, XCC chemokine ligand 9 (CXCL9), CXCL10, and CXCL12 were measured using cytometric bead array or enzyme-linked immunosorbent assay. Sorted OCPs were stimulated in culture by macrophage colony-stimulating factor and receptor activator of nuclear factor-κB ligand, and they were differentiated into mature osteoclasts that resorb bone. Selected chemokines (CCL2, CCL5, CXCL10, and CXCL12) were tested for their osteoclastogenic and chemotactic effects on circulatory OCPs in vitro.

Results: The OCP population was moderately enlarged among PB cells in RA and correlated with levels of tumor necrosis factor-α (TNF-α), rheumatoid factor, CCL2, and CCL5. Compared with PB, the RANK+ subpopulation was expanded in SF and correlated with the number of tender joints. Patients with PsA could be distinguished by increased RANK expression rather than total OCP population. OCPs from patients with arthritis had higher expression of CCR1, CCR2, CCR4, CXCR3, and CXCR4. In parallel, patients with RA had increased levels of CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10, with significant elevation in SF vs PB for CXCL10. The subset expressing CXCR4 positively correlated with TNF-α, bone resorption marker, and rheumatoid factor, and it was reduced in patients treated with disease-modifying antirheumatic drugs. The CCR4+ subset showed a significant negative trend during anti-TNF treatment. CCL2, CCL5, and CXCL10 had similar osteoclastogenic effects, with CCL5 showing the greatest chemotactic action on OCPs.

Conclusions: In our study, we identified distinct effects of selected chemokines on stimulation of OCP mobilization, tissue homing, and maturation. Novel insights into migratory behaviors and functional properties of circulatory OCPs in response to chemotactic signals could open ways to new therapeutic targets in RA.

Keywords: Osteoclast progenitor cells, Chemokines, Chemokine receptors, Osteoclasts, Rheumatoid arthritis, Peripheral blood, Synovial fluid, Cytokines, Inflammation, Bone loss

* Correspondence: danka.grcevic@mef.hr
1Croatian Institute for Brain Research, University of Zagreb School of Medicine, Salata 12, Zagreb HR 10000, Croatia
2Department of Physiology and Immunology, University of Zagreb School of Medicine, Salata 3b, Zagreb HR 10000, Croatia
Full list of author information is available at the end of the article

© The Author(s). 2017 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background
Rheumatoid arthritis (RA) is a systemic inflammatory disease manifesting predominantly as chronic affection of multiple joints and osteoedestruction, and involving complex interactions between the immune system, extracellular matrix, and bone cells [1]. Severe clinical symptoms and the chronic course of the disease, underpinned by persistent inflammation, progressive joint destruction, and constant pain, require prolonged drug use and result, despite treatment, in significant skeletal deformities and global disability [2]. With a prevalence of around 1%, RA is one of the most common autoimmune diseases in the human population and an important contributor to public health burden, lower quality of life, and reduced life expectancy. Although numerous studies have been focused on the pathogenesis of RA, especially disease etiology and onset, many questions remain unanswered, specifically the underlying causes of perpetuation of chronic inflammation and sustained renewal of bone-resorbing cells [3, 4].

Osteoclasts (OCs) are multinucleated cells with the unique ability to resorb bone. They develop by fusion of hematopoietic precursors belonging to the monocyte/macrophage lineage. OCs express receptors for two crucial regulators of their differentiation and maturation: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL, CD254). The receptor for colony-stimulating factor-1 (cFms, CD115, M-CSF receptor) appears at early developmental stages of the myeloid lineage, whereas receptor activator of nuclear factor-κB (RANK; CD265) is expressed on committed osteoclast progenitors (OCPs) and is crucial for OC survival and activation [5–7]. Physiologically, the main source of RANKL is osteoblast lineage cells, but RANKL has also been shown to be expressed by activated T lymphocytes, mature B lymphocytes, natural killer cells, and synoviocytes in inflammatory conditions. Furthermore, elevated levels of a wide range of proinflammatory cytokines (interleukin-1 [IL-1], IL-6, IL-15, IL-17, IL-18, IL-21, IL-22, IL-23, tumor necrosis factor-α [TNF-α]) and chemokines (CC chemokine ligand 2 [CCL2], CCL3, CCL4, CCL5) act in synergy with M-CSF/RANK signals to promote osteoclastogenesis [8, 9].

Because the life span of an individual OC is only a couple of weeks, OCs must be constantly replenished to sustain pathological bone resorption in the chronic course of RA [3, 10, 11]. The peripheral blood (PB) monocyte pool, originating from myeloid bone marrow progenitors, contains 1–2% of cells capable of differentiating in vitro into fully functional OCs in the presence of M-CSF and RANKL [3, 12]. Owing to monocyte/macrophage lineage heterogeneity and few consensual specific markers of human OCPs, researchers have used various monocyte markers in an attempt to better characterize this osteoclastogenic subpopulation in PB. On the basis of previous work of several groups, including our own, lymphoid marker-negative (CD3, CD19, CD56), CD11b and CD14 double-positive (DP) subpopulation of peripheral blood mononuclear cells (PBMCs) were identified as significantly OCP-enriched in patients with RA and patients with psoriatic arthritis (PsA) [3]. Furthermore, it has been proposed that the erosive phenotype of various forms of inflammatory arthritis could be a result of the plasticity of these hematopoietic monocytic progenitors, depending on the cytokine and chemokine milieu in the bone marrow, circulation, and synovium [13].

Chemokines are known to orchestrate the recruitment of immune cells in RA and other inflammatory rheumatic diseases [14]. They may also be involved in OCP trafficking because cells at various stages of OC lineage express chemokine receptors (CC chemokine receptor 1 [CCR1] to CCR4, CXC chemokine receptor 3 [CXCR3], CXCR4, or CX3C chemokine receptor 1) [15–18]. However, the contribution of chemotactic signals to homing of OCPs and to their differentiation into mature OCs has not been fully elucidated [3]. The aim of our present study was to define the chemokine receptor profile of peripheral OCPs specifically induced by arthritis as well as the susceptibility of this cell subset to chemotactic signals. Raised levels of chemokines and chemotactic gradient between plasma and synovial fluid (SF) seem to contribute not only to increased migration of OCPs but also to their enhanced differentiation. Thus, immunophenotyping of chemokine receptors as well as measurement of respective chemokine levels may represent useful parameters to assess arthritis severity, disease progression, or response to therapy.

Methods
Patients
Patients with arthritis (RA, n = 129; PsA, n = 53) admitted to Clinical Hospital Center “Sestre Milosrdnice”, Clinical Hospital “Sveti Duh”, and Clinical Hospital Center “Zagreb” were included in the study after approval was obtained from the ethics committees of those institutions and of the University of Zagreb School of Medicine, and informed consent was received from patients. Control subjects (CTRL; n = 110) were admitted to the same institutions for noninflammatory etiology and had no previous history of arthritic or autoimmune diseases. A rheumatology specialist established the diagnosis of RA on the basis of American College of Rheumatology 2010 criteria, whereas PsA was diagnosed according to the Moll and Wright criteria [19, 20]. PB samples were obtained from patients with RA and patients with PsA during routine clinical assessments and
processed for analysis. The following data were also collected from patients with arthritis: disease duration (years), patient and physician assessment of disease activity (visual analogue scale [VAS]), patient assessment of pain and fatigue (VAS), tender joint count (TJC; out of 28 joints), swollen joint count (SJC; out of 28 joints), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) level, Disease Activity Score in 28 joints (DAS28) calculated with ESR, Clinical Disease Activity Index (CDAI), treatment received (nonsteroidal anti-inflammatory drugs [NSAIDs], disease-modifying antirheumatic drugs [DMARDs], corticosteroids, and/or biological therapy), levels of rheumatoid factor (RF; determined by turbidimetry), and anti-citrullinated protein antibodies (ACPA; determined by enzyme-linked immunosorbent assay [ELISA]). For some analyses, patients with RA were grouped on the basis of their DAS28 into severe (DAS28 > 5.1) and low/moderate cases (DAS28 2.6–5.1) [21–23]. A cohort of patients with arthritis (n = 24) assigned for therapeutic arthrocentesis of the affected knee was included to obtain SF samples. A small subset of patients (n = 9) scheduled for biological treatment with TNF inhibitors (etanercept 50 mg weekly or adalimumab 40 mg every 2 weeks) was followed by taking PB samples immediately before and after 2, 4, and 6 months after onset of therapy.

