Inflammatory monocytes and Fcγ receptor IV on osteoclasts are critical for bone destruction during inflammatory arthritis in mice

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Destruction of bone tissue by osteoclasts represents a severe pathological phenotype during inflammatory arthritis and results in joint pain and bone malformations. Previous studies have established the essential role of cytokines including TNFα and receptor–ligand interactions, such as the receptor activator of nuclear factor-kappa B–receptor activator of nuclear factor-kappa B ligand interaction for osteoclast formation during joint inflammation. Moreover, autoantibodies contribute to joint inflammation in inflammatory arthritis by triggering cellular fragment crystallizable (Fc)γ receptors (FcγR), resulting in the release of proinflammatory cytokines and chemokines essential for recruitment and activation of innate immune effector cells. In contrast, little is known about the expression pattern and function of different FcγRs during osteoclast differentiation. This would allow osteoclasts to directly interact with autoantibody immune complexes, rather than being influenced indirectly via proinflammatory cytokines released upon immune complex binding to other FcγR-expressing innate immune cells. To address this question, we studied FcγR expression and function on osteoclasts during the steady state and during acute joint inflammation in a model of inflammatory arthritis. Our results suggest that osteoclastogenesis is directly influenced by IgG autoantibody binding to select activating FcγRs on immature osteoclasts, resulting in enhanced osteoclast generation and, ultimately, bone destruction.

Fc receptor | monocyte differentiation

Rheumatoid arthritis is a chronic autoimmune disease characterized by joint inflammation and bone and cartilage destruction (1, 2). There is convincing evidence that autoantibodies of the IgG isotype are critically involved in this process and that cellular fragment crystallizable (Fc)γ receptors (FcγRs) are essential for autoantibody-induced tissue damage (3–5). Thus, mice deficient in expression of functional FcγRs are protected from joint inflammation in both active and passive models of inflammatory arthritis (6–10). A variety of innate immune effector cells were demonstrated to be involved at different stages of the inflammatory process in vivo. Mast cells, for example, were shown to be critical for the initiation of autoantibody-induced arthritis development via the early production of IL-1 (11–16). Consistently, mice deficient in FcγRIII, which is the only activating Fcγ receptor expressed on mast cells, were nearly completely protected from joint inflammation and bone destruction (6). Apart from mast cells, deletion of neutrophils, monocytes, and macrophages was demonstrated to be critical for joint inflammation and pannus formation (7, 17–22). Interestingly, neutrophil recruitment was independent of FcγRIII, and it was demonstrated more recently that mouse activating FcγRIV, which is expressed on neutrophils, macrophages, and the Ly6C^{low} resident or nonclassical monocyte subset was responsible for pannus formation (10, 18, 23). In contrast, mice deficient in the high-affinity FcγRI were not protected from joint inflammation (6). Despite the considerable amount of knowledge about the role of IgG and FcγRs in activation and recruitment of innate immune effector cells to the inflamed joint, not much is known about further downstream effector cells and, most importantly, about osteoclasts that are directly involved in bone destruction. Osteoclasts normally reside within the bone tissue and are critical for the constantly ongoing bone remodeling. Upon recruitment of myeloid precursors of osteoclasts to the inflamed joint, the proinflammatory milieu supports the development of mature osteoclasts that start to resorb bone and are ultimately responsible for joint destruction (1, 24). Current strategies to block bone and joint destruction target key proinflammatory cytokines, such as TNFα or IL-6 (1, 2). In mouse models of arthritis blocking IL-1 and macrophage colony-stimulating factor (M-CSF) signaling was also efficient in ameliorating arthritis development (13, 17). Despite the great success of this therapeutic intervention, about 50% of patients with established arthritis may not respond to TNFα blockade, warranting further studies into the mechanism of autoantibody-dependent rheumatoid arthritis (25). FcγRs are a prime target for blocking autoantibody activity because they provide the direct link between the pathogenic antibody and innate effector cell activation (5, 26, 27). A prerequisite for targeting osteoclasts with such an approach, however, is a detailed understanding of FcγR expression and function on this cell type. By using a well-established passive model of murine inflammatory arthritis we show that the Ly6C^{high} inflammatory monocyte subset represents the major precursor cell population of osteoclasts in inflamed joints. Upon activation via receptor activator of nuclear factor-kappa B ligand (RANKL) these cells differentiate into mature osteoclasts paralleled by up-regulation of FcγRIV. Cross-linking of FcγRIV enhanced osteoclast differentiation, demonstrating that immune complex binding to osteoclasts is directly involved in the maturation process. Conversely, FcγRIV-deficient mice and mice with a targeted deletion of FcγRIV on osteoclasts were protected from autoantibody-dependent bone destruction and had lower numbers of osteoclasts in the inflamed joints. These results may suggest that interfering with the autoantibody–FcγR interaction on osteoclasts could represent a therapeutic strategy to block osteoclast-dependent joint destruction.

