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Osteoclast formation is strongly reduced both *in vivo* and *in vitro* in the absence of CD47/SIRPα-interaction

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Abstract

Physical interaction between the cell surface receptors CD47 and signal regulatory protein alpha (SIRPα) was reported to regulate cell migration, phagocytosis, cytokine production, and macrophage fusion. However, it is unclear if the CD47/SIRPα-interaction can also regulate macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor (NF)-κB ligand (RANKL)-stimulated formation of osteoclasts. Here, we show that functional blocking antibodies to either CD47 or SIRPα strongly reduced formation of multinucleated tartrate-resistant acid phosphatase $(TRAP)^+$ osteoclasts in cultures of murine hematopoietic cells, stimulated *in vitro* by M-CSF and RANKL. In addition, the numbers of osteoclasts formed in M-CSF/RANKL-stimulated bone marrow macrophage cultures from $CD47^{-/-}$ mice were strongly reduced, and bones of $CD47^{-/-}$ mice exhibited significantly reduced osteoclast numbers, as compared with wild-type controls. We conclude that the CD47/SIRPα interaction is important for M-CSF/RANKL-stimulated osteoclast formation both *in vivo* and *in vitro*, and that absence of CD47 results in decreased numbers of osteoclasts in $CD47^{-/-}$ mice.

Keywords: CD47; SIRPα; Osteoclast; Bone; M-CSF; RANKL

During physiological bone remodelling, there is a balance between the amount of bone resorbed by osteoclasts and the amount of bone formed by osteoblasts [1,2]. Most skeletal diseases, including periodontal disease, osteoporosis, rheumatoid arthritis and skeletal metastasis of malignant tumors, are associated with an imbalance in skeletal turnover so that osteoclastic bone resorption exceeds bone formation. Bone-resorptive osteoclasts are highly specialized polykaryonic cells, derived by fusion of cells from the monocyte/macrophage hematopoietic lineage [3]. Although the hematopoietic origin of osteoclasts has been know for three decades, it is not until recently the knowledge has emerged of which molecules are involved in the differentiation of the mononuclear osteoclast progenitor cells and their fusion to the active, bone resorbing, multinucleated osteoclasts.

Osteoclastogenesis requires an initial expansion of the number of hematopoietic progenitor cells induced by activation of c-Fms by macrophage colony-stimulating factor (M-CSF) released from stromal cells/osteoblasts. For differentiation of the progenitor cells along the osteoclastic pathway, three members of the tumor necrosis factor (TNF) ligand and receptor superfamilies have been identified as crucial molecules. The TNF related cytokine receptor activator of NF-κB ligand (RANKL), expressed on the surface of stromal cells/osteoblasts and to some extent also as soluble RANKL, activates its cognate receptor RANK, expressed on the surface of monocytes/osteoclast progenitor cells. Downstream RANK, activation of the NF-κB,

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AP-1 and phosphatidylinositol 3 kinase/Akt pathways and the subsequent induction of the master regulator of osteoclastogenesis, nuclear factor of activated T cells 2 (NFAT2/NFATc1), are important for osteoclast formation. The interaction between RANKL and RANK can be inhibited by osteoprotegerin (OPG), a TNF receptor related molecule expressed and released also by stromal cells/osteoblasts and which binds to RANKL as a decoy receptor. Mice with gene deletion of either *rankl, rank, nfat2, nf-κB, c-fms* and *m-csf* lack osteoclasts and exhibit an osteopetrotic phenotype, whereas mice rendered null for *opg* have abundant numbers of osteoclast and an early onset osteoporotic phenotype [1–3].

The discovery of the RANKL-RANK pathway provided insight into the long-sought for molecular mechanism in the cell-to-cell interaction between stromal cells/osteoblasts and osteoclast progenitor cells, as well as to the subsequent intracellular pathways involved in osteoclast progenitor cell differentiation. For the formation of the bone resorbing osteoclast to occur, the fusion of late osteoclast progenitor cells is required. Despite vast research in osteoclast differentiation, the mechanisms involved in cell-to-cell interaction between osteoclast progenitor cells during the late phases of osteoclast formation are essentially unknown, although DC-STAMP [4] and signaling through FcRγ/DAP12 [5] have been suggested to be involved.