### Isolation of mononuclear cells

Blood samples were obtained between 9:30 and 11:30 a.m. PBMCs and plasma were separated from PB by centrifugation (20 minutes at 600 × g) with Histopaque (Sigma-Aldrich, St. Louis, MO, USA). Aspirated SF samples were separated by sequential centrifugation (5 minutes at 100 × g) into acellular SF supernatants and SF-derived cells (synovial fluid mononuclear cells [SFMCs]). Plasma and SF samples were stored at −20 °C until a cytometric bead array (CBA) or ELISA was performed, whereas PBMCs and SFMCs were immediately used for flow cytometric phenotyping, RNA extraction, cell sorting, osteoclastogenic cultures, and migration assays.

### Flow cytometry

Phenotype characterization of OCPs from PB was performed using an InVitrogen Attune flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA). Cells were counted in a hemocytometer by trypan blue exclusion and labeled using a mix of commercially available monoclonal antibodies against lymphoid lineage markers (anti-CD3 fluorescein isothiocyanate [FITC] for T lymphocytes, anti-CD19 FITC for B lymphocytes, and anti-CD56 FITC for natural killer cells), myeloid lineage markers (anti-CD11b phycoerythrin [PE]-cyanine 7 [Cy7] and anti-CD14 peridinin-chlorophyll-cyanine 5.5), receptors for OC differentiation factors (receptor for M-CSF [biotinylated anti-CD115], RANK [anti-RANK PE]), and several chemokine receptors (anti-CCR1 allophycocyanin [APC], anti-CCR2 PE, anti-CCR4 APC, anti-CXCR3 APC, anti-CXCR4 PE) (all from eBioscience, San Diego, CA, USA). Suspensions were incubated on ice for 40 minutes, followed by washing in staining medium (PBS/2% FBS). As a second step (for biotinylated anti-CD115), cells were stained with streptavidin coupled to APC-eFluor 780 (Thermo Fisher Scientific) on ice for 40 minutes, followed by washing in staining medium, and afterward they were resuspended in staining medium for analysis. OCPs were defined as part of the monocytic subpopulation bearing the CD3−CD19−CD56−CD11b+CD14+ phenotype [3, 12]. The subpopulation was then analyzed for expression of CD115 and RANK as well as for subsequent chemokine receptor expression (CCR1, CCR2, CCR4, CXCR3, and CXCR4). Gates for the analysis were defined using unlabeled cells and "fluorescence minus one control".

### Cell sorting

For fluorescence-activated cell sorting (FACS), PBMCs of CTRL and RA samples were labeled using lymphoid lineage markers (anti-CD3 FITC for T lymphocytes, anti-CD19 FITC for B lymphocytes, anti-CD56 FITC for natural killer cells) and myeloid lineage markers (anti-CD11b APC and anti-CD14 PE-Cy7) and sorted using a BD FACSAria I cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, labeled cells were acquired at a speed of approximately 5000 cells/second, and singlets were delineated from the total cell population depicted on forward vs side scatter plots, followed by plotting lymphoid markers vs CD11b. Subsequently, lymphoid marker-negative cells, immediately positive for CD11b, were gated for CD14 expression. A defined OCP population (CD3−CD19−CD56−CD11b−CD14+) was sorted in 2-ml collection tubes containing α-minimal essential medium (α-MEM)/20% FBS and used for osteoclastogenic cultures. Sorting purity was determined by reanalysis of fractioned populations and was consistently greater than 99%.

### Osteoclast differentiation from sorted peripheral blood mononuclear cells

Sorted OCPs were plated at a density of 4 × 10⁴ cells per well in 96-well plates in α-MEM/10% FBS. For the first 3 days of cell culture, 60 ng/ml recombinant human (rh)M-CSF (R&D Systems, Minneapolis, MN, USA) was added to stimulate cell proliferation. The next 8–11 days, the cell culture medium was supplemented with 100 ng/ml rhRANKL (R&D Systems) and 30 ng/ml rhM-CSF to stimulate differentiation into mature OCs. At days 11–13 of culture, OCs were enumerated by staining for tartrate-resistant acid phosphatase (TRAP).
Migration assay

PBMCs were plated at a density of $10 \times 10^4$ per well in 48-well plates in α-MEM/10% FBS and cultured overnight with 35 ng/ml M-CSF. Nonadherent cells enriched in OCPs were harvested for further analyses as previously described [12]. Considering the heterogeneity of the PB monocyte/macrophage lineage, the nonadherent fraction obtained after short-term incubation was comprised of a mixture of lymphocytes and immature monocytes. Lymphocytes were mostly removed by subsequent media changes.

A portion of nonadherent cells was used in osteoclastogenic culture additionally treated with chemokines. These cells were plated at a density of 2.5 × 10^5 cells per well in 96-well plates in α-MEM/10% FBS supplemented by 100 ng/ml rhRANKL and 30 ng/ml rhM-CSF, with the addition of 40 ng/ml CCL2, 10 ng/ml CCL5, 20 ng/ml CXC chemokine ligand 10 (CXCL10) or 50 ng/ml CXCL12 (PeproTech, Rocky Hill, NJ, USA). Assays were performed on PB RA samples ($n = 6–12$ per chemokine treatment). After 10–11 days, cells were stained with TRAP for OC enumeration.

The remainder of nonadherent cells was used for the Transwell culture system. The cells were plated at a density of 5 × 10^4 cells per well in 48-well plates in α-MEM/10% FBS with 100 ng/ml RANKL and 30 ng/ml M-CSF. At day 2 of culture, adherent cells from each sample were harvested and seeded into the upper chambers of 24-well Costar Transwell plates (8.0-μm pore size; Corning Inc., Corning, NY, USA) at a density of 10^4 cells/well in 100 μl of α-MEM/10% FBS, and the lower chambers were filled with 40 ng/ml CCL2, 10 ng/ml CCL5, 20 ng/ml CXCL10, or 50 ng/ml CXCL12 (PeproTech) in 500 μl of α-MEM/10% FBS. After 3-h incubation at 37 °C with 5% CO₂, the upper surface of the membrane was carefully washed with PBS, and the remaining cells were removed with a cotton wool swab. The cells that had migrated to the bottom side of the Transwell membrane inserts were fixed with 4% paraformaldehyde and stained with 4’6-diamidino-2-phenylindole (DAPI). The migrated cells were counted (two wells per group, five central fields per Transwell) at × 200 magnification using a fluorescence microscope (Axiovert 200; Carl Zeiss AG, Oberkochen, Germany).