Results and Discussion

Expression of FcγRs on Osteoclast Precursor Cells and Mature Osteoclasts. Whereas it is clear that osteoclasts are essential for bone resorption

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during rheumatoid arthritis, it is largely unknown which FcγRs are expressed on osteoclasts (28, 29). In addition, there are conflicting results about the cell types that can give rise to osteoclasts during joint inflammation, although it seems clear that monocytes recruited to the inflamed joint have the potential to develop into osteoclasts (24, 30, 31). Because it was suggested that osteoclasts may develop from the Ly6C^{low} monocyte subset and our previous studies have shown that FcγRIV is selectively expressed on these cells, we first tested whether this monocyte subset indeed has the potential to develop into osteoclasts (32, 33). For this, FACS-sorted Ly6C^{low} and Ly6C^{high} monocyte subsets from mouse bone marrow and blood were cultured in the presence of RANKL and M-CSF and subsequently analyzed for the presence of multinucleated tartrate resistant acid phosphatase (TRAP)-positive cells. These experiments revealed that not the FcγRIV-positive Ly6C^{low}, but rather the FcγRIV-negative Ly6C^{high}, inflammatory (also referred to as classical monocytes) monocyte subset had the capacity to develop into osteoclasts under these conditions (Fig. 1*A*). Similar results were obtained with sorted human monocyte subsets (CD14^{low}/CD16^{high} vs. CD14^{high}/CD16^{neg}), firmly establishing that the inflammatory but not the resident monocyte subset (also referred to as nonclassical monocytes) can differentiate into osteoclasts (Fig. S1*A*) (24). To establish a role for inflammatory monocytes as osteoclast precursors in vivo, we used a C–C chemokine receptor type 2 (CCR2)-specific antibody to deplete this monocyte subset during the induction of inflammatory arthritis by passive transfer of K/BxN serum. As expected, this treatment resulted in a strong reduction of inflammatory monocytes and only mildly affected the resident monocyte subset (Fig. S2 *A* and