The ubiquitously expressed immunoglobulin (Ig)-superfamily member CD47 (integrin-associated protein/IAP) is a cell surface receptor known to associate with integrins and to stimulate chemotaxis, migration and activation of leukocytes [6]. CD47 can also function as a ligand for the Ig-superfamily cell surface receptor signal regulatory protein alpha (SIRPα) [7]. Although ubiquitously expressed, SIRPα is expressed highly only in the brain and on myeloid cells [7–9]. By binding to CD47 on other cells, SIRPα can inhibit phagocytosis in macrophages and related cells [10–12], but can also regulate cell migration [13], and stimulate macrophage–macrophage adhesion, fusion and formation of giant cells [14,15]. In this context, SIRPα was cloned and named macrophage fusion receptor [16]. Thus, using rat alveolar macrophages, cultured at high density and in the presence of human serum, it was found that the CD47/SIRPα-interaction was important for macrophage–macrophage fusion [14].

To determine the role of CD47 in osteoclast formation, both *in vitro* and *in vivo*, we here investigated if the CD47/SIRPα-interaction was also involved in the specific regulation of M-CSF/RANKL-induced osteoclast formation from murine hematopoietic progenitors, and if osteoclast formation was affected in CD47-deficient mice.

Materials and methods

Antibodies. Rat anti-murine SIRPα (mAb P84; rat IgG1) [17], rat anti-murine CD47 (mAb mIAP301; rat IgG2a) [18], were purified from hybridoma supernatants. Isotype controls were from Pharmingen, San Diego, CA, USA.

$CD47^{-/-}$mice. Generation of $CD47^{-/-}$ mice has been previously described [18]. Male $CD47^{-/-}$ C57BL/6J or Balb/c mice, backcrossed to C57BL/6J or Balb/c (The Jackson Laboratory, Bar Harbor, ME, USA) for 16 or more generations, and their wild-type homozygous littermates were from our own breeding colony. CsA mice were also from our own inbred colony. Animals were kept in accordance with local guidelines and maintained in a specific pathogen-free barrier facility. All animal procedures were approved by the Local Animal-Ethics Committee.

Spleen cell cultures. Spleen cells from 5 to 8 weeks old male wild-type CsA mice were incubated for 4–5 days with M-CSF and RANKL (25 and 100 ng/ml, respectively; R&D Systems, Abdingdon,UK), essentially as previously described [19]. Where indicated, mAb miap301, mAb P84, or isotype controls were included in the medium during the whole incubation period. At the end of culture, cells were washed and stained for TRAP according to manufacturer's instructions (Sigma Chemical Co., St. Louis, MO, USA). Multinucleated (no of nuclei $\geqslant 3$), $TRAP^+$ cells were counted as osteoclasts.

Cultures of bone marrow macrophage precursor cells (BMC). Bone marrow cells were isolated from femurs of CsA mice, or wild-type and $CD47^{-/-}$ C57BL/6J mice as previously described [20]. Briefly, the bone marrow cells were incubated for 2 h in tissue culture plastic dishes, after which the non-adherent cells were collected, centrifuged, resuspended in α-MEM with 10% FBS, L-glutamine, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin sulphate, and seeded at 10^6 cells/cm^2, on coverslips in 24-well plates. After incubation with M-CSF (25 ng/ml) and RANKL (100 ng/ml), with or without mAb miap301, mAb P84, or isotype control, for 4 days, the cells were fixed and stained for TRAP [21]. TRAP positive cells with three or more nuclei were counted as osteoclasts.