The cells that had migrated through the Transwell membrane to the lower chamber of the 24-well plate were cultured for the next 8–9 days in α-MEM/10% FBS supplemented with 100 ng/ml RANKL and 30 ng/ml M-CSF. At the end of the culture period, plates were stained for TRAP activity to determine the number of differentiated OCs.

Detection of osteoclasts

The number of OCs differentiated from PBMCs or the sorted OCP-enriched population was determined by TRAP staining at the culture endpoint (days 10–13). Osteoclastogenic cultures were grown as previously described. Staining for TRAP was performed using a commercially available set of chemicals according to the manufacturer’s instructions (leukocyte acid phosphatase kit; Sigma-Aldrich). OCs were identified and enumerated as TRAP⁺ cells with two or more nuclei per cell at × 200 magnification by light microscopy using the Axiovert 200 fluorescence microscope.

Vitronectin receptor (VNR) expression and bone resorption assays were performed in parallel with TRAP staining to confirm the identity of OCs. For VNR detection, osteoclastogenic cultures were grown as previously described. At culture days 14–16, cells were fixed in 4% paraformaldehyde for 10 minutes and blocked for 30 minutes in 2% bovine serum albumin/PBS. Monoclonal biotinylated CD51/CD61 (integrin αvβ3) antibody (1:80; eBioscience) was incubated for 2 h at room temperature. As a second-step reagent, streptavidin coupled to FITC was incubated for 1 h at room temperature. Cell nuclei were counterstained with DAPI. VNR expression was visualized at × 200 magnification using the Axiovert 200 fluorescence microscope.

For bone resorption assays, cells were plated over the bovine cortical bone slices using the same osteoclastogenic culture conditions as previously described. At culture days 16–18, cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Following TRAP staining using a commercially available set of chemicals (leukocyte acid phosphatase kit; Sigma-Aldrich), cells were removed from bone slices by sonication in 0.25 M NH₄OH for 5 minutes. Bone slices were then stained with 1% toluidine blue in 1% borax buffer for 2 minutes to visualize resorption pits at × 200 magnification using an Axiovert 200 light microscope.

Cytometric bead array

CBA was performed using the Human Chemokine CBA Kit (BD Biosciences, San Jose, CA, USA) to assess the levels of CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 in PB and SF samples. Plasma and SF supernatants were each diluted 1:4 using the supplied dilution buffer and incubated with capture beads. Each capture bead was coated with a capture antibody specific for each soluble protein and could be identified by its distinct fluorescence signature in two fluorescence channels on the flow cytometer (APC vs APC-Cy7). The samples were then washed and incubated with the detection reagent. The concentration of the bound analyte was determined by the intensity of the PE fluorescence, depending on the amount of bound PE-conjugated antibodies. After another washing step, complexes of capture beads, analytes, and detection reagents were analyzed using Invitrogen Attune flow cytometer. Concentrations of
chemokines were determined using FCAP Array version 3.0 software (BD Biosciences), based on standard curves generated by data acquired from provided protein standards.

**Gene expression analysis by quantitative polymerase chain reaction**

Total RNA was extracted from PBMCs using Invitrogen TRIzol reagent (Life Technologies, Carlsbad, CA, USA). For polymerase chain reaction (PCR) amplification, 1 μg of total RNA was converted to complementary DNA (cDNA) by reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and stored at −20 °C until further analysis. The amount of cDNA corresponding to 20 ng of reverse-transcribed RNA was amplified by quantitative polymerase chain reaction (qPCR) in an ABI Prism 7500 sequence detection system (Applied Biosystems). Gene expression of CCL2 (hs00234140_m1), CCL3 (hs00234142_m1), CCL4 (hs99999148_m1), CCL5 (hs00982282_m1), and CXCL12 (hs02668440_m1) was analyzed using commercially available TaqMan assays (Applied Biosystems). Each reaction was performed in duplicate in a 25-μl reaction volume. The relative quantities were calculated using the standard curve designed from six serial dilutions of the calibrator sample (CTRL PBMCs). According to the standard curve, the relative amounts of RNA for target genes were calculated as the ratio of the quantity of the target gene normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as the endogenous control.

**ELISA**

Plasma concentrations of TNF-α (Quantikine HS ELISA kit; R&D Systems), CXCL12 (Quantikine Immunoassay; R&D Systems), and carboxy-terminal telopeptide of type I collagen (CTX; Immunodiagnostic Systems, The Boldons, UK), as well as SF concentrations of RANKL (Quantikine Immunoassay; R&D Systems), were determined in accordance with the manufacturers’ instructions. Briefly, samples were incubated for 2–3 h at room temperature on monoclonal antibody precoated plates, washed three times, and incubated for the next 2 h with HRP-conjugated (CXCL12, RANKL) or alkaline phosphatase-conjugated (TNF-α, CTX) specific antibodies. After another three washes, the reactions were visualized with substrate solution (tetramethylbenzidine) (CXCL12, RANKL) or substrate and amplifier solutions (nicotinamide adenine dinucleotide phosphate and amplifier enzymes) (TNF-α, CTX) and then arrested with hydrochloric or sulfuric acid, respectively. Optical density was determined within 15 minutes on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) set to an excitation wavelength of 450 nm (CXCL12, RANKL) or 490 nm (TNF-α, CTX).

**Statistical analysis**

Data were presented as median with IQR for continuous variables (including clinical scores, laboratory measures, protein concentrations, cell numbers and frequencies) or categorized for categorical variables (including sex, DAS28 subgroup, and treatment). Statistical analysis of the group differences was performed using a nonparametric Mann-Whitney U test (for group-to-group comparisons) or the Kruskal-Wallis test followed by the Mann-Whitney U test (for multiple comparisons). Correlations were done using rank correlation and Spearman’s coefficient ρ with 95% CI. Trend analysis was performed on population frequencies during anti-TNF therapy by using one-way analysis of variance (ANOVA) with polynomial contrast type and testing for linear trends. Multiple logistic regression analysis was performed with the frequency of the PB OCP cells (CD3 + CD19 ’CD56 ’CD11b ’CD14’ ) as the dependent variable. Parameters were taken into consideration as independent variables if comparative analysis between CTRL and RA values showed a significant difference. An extensive search was then performed to see which three of the significantly different independent variables resulted in maximal adjusted $R^2$ values, with pairwise exclusion of missing values in the analysis. Statistical analyses were performed using MedCalc for Windows version 9.4.2.0 software (MedCalc Software, Ostend, Belgium). For all experiments, the α-level was set at 0.05.

**Results**

Patients with rheumatoid arthritis have highly active disease with elevated inflammatory, osteoresorptive, and autoimmune indicators

The demographic and clinical characteristics showed that the majority of patients with RA included in the study had a chronic course of active disease (median DAS28 5.97; IQR 4.99–6.71) (Table 1) with multiple joints affected and enhanced osteoresorption (median CTX 0.38 [IQR 0.26–0.49] pg/ml in RA vs 0.28 [IQR 0.21–0.32] pg/ml in CTRL). The majority of patients were seropositive with elevated inflammatory indicators (ESR and CRP). Although patients were actively treated (by NSAID, DMARD, glucocorticoid, or biologic therapy), DAS28 and other clinical parameters (SJC, TJC, CDAI) remained high, indicating poor regulation and disease progression. Compared with CTRL subjects, there were no differences between groups in terms of age (median age 65 [IQR 48–68] years for CTRL vs 65 [IQR 51–74] years for RA) or sex (male/female ratio 21/89 for CTRL vs 15/114 for RA).