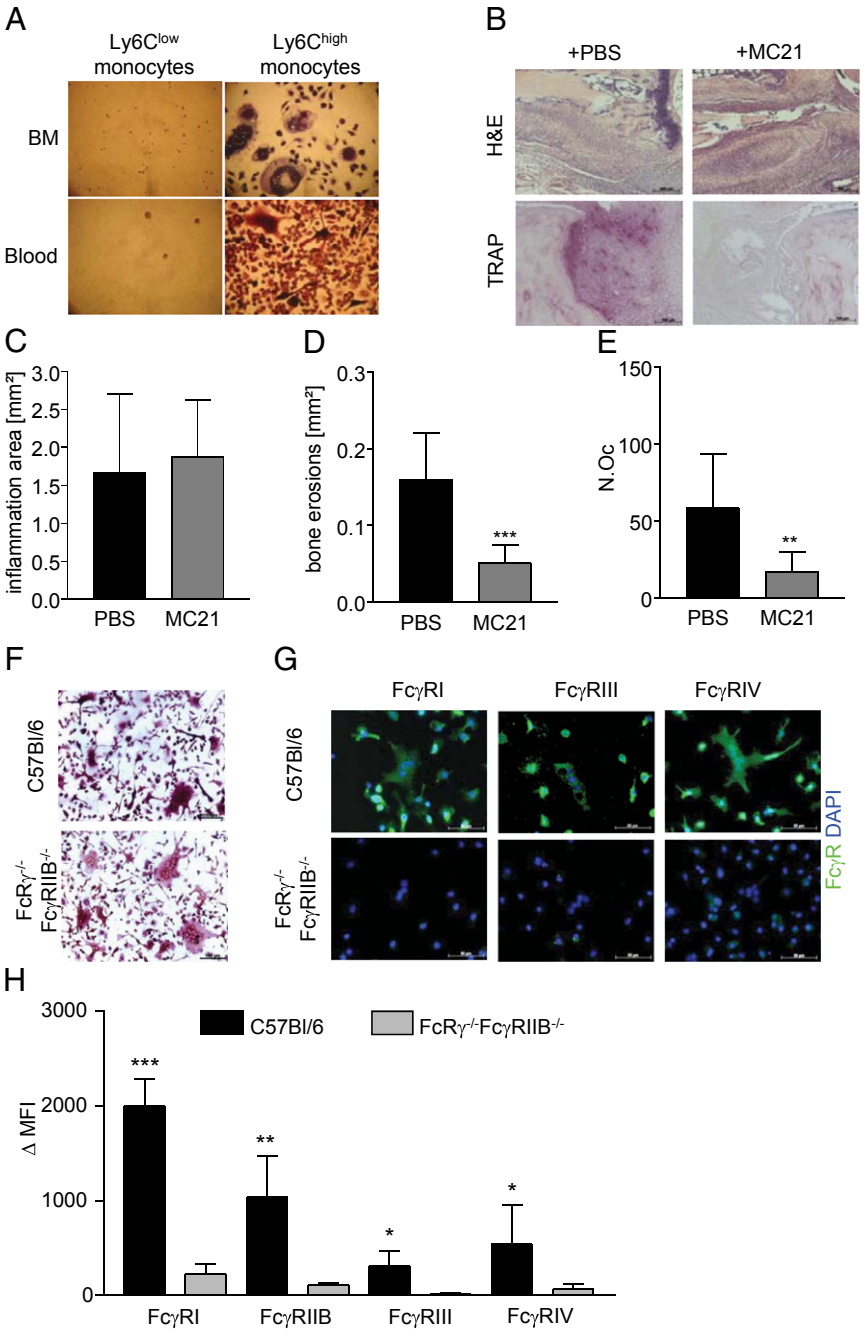

Fig. 1. Osteoclast precursor cells and FcγR expression on osteoclasts. (*A*) Ly6C^{low} and Ly6C^{high} monocyte subsets were sorted from mouse bone marrow (BM) and peripheral blood and cultivated in osteoclast differentiation medium for 7 d. Shown are representative pictures from at least three independent experiments of TRAP-stained Ly6C^{low} and Ly6C^{high} monocyte subset cultures at day 7 of culture in osteoclast differentiation medium. (*B–E*) Effect of Ly6C^{high} monocyte depletion on the development of osteoclasts, joint inflammation, and bone destruction during inflammatory arthritis. Shown are representative H&E- and TRAP-stained sections of inflamed joints at day 7 after induction of inflammatory arthritis in mice treated with daily injections of PBS or the CCR2-specific antibody MC21 to deplete the Ly6C^{high} monocyte subset (*B*). The severity of joint inflammation and bone destruction was evaluated by measuring the area of the inflammatory infiltrate (*C*) and of bone erosions (*D*) and by counting the number of osteoclasts (N.Oc.) at the sites of bone erosions (*E*). Values in C–E are the mean ± SD. The experimental groups consisted of five 12-wk-old C57BL/6 mice and the experiment was performed twice with comparable results. (*F–H*) FcγR expression on osteoclasts. (*F*) Shown are representative TRAP-stained pictures derived from three independent experiments of osteoclast cultures generated from the bone marrow of C57BL/6 or FcRγ/FcγRIIB double-deficient mice (FcRγ $^{-/-}$FcγRIIB$^{-/-}$). (*G*) Representative immunofluorescence stainings of osteoclast cultures from the bone marrow of C57BL/6 or FcRγ/FcγRIIB double-deficient mice using either FcγRI-, FcγRIII-, or FcγRIV-specific antibodies in combination with DAPI to detect cell nuclei. (*H*) Quantification of FcγRI-, FcγRIIB-, FcγRIII-, and FcγRIV expression on F4/80+ OSCAR+ cells differentiated from the bone marrow of C57BL/6 (black colums) or FcRγ/FcγRIIB knockout mice (gray columns) by FACS analysis. Shown is the delta median fluorescence intensity (Δ MFI) ± SD compared with unstained samples. Experiments shown in *F–H* were performed three times with comparable results. Pictures shown in *F* and *G* were taken at 200× magnification (TRAP staining) or 400× magnification (immunofluorescence). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

B). As shown in Fig. 1 *B–E*, this did not result in changes in the size of the inflammatory infiltrate, which is dominated by neutrophils, but did virtually abrogate bone destruction and resulted in a dramatic decrease of osteoclast formation.

We next assessed the expression of activating FcγRs on immature and mature osteoclasts, revealing that FcγRI and FcγRIII expression, which is already present on the Ly6C^{high} monocyte precursor cell, is maintained (32). In addition, however, FcγRIV becomes up-regulated during osteoclast maturation, potentially enabling them to interact with mouse IgG2a autoantibodies present in the inflammatory arthritis-inducing serum (Fig. 1 *F–H*) (10). Besides activating FcγRs, expression of the inhibitory FcγRIIB, already present on inflammatory monocytes, was maintained and comparable between different strains of activating FcγR-deficient mice (Fig. S2*C*). Consistent with these results in mice, also human osteoclasts expressed the full set of human FcγRs and up-regulated FcγRIIIA (CD16) upon differentiation from FcγRIIIA^{negative} CD14^{high} inflammatory monocytes into osteoclasts (Fig. S1*B*).

Impact of FcγR Deletion on Bone Homeostasis and Osteoclast Development During the Steady State. Having established that all activating FcγRs are expressed on mouse and human osteoclasts, we next investigated whether the absence of individual or all functional activating FcγRs has an impact on bone homeostasis and osteoclast development in the absence of inflammation. As depicted in Fig. S3 *A–C*, quantification of osteoclast size and osteoclast numbers in the tibia of the different FcγR knockout mice did not show major differences among the strains. These results were further confirmed by microcomputerized tomography (μCT) analysis showing that the bone volume, the trabecular separation, numbers, and thickness were comparable in all strains (Fig. S3 *D–H*), supporting the notion that the absence of individual or all activating FcγRs has no major impact on osteoclast development and bone homeostasis during the steady state. This is consistent with previous studies showing that deletion of the FcRγ-chain alone has no effect on bone homeostasis but requires additional signals transmitted via DNAX-activating protein of 12 kDa (34).