Tissue collection and analysis of osteoclasts in vivo. At 18 weeks of age, wild-type and $CD47^{-/-}$ mice were euthanized by cervical dislocation. Both femurs were excised and bisected transversely at the midpoint of the shaft. The distal halves of the right femurs were fixed and embedded, undecalcified, in methyl-methacrylate resin (Medim-Medizinische Diagnostik, Giessen, Germany), and 5-μm sagittal sections were prepared for analysis using Q Win software (Leica Microsystems Pty Ltd., Sydney, Australia). For measurements of osteoclast surface and osteoclast number, sections were stained for TRAP activity as described previously [22].

Statistical analyses. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Levene's homogenicity test, and post-hoc Bonferroni's, or where appropriate, Dunnett's T3 test (SPSS 12.0.1), or Student's *t*-test, as indicated in the legends to figures. All experiments were performed at least twice with comparable results and all data are presented as means ± SEM.

Results

Antibodies to CD47 or SIRPα block osteoclast formation in vitro

To elucidate the role of CD47 and SIRPα in osteoclastogenesis, we initially studied the effects of functional blocking monoclonal antibodies (mAb) to SIRPα (mAb P84) [10] or CD47 (mAb miap301) [11], on the formation of $TRAP^+$ multinucleated osteoclasts *in vitro*. The mAbs P84 and miap301 can both block the CD47/SIRPα-interaction [10,11]. In mouse spleen cell cultures stimulated with 25 ng/ml M-CSF and 100 ng/ml RANKL, both mAb P84 and mAb miap301 at 10 μg/ml inhibited osteoclast formation by $60 \pm 4\%$ and $70 \pm 2\%$, respectively ($P < 0.001$; Fig. 1A). We found no significant effects of isotype control antibodies (Fig. 1A). Since osteoclast formation can be studied *in vitro* using several different techniques, we wanted to confirm our findings using hematopoietic progenitor cells isolated from another tissue. Therefore, the effects of