To distinguish patients with RA on the basis of disease activity and to set the frame for further comparisons with laboratory and experimental data, we grouped disease parameters into variables reflecting the intensity of
Subsequently, a population of lymphoid-negative cells were analyzed by plotting lymphoid markers vs CD11b. Total PBMCs, depicted on forward vs side scatter plots, using the gating strategy presented in Fig. 2a. Briefly, by FACS to determine the frequency of OCP populations CTRL subjects and subjects with arthritis were analyzed. On the basis of our previous findings [3, 12], PBMCs of patients have distinct functional and phenotypic features Circulating osteoclast progenitor cells from arthritic patients have distinct functional and phenotypic features. In addition to PBMCs, SFMCs were analyzed using a similar gating strategy to determine specific systemic-to-local differences between myeloid cell subsets.

We presumed that the inclusion of an RA cohort with a significant proportion of highly active disease (sustained inflammation and joint destruction) would allow for a clearer detection of distinct OCP subpopulations induced by arthritis. Therefore, we first determined differences in PB of CTRL and RA samples. The DP (CD11b⁺CD14⁻) subset of PB monocytes was moderately expanded in patients with RA compared with CTRL subjects (Fig. 2b). In RA, the proportion of these cells was positively associated with plasma levels of TNF-α and RF (Fig. 2c) but not with the level of CTX (not shown). The DP PB population possesses osteoclastogenic potential because we were able to obtain fully functional bone-resorbing OCs from sorted cells under stimulation by M-CSF and RANKL (Fig. 2d). However, this population generated numbers of differentiated TRAP⁺ OCs similar to those of CTRL subjects when seeded at the same densities. In line with the latter finding, the frequency of PB DP cells expressing corresponding receptors—CD115 (M-CSF receptor) and RANK (RANKL receptor)—was comparable between CTRL subjects and patients with RA, indicating similar responsiveness to the respective ligands (Fig. 2b).

We further assessed differences between the circulatory and synovial compartments of patients with RA and found that the proportion of DP monocytes was comparable between PB and SF samples (Fig. 2b). Because it was not feasible to obtain appropriate CTRL SF samples (owing to

| Table 1 Demographic and clinical characteristics of patients with rheumatoid arthritis and psoriatic arthritis |
|-------------------------------------------------|-----------------|
| Age, years                                      | RA              |
|                                                | PsA             |
| Sex, male/female                                | 65 [51–74]      | 56 [44–74] |
| DAS28-ESR                                       | 15/114          |
| DAS28-ESR >5.1/DAS28-ESR ≥5.1, %                | 5.97 [4.99–6.71]|
| CDAI                                            | 5.50 [4.05–6.78]|
| Disease duration, years                         | 25.0%/75.0%     |
| ESR, mm/h                                       | 37.0 [24.0–44.1]|
| CRP, mg/L                                       | 33.6 [14.0–52.1]|
| RF, IU/L                                        | 18.5 [9.0–29.0] |
| ACPA, EU/L                                      | 27.5 [19.0–39.5]|
| Tender joint count (out of 28)                  | 27.5 [19.0–39.5]|
| Swollen joint count (out of 28)                 | 37.0 [24.0–44.1]|
| Disease activity, cm, physician’s VAS assessment | 79.7 [15.0–239.0]|
| Disease activity, cm, patient’s VAS assessment  | N/A             |

Abbreviations: RA Rheumatoid arthritis, PsA Psoriatic arthritis, ESR Erythrocyte sedimentation rate, CRP C-reactive protein, CDAI Clinical Disease Activity Index, DAS28-ESR Disease Activity Score in 28 joints based on erythrocyte sedimentation rate, VAS Visual analogue scale, RF Rheumatoid factor, ACPA Anticitrullinated protein antibodies, EU ELISA units, N/A Not applicable

Values are presented as median with IQR, except DAS28 and sex categories.
the limited amount and low cellularity of SFs in healthy synovial joints, as well as ethical unacceptability), we could not compare the SFMC profile between CTRL subjects and patients with RA. However, the proportion of DP SFMCs in RA compared with DP PBMCs in CTRL subjects was statistically higher, indicating a general increase of the DP population associated with RA. Expression of CD115 was lower in DP SFMCs than in the PBMC subset of patients with arthritis, which can be explained by enhanced shedding of membrane receptors for proinflammatory mediators seen as a negative feedback loop in inflamed tissues [25]. Nevertheless, the population of DP monocytes expressing RANK, considered to represent progenitors committed to OC lineage [3], was enlarged in the synovial vs the circulatory compartment, showing a positive correlation with TJC (Fig. 2c).

At this point, we also aimed to assess whether observed changes were specifically associated with RA compared with other forms of arthritis, as well as to analyze another group of SFMC samples (not possible to obtain from CTRL subjects). We included patients with PsA with affected peripheral joints; they resemble RA by having osteodestructive chronic inflammatory polyarthritis but are almost always seronegative with distinct pathogenic and clinical features. In general, the frequency of osteoclastogenic subpopulations followed a pattern in patients with PsA similar to that in patients with RA. Although the increase in proportion of DP monocytes did not reach significance, the proportion of these cells expressing RANK was significantly larger in RA than in CTRL subjects (Additional file 1: Figure S1a). In our previous study, we indicated that the RANKL–RANK axis may be specifically associated with PsA compared with RA pathogenesis [12]. However, the level of soluble RANKL was not significantly higher in PsA SF than in RA SF (p = 0.355), possibly owing to the small sample size (Additional file 1: Figure S1b).

Circulating osteoclast progenitors expressing chemokine receptors are exposed to higher plasma chemokine levels

After a functional confirmation of osteoclastogenic potential for CD3<sup>−</sup>CD19<sup>−</sup>CD56<sup>−</sup>CD11b<sup>+</sup>CD14<sup>+ </sup>cells, but without increases in the number of differentiated TRAP<sup>+</sup> OCs under M-CSF/RANKL stimulation in patients with arthritis, we hypothesized that the enhanced OCP activity might be caused by the complementary proinflammatory/osteoclastogenic stimuli, namely chemokine signals [3]. Monocytes, which comprise OCPs, are known to express a variety of chemokine receptors, which, upon ligand binding, initiate multiple cellular responses, including migration, proliferation, differentiation, and activation [26]. Proportions of DP subpopulations in PB were higher for several chemokine receptors in patients with RA (CCR1, CCR2, CCR4, CXCR4) (Fig. 3a) and in patients with PsA (CCR1, CXCR4) (Additional file 1: Figure S1c). In addition, receptor expression for CCR1 and CXCR4 was significantly higher in SFMCs than in PBMCs of patients with RA.

Upregulation of the distinct set of chemokine receptors on OCPs suggested their high susceptibility to chemokine signals and prompted us to determine the chemokine ligand profile in plasma and SF of patients with RA. Using the flow cytometry-based, predesigned Human Chemokine CBA Kit, we detected higher levels of CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 in arthritis than in CTRL plasma (Fig. 3b). Moreover, CXCL10 was significantly higher in SF than in plasma of patients with RA, indicating a chemotactic gradient for cell migration from the circulation to the synovial compartment. The increase of CCL2, CCL3 and CCL4 levels in SF of patients with RA was significant compared with
Expanded osteoclast progenitor (OCP) population correlates with indicators of disease activity in rheumatoid arthritis (RA).

**A** Peripheral blood mononuclear cells were analyzed by flow cytometry using the gating strategy for OCPs as nonlymphoid (CD3⁻CD19⁻CD56⁻) cells double-positive for monocyte markers CD11b and CD14, and they were further assessed for the expression of macrophage colony-stimulating factor receptor (CD115) and receptor activator of nuclear factor-κB (RANK).