Impact of FcγR Cross-Linking on Osteoclast Differentiation and Activity in Vitro. To study whether signaling via individual activating FcγRs can have an impact on osteoclast differentiation and activity we performed a series of in vitro experiments. For this, osteoclast cultures were differentiated for 4 d in the presence of M-CSF and RANKL to allow expression of all activating FcγRs on immature osteoclasts. Cross-linking of the individual FcγRs was achieved by adding FcγRI-, FcγRIII- or FcγRIV-specific biotinylated antibodies followed by the addition of streptavidin to cross-link the receptors. As controls, osteoclast cultures were generated from the respective FcγR-deficient animals. As depicted in Fig. 2*A*, this resulted in enhanced generation of osteoclasts if FcγRI and FcγRIV, but not if FcγRIII, was cross-linked. In contrast, no increase in osteoclast activity was observed upon activating FcγR cross-linking, suggesting that signaling via the activating FcγRs I and IV has an impact on osteoclast development but not on their functional activity in vitro (Fig. 2*B*). Similar results were obtained with human osteoclast cultures showing that cross-linking of activating FcγRs did stimulate osteoclast differentiation but did not increase their activity (Fig. S1 *C* and *D*). Of note, compared with the mouse system cross-linking of all activating FcγRs did enhance osteoclastogenesis.

Role of FcγRI and FcγRIV for Osteoclast Development and Bone Destruction During Inflammatory Arthritis in Vivo. After having established that cross-linking of FcγRI and IV can have an impact on osteoclastogenesis in vitro, we next wanted to confirm a role of these activating FcγRs during joint inflammation in vivo. To study this, we used the well-established K/BxN serum transfer arthritis model (6). Previous studies have shown that FcγRIII deletion abrogates development of arthritis, possibly due to the critical role of immune complex-mediated activation

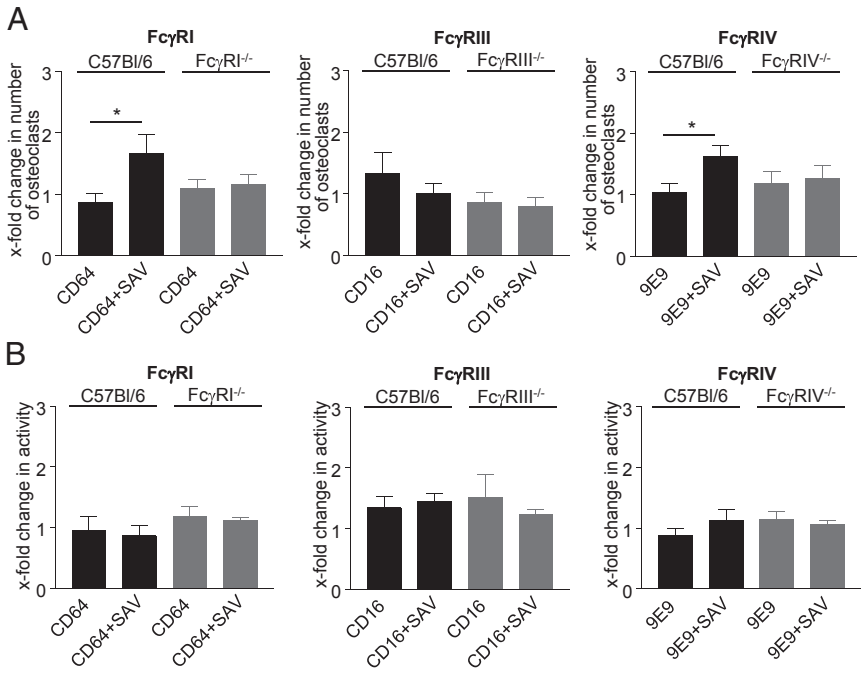

Fig. 2. Impact of activating FcγRs on osteoclast development and activity in vitro. Osteoclasts of the indicated mouse strains were generated from bone marrow cells in the presence of M-CSF and RANKL. At day 4 of the culture biotinylated FcγRI (CD64)-, FcγRIII (CD16)-, or FcγRIV (9E9)-specific antibodies were added to the culture either alone or in combination with streptavidin (+SAV) to cross-link the respective activating FcγRs. Shown is the change in osteoclast numbers (*A*) or osteoclast activity (*B*) under the indicated conditions compared with numbers and activity of osteoclasts generated without the addition of antibodies specific for activating FcγRs. Data shown in the individual histograms consist of at least four independent experiments. Values are the mean ± SD, $*P < 0.05$.