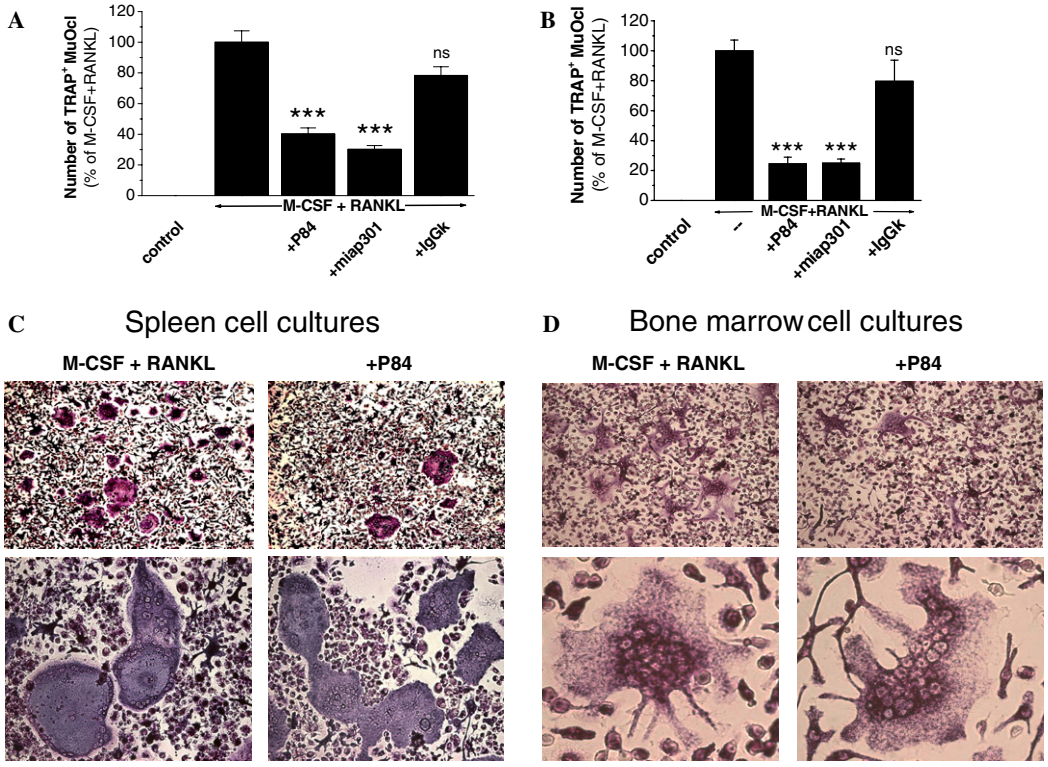

Fig. 1. Osteoclast formation is strongly reduced by blocking the CD47/SIRPα-interaction. (A) Functional blocking antibodies to SIRPα (P84; 10 μg/ml) or CD47 (miap301; 10 μg/ml) inhibit M-CSF/RANKL-stimulated formation of multinuclear $TRAP^+$ osteoclasts (MuOcl) in CsA spleen cell cultures. (B) Anti-SIRPα and anti-CD47 mAbs also inhibited formation of MuOcl in CsA BMC cultures. Data on osteoclast formation in (A) represents the mean ± SEM of 11–14 wells per treatment group pooled from two individual experiments, where the numbers of $TRAP^+$MuOcl in M-CSF/RANKL-stimulated cultures without antibody was 93 ± 6.9 and set to 100%. In (B), data represents the mean ± SEM of 9-wells per treatment group pooled from two individual experiments, where the numbers of $TRAP^+$MuOcl in M-CSF/RANKL-stimulated cultures without antibody was 273 ± 19.7 and set to 100%. $***P < 0.001$, as compared with M-CSF/RANKL-stimulated cultures without antibody, using one-way analysis of variance (ANOVA) with Levene's homogenicity test, and post-hoc Bonferroni's, or where appropriate, Dunnett's T3 test. Representative photomicrographs, showing no difference in mononuclear cell density or TRAP-staining, and no difference in osteoclast morphology, in spleen cell cultures (C) and BMC cultures (D), incubated with M-CSF/RANKL in the absence or presence of 10 μg/ml P84. Magnifications in (C) are 10× and 20× in the upper and lower panels, respectively. In (D), the magnifications are 20× and 40× in the upper and lower panels, respectively.

the antibodies were also studied in M-CSF/RANKL-stimulated cultures of BMC. Similar to the observation in spleen cell cultures, osteoclast formation in BMC cultures stimulated by M-CSF/RANKL was strongly inhibited by 10 μg/ml of mAb P84 or mAb miap301 (75.4 ± 4.4% and 75.0 ± 2.7% inhibition, respectively; $P < 0.001$; Fig. 1B). Despite the strong inhibitory effect of the antibodies on osteoclast numbers in these cultures, we found that the mononuclear cell density, the size of the osteoclasts, or the number of nuclei per osteoclast formed in spleen cell cultures (Fig. 1C) or in BMC cultures (Fig. 1D) were not affected by mAb P84.

CD47-deficiency results in reduced osteoclast formation in vitro

Based on our findings that interference with the CD47/SIRPα interaction by monoclonal antibodies could strongly reduce M-CSF/RANKL-induced osteoclast formation *in vitro*, we next studied osteoclast formation in cultures of BMC from wild-type or $CD47^{-/-}$ mice. Experiments using M-CSF/RANKL-stimulated BMC cultures revealed that the number of $TRAP^+$ osteoclasts was strongly reduced in $CD47^{-/-}$ cultures, as compared to that in wild-type cultures ($P < 0.001$; Fig. 2A). Similar to the observation in the experiments described above using blocking mAbs, no difference in mononuclear cell density or osteoclast morphology could be observed in $CD47^{-/-}$ or wild-type BMC cultures (not shown).