**B** Proportion of OCPs, and CD115⁺ and RANK⁺ subsets in peripheral blood control (CTRL) subjects and patients with RA, as well as RA synovial fluid (SF) samples. Gates for the analysis were defined using unlabeled cells and "fluorescence minus one control". Association of the proportion of circulating OCPs with plasma levels of rheumatoid factor (RF) and tumor necrosis factor-α (TNF-α), as well as association of the proportion of RANK⁺ OCPs with tender joint count (TJC).

**D** Quantification, morphology, and bone-resorbing activity of osteoclasts (OCs) differentiated from sorted peripheral blood OCPs in RA and CTRL samples. Osteoclastogenic potential of circulating OCPs was evaluated in vitro by stimulating sorted CD3⁻CD19⁻CD56⁻CD11b⁺CD14⁺ cells with macrophage colony-stimulating factor (30 ng/ml) and RANK ligand (100 ng/ml). Multinuclear cells positive for tartrate-resistant acid phosphatase (TRAP) were counted as mature OCs. Bone-resorbing activity was confirmed for sorted cells by pit formation assay under the same culture conditions. TRAP-stained mature OCs and bovine cortical bone slices from CTRL and RA cultures were scanned using light microscopy at x 200 magnification. Red arrows indicate bone resorption pits, formed by active mature osteoclasts. Values are presented as medians (middle lines), with boxes representing IQR, whiskers representing 1.5 times the IQR, and squares or circles representing outliers. Group-to-group comparisons were performed using a nonparametric Mann-Whitney U test, whereas correlations were done using rank correlation and Spearman’s coefficient. p Values <0.05 are shown.
CTRL plasma, considered as the basal level. However, the difference was not significant compared with RA plasma, probably owing to the small number of SF samples and high variability (with part of the measurements below the detection limit of the CBA). Additionally, CXCL12 was measured by ELISA, with no difference found between groups (Fig. 3c). Indirectly, we confirmed that PBMCs were not the major source of chemokines by analyzing their gene expression using qPCR (Additional file 2: Figure S2). With the exception of CCL5, which was abundant at protein and gene expression levels, chemokines were barely detectable by qPCR, and there were no differences between groups, suggesting that they are produced by cells other than PBMCs and egress into the circulation to exert chemotactic effects.
Chemokine receptor/ligand levels are associated with clinical variables and indicate biological significance for disease pathogenesis and progression

To this point, our data confirmed increased chemokine levels in patients with RA, which may be responsible for enhanced migration of OCPs expressing appropriate receptors. Therefore, we further assessed the association between the frequency of OCPs and their chemokine receptor expression with the level of proinflammatory mediators and clinical parameters of the disease. The proportion of PB DP monocytes was positively associated with plasma levels of CCL2 and CCL5 (Fig. 4a), and both ligands were positively associated with TNF-α ($\rho = 0.549, p < 0.001$ for CCL2; $\rho = 0.554, p < 0.001$ for CCL5). In addition, the proportion of DP subsets expressing CCR1 and CCR4 was significantly associated with plasma levels of corresponding chemokine ligands ($\rho = 0.477, p = 0.006$ for CCR1 with CCL3; $\rho = 0.655, p < 0.001$ for CCR4 with CCL2; $\rho = 0.420, p = 0.010$ for CCR4 with CCL3). Besides findings in patients with RA, the proportion of PB DP monocytes was positively associated with ESR in patients with PsA ($\rho = 0.822, p < 0.001$).

Among analyzed chemokine receptors, the DP subset expressing CXCR4 showed the most significant associations with inflammatory indicators and disease activity, including TNF-α concentration, duration of arthritis, autoantibody levels, and bone resorption (Fig. 4b). Moreover, the proportion of the DP subset expressing CXCR4 was significantly lower in patients treated with DMARD (Fig. 4c), whom we showed to also have a lower bone resorption rate (Fig. 1b). Therefore, we concluded that the highly osteoclastogenic DP population of PBMCs, especially the subset expressing CXCR4, may be used as an indicator of disease activity and response to therapy.

Finally, we assessed the contribution of analyzed data to the frequency of PB DP cells and found that multiple logistic regression was able to explain 79% of the variability of the DP subpopulation frequency (adjusted $R^2 = 0.790$) from CCL5, CXCL10, and CXCR4 values. These variables significantly predicted the frequency of DP monocytes ($p < 0.001$), adding to the prediction in the following order: CCL5 ($\beta = 0.527, p < 0.0001$), CXCL10 ($\beta = 0.522, p < 0.0001$), and CXCR4 ($\beta = 0.284, p = 0.015$).

Chemokine receptor expression is significantly affected by anti-TNF therapy, indicating involvement of chemokine signals in anti-TNF response

Therapeutic targeting of proinflammatory mediators has been approved for arthritis treatment to suppress inflammatory responses as well as subsequent local and systemic bone loss. Our preliminary data on PsA samples ($n = 4$) showed a positive association between the proportion of PB DP monocytes and DAS28, as well as...
reduction of both variables along with the anti-TNF treatment in two of four patients [3].

In this study, the small cohort of patients (RA, n = 4; PsA, n = 5) receiving anti-TNF therapy was followed to 6 months after the initial application. PBMCs were analyzed as previously described for patients with RA and patients with PsA (Fig. 2a). The total DP population was not suppressed by the treatment, and neither were the subsets expressing receptors for crucial osteoclastogenic factors: CD115 and RANK (Fig. 5a). In general, individual values were highly variable even at the starting point, which may be due to the fact that included patients were already in the chronic phase of the disease, having had arthritis for more than 10 years and having previously been treated with different combinations of NSAIDs, glucocorticoids, and DMARDs. However, the subpopulation of DP monocytes expressing CCR4 showed significant dynamic changes in response to anti-TNF therapy, exhibiting a significant negative trend during the anti-TNF treatment in patients with RA and patients with PsA, offering the possibility of using it as an indicator of a good therapeutic response (Fig. 5b). We observed elevated plasma levels of major CCR4 ligands (CCL2, CCL3, CCL5) and a significant positive correlation of CCR4 expression with CCL2 and CCL3 in patients with RA. Therefore, the downregulation of CCR4 may be important to reducing the susceptibility of OCPs to the chemokine gradient.

**Functional in vitro confirmation of direct osteoclastogenic and chemotactic effect of chemokines on circulating osteoclast progenitors**

Finally, we functionally tested the effects of chemokines on circulatory monocytes in vitro for their chemotactic and osteoclastogenic properties. PBMCs from individual samples of RA patients were first stimulated by M-CSF overnight, then nonadherent cells were harvested and split into two groups: One was grown further in osteoclastogenic culture conditions under chemokine treatment to assess direct osteoclastogenic effects, and the other was plated in a Transwell culture system to determine chemotaxis of OCPs toward the chemokine gradient (Fig. 6a). The latter migration assay was further analyzed for the number of attracted cells and for their osteoclastogenic potential. For these experiments, we selected CCL2, CCL5, and CXCL10 on the basis of receptor/ligand profiling in PB samples (Fig. 3). Besides

---

**Fig. 5 Alteration of chemokine receptor expression on circulatory osteoclast progenitors (OCPs) in follow-up of anti-TNF therapy.**