of mast cells via this activating FcγR (6, 12). In contrast, FcγRI deletion did not affect joint inflammation and pannus formation, whereas mice deficient in FcγRIV showed a reduced inflammatory infiltrate (6, 10). Because FcγRI and FcγRIV are expressed on osteoclasts we hypothesized that irrespective of joint inflammation deletion of one of these receptors might affect osteoclast-dependent bone resorption. As shown in Fig. 3 *A* and *B*, however, FcγRI-deficient animals were indistinguishable from C57BL/6 animals with respect to the size of the inflammatory infiltrate. Moreover, the number of osteoclasts at the sites of inflammation and the area of bone erosions were not different from those in the control animals (Fig. 3 *B–D*). Similar results were obtained at distant sites in the tibia of the animals, suggesting that FcγRI, despite being expressed on osteoclasts and able to trigger osteoclast differentiation in vitro, is not involved in autoantibody-dependent osteoclast differentiation in vivo (Fig. 3 *E–H*).

In contrast, FcγRIV-deficient mice had a reduced size of the inflammatory infiltrate as described before (10) and much smaller areas of bone erosions (Fig. 4 *A–C*). Consistent with the in vitro data, a reduced number of osteoclasts could be observed at the sites of inflammation and a lower number of collagen type I fragments could be detected in the serum (Fig. 4 *D* and *E*). This phenotype was specific for the affected joints because no change in bone density, trabecular number, thickness or separation was observed at uninflamed sites in the tibia of mice (Fig. S4). To exclude that this reduction in bone erosions was the result of the ubiquitous deletion of FcγRIV on monocytes, macrophages, and neutrophils in FcγRIV knockout mice, we generated a mouse with an osteoclast-specific deletion of FcγRIV by crossing FcγRIV-floxed mice with cathepsin K–cre animals (CtskCreR4flox) (35). As shown in Fig. S5 *A–D*, this resulted in the deletion of FcγRIV specifically on osteoclasts but not on the Ly6C^{low} resident monocyte subset, on neutrophils, or on tissue-resident macrophages. Consistent with the data obtained with osteoclast cultures from FcγRIV-deficient mice, osteoclasts from FcγRIV-floxed mice did not show enhanced osteoclastogenesis or enhanced osteoclast activity upon addition of FcγRIV cross-linking antibodies (Fig. S5 *E–G*). Because osteoclasts are the only cells lacking FcγRIV in this in vitro culture system, this indeed suggests that stimulating FcγRIV directly on osteoclasts is responsible for enhanced osteoclastogenesis. In contrast to the reduction of joint swelling observed in FcγRIV-deficient mice, the animals with a targeted deletion of this receptor on osteoclasts showed no reduction of the clinical signs of arthritis (Fig. 4 *A* and *B*). The level of bone erosions, the number of osteoclasts, and the amount of collagen type I fragments in the serum, however, were reduced to a level comparable to that in FcγRIV-deficient mice, suggesting that indeed autoantibody immune complex binding to FcγRIV on osteoclasts is critical for the bone destruction in this passive model of inflammatory arthritis in mice (Fig. 4 *C–E*). Importantly, the amount of osteoclast precursor cells and of other monocyte subpopulations was not different in

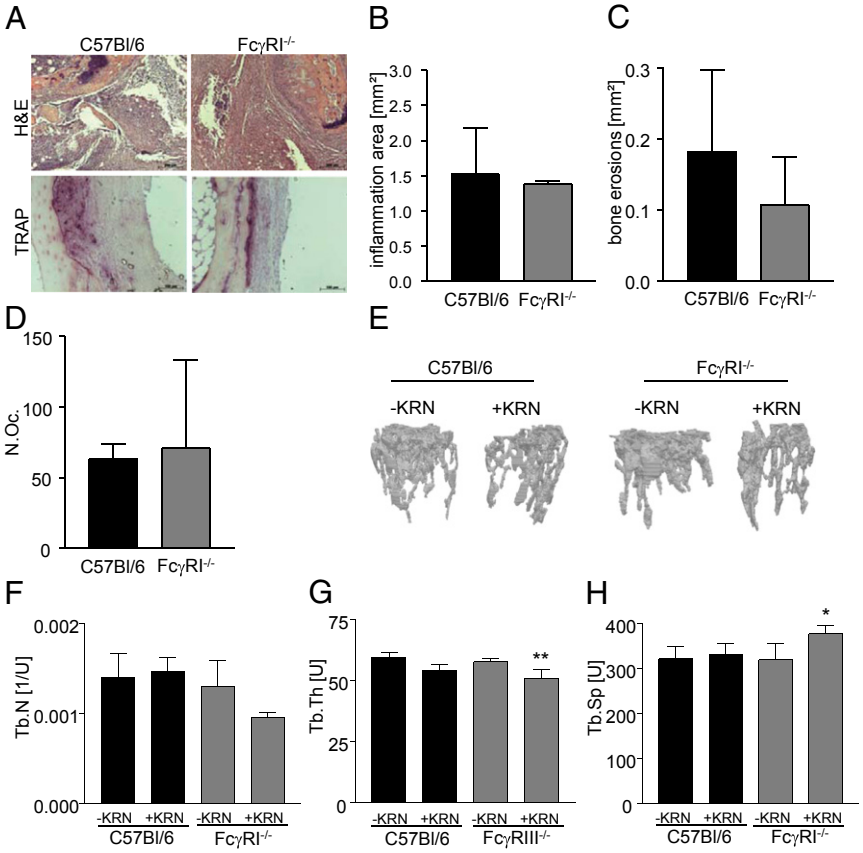

Fig. 3. Impact of FcγRI on osteoclast development and bone homeostasis during inflammatory arthritis in vivo. (*A*) Shown are representative tissue sections of joints depicting pannus formation and development of osteoclasts via TRAP staining in C57BL/6 and FcγRI knockout mice 10 d after injection of arthritogenic serum. (*B–D*) Quantification of the inflammation area (*B*) and the area of bone erosions (*C*) and the number of osteoclasts (N.Oc.) (*D*) in inflamed joints of the indicated mouse strains. (*E–H*) Evaluation of systemic effects on bone homeostasis in FcγRI-deficient mice by μCT analysis. Shown are representative 3D reconstitutions of the tibia bone structure in the absence (–KRN) or presence (+KRN) of joint inflammation in the indicated mouse strains (*E*) and a quantification of the trabecular number (Tb.N) (*F*), trabecular thickness (Tb.Th) (*G*), and trabecular separation (Tb.Sp) (*H*). Values are the mean ± SD. Groups consisted of three to five 12-wk-old mice. Shown is one representative out of three independent experiments. $*P < 0.05$, $**P < 0.01$.

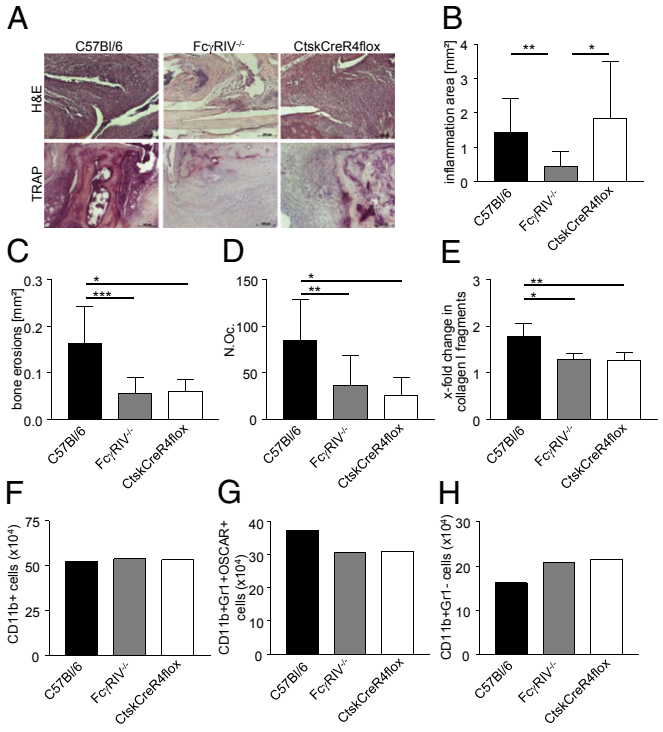

Fig. 4. Impact of FcγRIV on osteoclast development and bone homeostasis during inflammatory arthritis in vivo. (*A*) Shown are representative tissue sections of joints depicting pannus formation and development of osteoclasts via TRAP staining in C57BL/6, FcγRIV-deficient and cathepsin K–cre/FcγRIV-floxed (CtskCreR4flox) mice 10 d after injection of KRN serum. (*B–D*) Quantification of the inflammation area (*B*), the area of bone erosions (*C*), and the number of osteoclasts (N.Oc.) (*D*) in inflamed joints of the indicated mouse strains. (*E*) Shown is the change in the level of collagen type I fragments in the serum of the indicated mouse strains 10 d after induction of arthritis compared with mice without arthritis. (*F–H*) Determination of the absolute numbers of CD11B-positive cells (*F*), immature osteoclasts [positive for CD11B, Ly6C (GR1) and OSCAR] (*G*), and resident monocytes [CD11B-positive, Ly6C (GR1)-negative] (*H*) in joints with clinical signs of arthritis in the indicated mouse strains. One representative out of three independent experiments is shown. Values in *B–E* are the mean ± SD. Groups consisted of at least four to five 12-wk-old mice. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

inflamed joints of wild-type, FcγRIV-deficient and CtskCreR4-floxed mice, further arguing for a mechanism in which autoantibody immune complexes have a direct positive effect on osteoclast maturation during inflammatory arthritis (Fig. 4 *F–H*).