(a) Proportion of OCPs (CD3+CD19−CD56−CD11b+CD14+) and their subsets expressing macrophage colony-stimulating factor receptor (CD115) and receptor activator of nuclear factor-κB (RANK) in peripheral blood of patients with rheumatoid arthritis (RA) and patients with psoriatic arthritis (PsA) before (0) and 2, 4, and 6 months after induction of anti-TNF therapy (etanercept 50 mg weekly or adalimumab 40 mg every 2 weeks). (b) Subsets of OCPs expressing chemokine receptors with regard to duration of anti-TNF therapy in patients with RA and patients with PsA. Values are presented as median with IQR for each time point. Analysis for linear trend was performed using one-way analysis of variance with polynomial contrast type. p Values <0.05 are shown. CCR CC chemokine receptor, CXCR CXC chemokine receptor.
higher CCR1, CCR2, and CCR4 (that bind CCL2 and CCL5), we found that CXCR3 (receptor for CXCL10) was also increased on OCPs in patients with RA (median 43.1% [IQR 33.3–54.2] in CTRL vs 61.5% [IQR 53.3–69.2] in RA, \( p = 0.009 \)). CCL2 was taken as representative of a chemokine involved only in monocyte recruitment, whereas CXCL10 was taken as a chemokine that nonselectively attracts both myeloid and lymphoid lineages. In addition, we tested CCL5, which was found to be elevated in plasma but lower in SF, indicating its role in systemic activation rather than in joint-specific homing [27]. On the basis of CXCR4 expression (Fig. 3a), CXCL12 was also selected for in vitro migration assays, but in our culture conditions, the effects on osteoclastogenesis or chemotaxis were marginal or not observed, respectively (median ratio 1.31 [IQR 1.21–1.38] for the number of OCs in CXCL12-treated vs nontreated cultures, \( p = 0.106 \); ratio 0.97 [IQR 0.77–1.56] for the number of migrated cells in CXCL12-treated vs nontreated cultures, \( p = 1.000 \)).

We expected great susceptibility of PB monocytes to chemokine signals in RA, considering their higher...
expression of chemokine receptors than CTRL (Fig. 6b). In general, CCL2, CCL5, and CXCL10 showed measurable osteoclastogenic as well as chemotactic effects. In the direct osteoclastogenic effect, all tested chemokines showed comparable potential (doses were optimized in accordance with the median effective dose provided by the manufacturer) and generated similar numbers of TRAP⁺ OCs (Fig. 6c). In addition, the identity of differentiated OCs was further confirmed by expression of VNR and bone-resorbing activity (Additional file 3: Figure S3). Interestingly, CCL5 showed the greatest chemotactic effects among tested chemokines by attracting the highest number of cells in the migration assay (Fig. 6d). In addition, these migrated cells possessed greater osteoclastogenic potential when stimulated by RANKL and M-CSF than untreated cells and cells attracted to CCL2 and CXCL10 gradient (Fig. 6e). These results imply that chemokine signals not only stimulate chemotaxis of OCPs but also act synergistically with RANKL and M-CSF to enhance their differentiation into mature OCs.

Discussion

Our study provides evidence of the importance of chemokine signals to complement the canonical M-CSF/RANKL-mediated OC differentiation pathway by stimulation of OCP migration and maturation. Although genetic experiments in mice and primary human cell cultures have helped define the regulation of OC differentiation under physiological conditions, alternative pathways of OC stimulation, especially in patients with inflammatory conditions, are not yet clear. The complex environment that orchestrates pathological osteoresorption involves a variety of mediators and several cell types, both as sources and targets of these secreted factors [28]. Our study revealed specific roles for several chemokines. Namely, CCL2, CCL5, and CXCL10 exhibit a similar magnitude of direct osteoclastogenic action, whereas when tested in vitro, CCL5 has the strongest chemotactic effect on OCPs. However, CCL5 was lower in SF than in plasma of patients with RA in vivo, suggesting its significance for the systemic mobilization of myeloid cells rather than their accumulation in the synovial compartment.

The susceptibility of OCPs to chemotactic signals and the increased production of respective chemokines create optimal conditions for their enhanced homing not only to affected joints but also at the systemic level [29]. Immunophenotyping of PB cells from patients with RA showed a moderate expansion of the total monocyte pool (CD11b⁺CD14⁺), with high osteoclastogenic activity. Furthermore, frequencies of OCP subpopulations expressing chemokine receptors (CCR1, CCR2, CCR4, CXCR3, CXCR4) were significantly increased. These subpopulations, specifically CD3⁺CD19⁻CD56⁻CD11b⁻CD14⁺CXCR4⁺ cells, were positively associated with the indicators of disease severity (i.e., levels of TNF-α, chemokines, autoantibodies, and CTX) and further expanded in the synovial compartment. Moreover, the reduction of the CXCR4⁺ subset in patients treated with DMARDs pointed to the potential application of its frequency as an indicator of a good therapeutic response. Inactivation of CXCR4 has been shown to effectively ameliorate symptoms in murine collagen-induced arthritis, a mouse model of RA [30]. Although CXCL12, an exclusive CXCR4 ligand, has also been implicated in the pathogenesis of several skeletal disorders, including RA [31], we found no difference in CXCL12 levels between plasma and SF of patients with RA, nor did we find a difference compared with the CTRL group. In our in vitro assay, CXCL12 did not show a significant osteoclastogenic or chemotactic effect. Studies in which researchers observed a chemotactic effect of CXCL12 were focused mainly on bone marrow and not on PB OCPs [32, 33]. Thus, in the systemic circulation, involvement of the CXCL12-CXCR4 axis in RA seems to depend on receptor rather than ligand modulation.

In contrast to CXCL12, several tested chemokines (CCL2, CCL4, CXCL10) were elevated in plasma and enriched in SF, indicating that they are produced by cells within the synovial compartment, from whence they spill over into the circulation proportionately to the intensity of the disease [34]. Although aware that a comparison between arthritic and CTRL SF would provide better insights into the changes of the chemokine ligand/receptor profile associated with arthritis, we were unable to obtain appropriate CTRL SF, owing to the limited amount and low cellularity in healthy synovial joints. In addition, arthrocentesis in patients with arthritis was performed for therapeutic evacuation of SF, whereas SF aspiration from healthy subjects has no ethical justification, considering the pain and discomfort involved as well as the risk of articular tissue damage, infection, and joint dysfunction [35]. On the basis of increases in proportions of CCR1⁺ and CCR2⁺ DP subsets, we propose that the CCR1- and CCR2-mediated chemotaxis (by CCL2 and CCL4 ligands) is important for joint-specific recruitment of OCPs. CCR2, expressed in lymphoid and myeloid cells, is important for the recruitment of cells from several lineages, including monocytes, into the joints [27, 36, 37]. In an in vitro study, Lebre et al. suggested that CCR1 blockade might be effective in inhibiting the migration of monocytes toward the synovial compartment in RA [27]. Among DP SFMCs, the CCR4⁺ population was not enriched, indicating that CCR4 is not crucial for accumulation of monocytes in arthritic joints, as opposed to its importance for T lymphocytes [38]. However, circulatory CCR4⁺ OCPs were significantly suppressed during anti-TNF therapy. There are several
functional links between TNF-α and chemokine effects that can be modulated by TNF blockade, including TNF-mediated vascular activation that promotes leukocyte adhesion and chemotaxis [39], as well as TNF-induced chemokine secretion by resident and infiltrating inflammatory cells [27]. The dynamic changes of the chemokine receptor profile by anti-TNF treatment observed in our study may reduce OCP susceptibility to chemokine signals and offer a powerful tool to control disease progression.