Taken together, our results reveal an unexpected role of FcγRs for osteoclast development during joint inflammation. Whereas during the steady state none of the mice with deficiencies in select or all activating FcγRs showed a difference in bone homeostasis, FcγRIV-deficient and CtskCreR4flox mice with an osteoclast-specific deletion of this receptor displayed a strong reduction in the generation of osteoclasts and a much lower level of bone erosions in the inflamed joints. Because the same number of osteoclast precursor cells was present in the inflamed joints, this indicates that in addition to the crucial role of cytokines such as M-CSF, TNFα, and the receptor activator of nuclear factor-kappa B–RANKL interaction, the cross-linking of activating FcγRIV by autoantibody immune complexes is critical for osteoclast development. In contrast, FcγRI, despite being expressed on osteoclasts and able to bind IgG2a antibodies, was not required for osteoclast development in vivo. At present we can only speculate about a role for FcγRIII in immune complex-dependent activation of osteoclasts in vivo because this receptor is critical for the very first steps of development of inflammation via mast cells and macrophages. Again, cell type-specific deletion strategies would be required to address this question in more detail. Consistent with the mouse system, human osteoclasts developed from the same monocyte precursor and up-regulated human FcγRIIIA (CD16) upon differentiation into the osteoclast lineage. Further confirming the mouse data, cross-linking of all human activating FcγRs also had a specific effect on osteoclast development and not on osteoclast activity. Whether these findings may also be applicable for patients with human arthritis is unclear at present, although there is evidence that arthritis patients carrying the FcγRIIIA-158V allele, conferring increased affinity for the human IgG1 subclass, showed more pronounced bone erosions compared with individuals positive for the low-affinity allele of this activating FcγR (36).

Materials and Methods

Mice. C57BL/6 mice were obtained from Elevage Janvier. KRN mice were provided by Diane Mathis (Harvard Medical School, Boston). FcRγ$^{-/-}$FcγRIIB$^{-/-}$, FcγRI, FcγRIIB, FcγRIII, FcγRIV, FcγRIV-floxed, and FcRγ-deficient mice on the C57BL/6 background were provided by Jeffrey Ravetch (The Rockefeller University, New York). CtskCre mice were provided by R. A. Davey (University of Melbourne, Melbourne, Australia). All animals were maintained under specific pathogen-free conditions. All experiments were performed with the approval of the local ethics authorities (Tierschutzbeauftragter of the University of Erlangen-Nürnberg and the Government of Mittelfranken, Ansbach, Germany) and according to the rules and regulations of the animal facilities in Germany and the United States.

Antibodies. The antibodies used for cell sorting, flow cytometry, and immunfluorescence analysis and in vitro cross-linking of FcγRs on osteoclasts are summarized in Table S1. The CCR2-specific antibody (MC21) for in vivo depletion of inflammatory monocytes was kindly provided by Matthias Mack (University of Regensburg, Regensburg, Germany).

Isolation of Resident and Inflammatory Monocytes via FACS Sorting. Sterile bone marrow preparations were obtained by flushing femurs and tibias with complete α-MEM medium. After filtering, red blood cells were lysed and the cells were washed twice with PBS. Cells were stained with an antibody mixture consisting of antibodies specific for GR-1, CD62L, CD45, Ly6G, NK1.1, and CD11b for 20 min on ice in the presence of Fc-block. Human peripheral blood lymphocytes were isolated from buffy coats via a ficoll gradient, followed by lysis of red blood cells and staining with an antibody mixture against CD19, CD3, CD56, CD66c, CD14, and CD33. Dead cells were excluded by DAPI staining and the cell populations of interest were sorted on a FACS Aria III (BD Biosciences).

Osteoclast Cultures. Total bone marrow or blood (after red blood cell lysis) was cultured overnight with 30 ng/mL of M-CSF. One million nonadherent cells or 100,000 sorted cells were cultured further in 24- or 96-well plates in α-MEM medium supplemented with 10% heat-inactivated FCS, glutamine, penicillin, and streptomycin (all from Invitrogen). M-CSF (30 ng/mL) and RANKL (50 ng/mL, mouse or human) (PeproTech) were added for induction of osteoclast differentiation. After 7 d of culture, the cells were either stained for TRAP by using the leukocyte acid phosphatase kit 386A (Sigma) to identify osteoclasts or analyzed for the expression of FcγRs by flow cytometry. For this, differentiated osteoclasts were detached from wells by incubation with 0.5 mM EDTA for 10 min at 37 °C. After filtration, cells were stained with DAPI and F4/80-, osteoclast associated receptor- (OSCAR-), and either FcγRI-, FcγRIIB-, FcγRIII-, or FcγRIV-specific antibodies. A mixture of DAPI and OSCAR-, CD14-, CD33-, and FcγR-specific antibodies was used to stain human osteoclasts. Data acquisition and analysis was performed with the FACS Diva software (BD Biosciences).

Immunofluorescence Staining. For immunofluorescence analysis, osteoclasts were cultured on glass slides followed by fixation with 4% (vol/vol) paraformaldehyde in PBS, blocking with PBS/2% (vol/wt) BSA and staining for 1 h at room temperature with antibodies specific for FcγRI, FcγRIII, and FcγRIV diluted in PBS/2% (vol/wt) BSA. DAPI was used to counterstain nuclei. To stain for FcγRIV expression in the spleen, 6-μM tissue sections were fixed for 2 min in cold acetone followed by blocking for 30 min with 5% (vol/vol) goat serum/PBS. Finally an antibody mixture against B220, FcγRIV, and F4/80 was added in blocking buffer to detect B cells and FcγRIV-positive macrophage populations in the splenic red pulp. Cells were visualized on a Zeiss Axiovert 200M microscope equipped with the Zeiss Axiovision software (Carl Zeiss).

Cross-Linking of FcγRs on Osteoclasts in Vitro. Nonadherent cells (750,000) were differentiated on glass slides in the presence of 30 ng/mL of M-CSF and 10 ng/mL of RANKL. On day 4 (murine) or day 5 (human) cells were washed with α-MEM and incubated for 15 min at room temperature with 2 μg of biotinylated FcγR-specific antibodies followed by addition of 2 μg of streptavidin after removing unbound antibodies by washing with medium. Osteoclast cultures without addition of FcγR-specific antibodies or without addition of streptavidin served as controls. Cells were stained for TRAP after two additional days of culture in medium containing 30 ng/mL of M-CSF and 10 ng/mL of RANKL. The number of osteoclasts/field of vision was determined using a Zeiss Axiovert 200M microscope by counting the number of TRAP+ multinucleated (≤3 nuclei) cells in 25 fields of vision. To determine osteoclast activity, the OsteoLyse Assay Kit (Lonza) was used according to manufacturer's instructions. Briefly, 100,000 nonadherent cells were seeded per well and cultured for 4 or 5 d followed by cross-linking of FcγRs as described above. After 7 d the supernatant was collected and the released fluorescence in the medium was measured using a time-resolved fluorescence fluorimeter (Wallac Victor3; Perkin-Elmer).

K/BxN Serum Transfer. Arthritis in male mice was induced by injection of 300 μL of a pooled serum from K/BxN mice essentially as described (6). Before injection of KRN serum and at the last day of the experiment serum was collected and the levels of murine fragments of C-terminal telopeptides of the α1 chain of type I collagen were analyzed by ELISA according to the manufacturer's instructions (RatLaps; IDS). To deplete Ly6C^{high} inflammatory monocytes, 10 μg of a CCR2-specific antibody (MC21) was injected in 200 μL of sterile PBS once per day during the whole course of the experiment. As a control, mice received PBS, given at the same intervals.

Collagenase D Digestion of Joint Tissue. To analyze joint infiltrates, the skin from the left hind paw was removed and the whole tissue surrounding the mid-paw was prepared and transferred into HBSS containing magnesium, calcium, 0.02% sodium acid, and 1 mg/mL of collagenase D. After 2 h at 37 °C with occasional mixing of the probe cells were washed, filtered through a 40-μm cell strainer, and analyzed by flow cytometry. The absolute cell numbers were determined by counting an aliquot of the sample.

Microcomputerized Tomography. Femurs of the left hind leg were fixed 4–6 h in 4% (vol/vol) formalin and then stored in 70% (vol/vol) ethanol until μCT. Images were acquired on a laboratory cone-beam μCT scanner for ultra-high-resolution imaging developed at the Institute of Medical Physics of the University of Erlangen-Nuremberg, at 6-μm resolution (1174; Skyscan).

Bone Histomorphometry. Histological analysis was performed on 4% (vol/vol) formalin-fixed front or hind legs. The complete front legs were decalcified for 2 d in formic acid bone decalcifier (Immunocal; Decal Chemical Corp.) and 5-μm paraffin sections were stained with H&E for quantification of the inflammation area. To quantify bone erosions, osteoclast numbers, and osteoclast size, all tissue was removed from hind legs followed by decalcification for 2 wk in 14% (wt/vol) EDTA (pH adjusted to 7.2 by addition of ammonium hydroxide). Five-micrometer paraffin sections of the tibia and the paw were stained for TRAP and quantification of the different parameters was done by digital image analysis (OsteoMeasure; OsteoMetrics).

Statistical Analysis. For calculation of statistical significance SPSS (IBM) was used. Data were analyzed using Student *t* test or one-way ANOVA followed by Tukey's honestly significant difference test or Dunett's T3 in case of unequal variances. *P* values less than 0.05 were considered significant.

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