In addition to providing chemotactic signals, chemokines may act additively to M-CSF/RANKL stimulation to enhance OC differentiation. In our in vitro conditions, we observed an increased number of differentiated bone-resorbing OCs under CCL2, CCL5, and CXCL10 treatment. Studies on isolated human PB monocytes using different culture systems pointed to a direct osteoclastogenic effect of several chemokines, including CCL2 and CCL5, through autocrine and paracrine loops [12, 40, 41]. Besides a direct osteoclastogenic effect, chemokines may be involved in ACPA-mediated OC differentiation [42]. These noncanonical pathways of OC differentiation are specifically important in inflammatory settings, when chemokines as well as proinflammatory cytokines (TNF-α and IL-1) substitute RANKL and M-CSF to augment OC differentiation [43]. A RANKL surplus has not been observed in some animal models of inflammation-induced bone loss, explaining why blocking of the canonical M-CSF/RANKL-mediated osteoclastogenic pathway does not achieve clinical benefit [13]. Negative feedback mechanisms initiated during excessive inflammation may cause refractoriness to M-CSF by ectodomain shedding of CD115 on early human OCPs [25]. This is in line with our observation of significantly reduced CD115 membrane expression in SF-derived monocytes from patients with RA as well as patients with PsA. However, reduced M-CSF expression was not associated with downregulation of RANK expression, suggesting that OC differentiation proceeded despite negative feedback, which was possibly overridden by the excess of other proinflammatory osteoclastogenic signals.

Chemotactic signaling is thought to be important in the pathogenesis of RA, and various chemokines, along with their respective receptors, have been implicated in leukocyte ingress into inflamed synovia [8]. In spite of tremendous efforts to identify immunological abnormalities in RA, a clear association between specific immune cell populations and RA disease activity measures has not been identified, especially for innate immune cells. The majority of studies have dealt with cells involved in adaptive immunity, especially CD4+ T lymphocytes [44]. Substantial expression of chemokine receptors on PB monocytes, and more specifically on OCPs, may be particularly important for RA pathogenesis because chemokines initiate several cellular responses crucial for the destructive effects on bone tissue. Although much is known regarding chemokine ligand and receptor profiles in patients with RA, blocking therapy generally did not yield the expected results, thus indicating that more data are required regarding cell- and disease-specific details. The relevant myeloid subpopulations might be more heterogeneous than currently appreciated, with unique markers of OCP subpopulations in various forms of arthritis [13, 28]. By immunophenotyping the chemokine receptor profile on OCPs, and by assessing the in vivo chemokine milieu as well as its in vitro effects, we were able to add to the understanding of the complex inflammatory network present in patients with RA.

Conclusions
Our results reveal the role of chemokines as potent proinflammatory, chemotactic, and osteoclastogenic factors that may be important for systemic stimulation of OCPs, as well as their increased migration, subsequent tissue homing, and in situ maturation. In addition, we identified arthritis-associated OCP subsets expressing chemokine receptors, which are therefore susceptible to the chemokine gradient, explaining local as well as generalized bone loss seen in RA. Novel insights with regard to the migratory behavior and functional properties of OCPs in response to chemokine signals could open a doorway to new therapeutic targets in RA.

Additional files

Additional file 1: Figure S1. Increased frequency of osteoclast progenitor cells and subsets expressing chemokine receptors in peripheral blood and synovial fluid samples of patients with RA and PsA. a Proportion of OCPs, subsets expressing macrophage colony-stimulating factor receptor (CD115) and RANK in peripheral blood of CTRL subjects and patients with arthritis, and SF samples (SF RA, SF PsA), assessed by flow cytometry. b Concentrations of soluble RANKL in SF of arthritic patients, measured by ELISA. c Chemokine receptor expression on OCPs in peripheral blood of CTRL subjects and patients with arthritis, and SF of patients with arthritis, assessed by flow cytometry. Values are presented as medians (middle line), with boxes representing IQR, whiskers representing 1.5 times the IQR, and squares or circles representing outliers. Group-to-group comparisons were performed using a nonparametric Mann-Whitney U test, and p values <0.05 are shown for comparisons made between PsA and other groups. Previously shown p values for comparisons between RA and CTRL groups (Figs. 2 and 3) are not shown again for the sake of visual clarity. (TIF 1768 kb)

Additional file 2: Figure S2. Low level of chemokine gene expression in PBMCs of patients with RA. qPCR analysis of the expression of chemokine genes in PBMCs of CTRL patients and patients with RA, presented as RNA RQ. Values are presented as medians (middle line), with boxes representing IQR, whiskers representing 1.5 times the IQR and squares or circles representing outliers. Group-to-group comparisons were performed using a nonparametric Mann-Whitney U test, p values <0.05 are shown. (TIF 987 kb)

Additional file 3: Figure S3. OCs differentiated from PBMCs exhibit OC-specific phenotype and bone-resorbing activity. The number of
multinucleated cells expressing TRAP was used for quantification of differentiated OCs under stimulation by M-CSF and RANKL. To further confirm the identity of OCs, parallel cultures were performed and stained for VNR expression. Functional bone-resorbing activity of differentiated OCs was confirmed by pit formation assays using bovine cortical bone slices under the same culture conditions. Presented are representative images of osteoclastogenic cultures of PBMCs from control subjects and patients with RA. In addition, cultures from patients with RA stimulated with CCL5 (10 ng/ml) are shown to confirm the osteoclastogenic effect of CCL5. Red arrows indicate bone resorption pits, formed by active mature osteoclasts. TRAP-stained mature OCs and bovine cortical bone slices were imaged under light microscopy at × 200 magnification. VNR-expressing mature OCs were imaged using a fluorescence microscope at × 200 magnification. (TIF 4477 kb)

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Only group data are presented, and they are not attributable/traceable to any individual person. Consent to use and publish the data collected in this way was obtained from all participants.

Ethics approval and consent to participate
The study was performed in accordance with the Declaration of Helsinki and was approved by the University of Zagreb School of Medicine Ethics Committee (525-10/0255-15-3, given on the basis of prior approval of all three involved clinical departments). Informed consent to participate in the study was obtained from all participants.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1Croatian Institute for Brain Research, University of Zagreb School of Medicine, Salata 12, Zagreb HR 10000, Croatia. 2Department of Physiology and Immunology, University of Zagreb School of Medicine, Salata 3b, Zagreb HR 10000, Croatia. 3Department of Rheumatology, Physical Medicine and Rehabilitation, Clinical Hospital Center “Sestre Milosrdnice”, University of Zagreb School of Medicine, Vngrodaska cesta 29, Zagreb HR 10000, Croatia. 4Department of Clinical Immunology and Pulmonology, Clinical Hospital “Sveti Duh”, Sveti Duh HR 10000, Croatia. 5Department of Clinical Immunology and Rheumatology, Clinical Hospital Center “Zagreb”, Kipsaticeva 12, Zagreb HR 10000, Croatia. 6Department of Anatomy, University of Zagreb School of Medicine, Salata 11, Zagreb HR 10000, Croatia.

Received: 6 February 2017 Accepted: 16 May 2017
Published online: 15 June 2017

References
1. Jung SM, Kim KW, Yang CW, Park SH, Ju JH. Cytokine-mediated bone destruction in rheumatoid arthritis. J Immunol Res. 2014;2014:263825.
2. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The global burden of rheumatoid arthritis: estimates from the Global Burden of Disease 2010 study. Ann Rheum Dis. 2014;73(7):1316–22.
3. Sucur A, Katavic V, Kelava T, Jajić Z, Kovacic N, Grecovic D. Induction of osteoclast progenitors in inflammatory conditions: key to bone destruction in arthiritis. Int Orthop. 2014;38(9):1893–903.
4. Goldring SR, Purdue PE, Cotti TN, Shen Z, Flannery MR, Binder NB, et al. Bone remodelling in inflammatory arthritis. Ann Rheum Dis. 2013;72 Suppl 2:i62–5.
5. Schett G. Osteoimmunology in rheumatic diseases. Arthritis Res Ther. 2009;11(2):210.
6. Takayangi H. Osteoimmunology and the effects of the immune system on bone. Nat Rev Rheumatol. 2009;5(12):667–76.
7. Arau F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasil K, et al. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor-κB (RANK) receptors. J Exp Med. 1999;190(12):1741–54.
8. Braun T, Zvejina J. Positive regulators of osteoclastogenesis and bone resorption in rheumatoid arthritis. Arthritis Res Ther. 2011;13(4):235.
9. Souza PP, Lerner UH. The role of cytokines in inflammatory bone loss. Immuno Invest. 2013;42(7):555–622.
10. Del Fattore A, Tett A,ucci N. Bone cells and the mechanisms of bone remodelling. Front Biosci. 2012;24:2302–21.
11. Deal C. Bone loss in rheumatoid arthritis: systemic, periarticular, and focal. Curr Rheumatol Rep. 2012;14(3):231–7.
12. Ilic M, Jajić Z, Lazic E, Ivicevic S, Grubisic F, Marusic A, et al. Association of systemic and intra-articular osteoclastogenic potential, pro-inflammatory mediators and disease activity with the form of inflammatory arthritis. Int Orthop. 2013;37(11):1883–92.
13. Adamopoulos IE, Mellins ED. Alternative pathways of osteoclastogenesis in inflammatory arthritis. Nat Rev Rheumatol. 2015;11(3):189–94.
14. Szekeczey Z, Koch AE. Successes and failures of chemokine-pathway targeting in rheumatoid arthritis. Nat Rev Rheumatol. 2016;12(1):5–13.
15. Galliera E, Locati M, Mantovani A, Corsi MM. Chemokines and bone remodeling. Int J Immunopathol Pharmacol. 2008;21(3):485–91.
16. Khan UA, Hashimi SM, Khan S, Quan J, Bakr MM, Forwood MR, et al. Differential expression of chemokines, chemokine receptors and proteinases by foreign body giant cells (FBGCs) and osteoclasts. J Cell Biochem. 2014;115(7):1200–8.
17. Hoshino A, Ueha S, Hanada S, Imai T, Ito M, Yamamoto K, et al. Roles of chemokine receptor CXCR1 in maintaining mature bone homeostasis through the regulation of both osteoblasts and osteoclasts. J Cell Sci. 2013;126(Pt 4):1032–45.
18. Kim MS, Magno CL, Day CJ, Morrison NA. Induction of chemokines and chemokine receptors CCR2b and CCR4 in authentic human osteoclasts differentiated with RANKL and osteoclast like cells differentiated by MCP-1 and RANTES. J Cell Biochem. 2006;97(3):152–8.
19. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham 3rd CO, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62(9):2569–81.
20. Moll JM, Wright V. Psaoriatic arthritis. Semin Arthritis Rheum. 1973;3(1):55–78.
21. Fransen J, van Riel PL. The Disease Activity Score and the EULAR response criteria. Rheum Dis Clin North Am. 2009;35(4):745–57.
22. Anderson J, Caplan L, Yazdany J, Robbins ML, Neogi T, Michaud K, et al. Rheumatoid arthritis disease activity measures: American College of Rheumatology recommendations for use in clinical practice. Arthritis Care Res (Hoboken). 2012;64(5):640–7.
23. Jajić J, Jajić Z. The development of rheumatology through two millennia. Zagreb, Croatia: Bitroisak; 2008.
24. Wisłowska M, Jakubicz D, Stepien K, Cicha M. Serum concentrations of formation (PINP) and resorption (Ctx) bone turnover markers in rheumatoid arthritis. Rheumatol Int. 2009;29(12):1403–9.
25. Ivashkiv LB, Zhao B, Park-Min KH, Takami M. Feedback inhibition of CXCL10 expression in synovium is associated with disease activity and bone and joint destruction in patients with rheumatoid arthritis treated with golimumab. Mod Rheumatol. 2016;26(1):46–50.
26. Zhang Q, Guo R, Schwarz EM, Boyce BF, Xing L. TNF inhibits production of stromal cell-derived factor 1 by bone stromal cells and increases osteoclast precursor mobilization from bone marrow to peripheral blood. Arthritis Res Ther. 2008;10(2):R87.
27. Wright LM, Maloney W, Yu X, Kindle L, Collin-Osdoby P, Osdoby P. Stromal cell-derived factor-1 binding to its chemokine receptor CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human, osteoclasts. Bone. 2005;36(5):840–53.
28. Pandya JM, Lundell AC, Andersson K, Nordström I, Theander E, Rudin A. Blood cell mobilization in untreated early rheumatoid arthritis. CXCL10 as a disease activity marker. Arthritis Res Ther. 2017;19:20.
29. Hui AY, McCarty WJ, Masuda K, Firestein GS, Sah RL. A systems biology approach to synovial joint inflammation in health, injury, and disease. Wiley Interdiscip Rev Syst Biol Med. 2012;4(1):15–37.
30. Talbot J, Blanchini FJ, Nascimento DC, Oliveira RD, Souto FO, Pinto LG, et al. CCR2 expression in neutrophils plays a critical role in their migration into the joints in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(1):1751–9.
31. Brühl H, Cihák J, Plachý J, Kunz-Schughart L, Niedermeier M, Denzel A, et al. Targeting of Gr-1+, CCR2+ monocytes in collagen-induced arthritis. Arthritis Rheum. 2007;56(9):2975–85.
32. Flytlik HA, Hvid M, Lindgreen E, Kofoed-Olsen E, Petersen EL, Jørgensen A, et al. Expression of MDC/CCL22 and its receptor CCR4 in rheumatoid arthritis, psoriatic arthritis and osteoarthritis. Cytokine. 2010;49(1):24–9.
33. Obereci R, Schuetz, J, Schuetz H, Koch AK, Luchtefeld M, Grote K, et al. Targeting tumor necrosis factor-α with adalimumab results in endothelial activation and monocyte adhesion and are induced by inflammatory cytokines in osteoblasts. J Bone Miner Res. 2004;19(12):2065–77.
34. Krishnamurthy A, Joshua V, Raj Hensolov A, Jin T, Sun M, Vvar N, et al. Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss. Ann Rheum Dis. 2016;75(4):721–9.
35. Kim HR, Kim KW, Kim BM, Jung HG, Cho ML, Lee SH. Reciprocal activation of CD4+ T cells and synovial fibroblasts by stromal cell-derived factor 1 promotes RANKL expression and osteoclastogenesis in rheumatoid arthritis. Arthritis Rheum. 2014;66(3):538–48.
36. Nagafuchi Y, Shoda H, Sumitomo S, Nakachi S, Kato R, Tsuchiya Y, et al. Immunophenotyping of rheumatoid arthritis reveals a leakage between HLA-DRB1 genotype, CXCR4 expression on memory CD4+ T cells, and disease activity. Sci Rep. 2016;6:20338.

Submit your next manuscript to BioMed Central and we will help you at every step:

• We accept pre-submission inquiries
• Our selector tool helps you to find the most relevant journal
• We provide round the clock customer support
• Convenient online submission
• Thorough peer review
• Inclusion in PubMed and all major indexing services
• Maximum visibility for your research

Submit your manuscript at www.biomedcentral.com/submit