CD47-deficiency results in reduced osteoclast formation in vivo

To investigate the *in vivo* impact of our *in vitro* observations, we next analyzed the $TRAP^+$ osteoclast density in femoral bones of 18 weeks old male $CD47^{-/-}$ or wild-type mice. Histological examination showed reduced numbers of $TRAP^+$ osteoclasts in $CD47^{-/-}$ bone specimens, as compared with that in wild-type mice (Figs. 2B and C). Detailed analyses revealed a 37% reduction in osteoclast surface ($P < 0.01$; Fig. 2D) and a 29% reduction in osteoclast number ($P < 0.05$; Fig. 2E) in the distal femoral

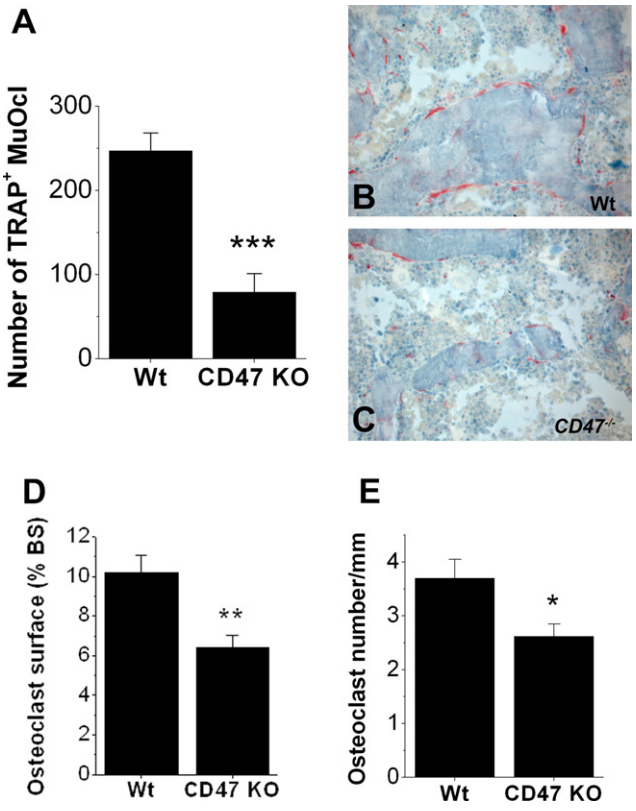

Fig. 2. CD47-deficiency inhibits osteoclast formation *in vitro* and *in vivo*. (A) Strongly reduced formation of $TRAP^+$ MuOcl in M-CSF/RANKL-stimulated BMC cultures from $CD47^{-/-}$ mice, as compared with that in BMC cultures from wild-type mice. Data are means ± SEM of 5–6 wells per treatment group in one representative experiment out of three with similar results. $^{***}P < 0.001$, as compared with that in wild-type cultures, using one-way analysis of variance (ANOVA). Photomicrographs show representative TRAP-stained sections of cancellous bone from (B) wild-type or (C) $CD47^{-/-}$ mice (20× magnification). (D) Reduced osteoclast surface in femoral trabecular bone of $CD47^{-/-}$ mice, as compared with that in wild-type controls. Data are mean ± SEM of 7 mice in each group. $^{**}P < 0.01$, as compared with that in wild-type cultures, using Student's *t*-test. (E) Reduced osteoclast number in femoral trabecular bone of $CD47^{-/-}$ mice, as compared with that in wild-type controls. Data are means ± SEM of 7 mice in each group. $^{*}P < 0.05$, as compared with that in wild-type mice, using Student's *t*-test.

metaphysis of $CD47^{-/-}$ mice, consistent with the reduction in the number of osteoclasts observed *in vitro* after stimulation with M-CSF and RANKL.

Discussion

We here show for the first time, that the interaction between CD47 and SIRPα is of significant importance in regulating osteoclast formation, both *in vivo* and *in vitro*. By using well characterized monoclonal antibodies against CD47 or SIRPα, each of which can block the interaction between these two cell surface glycoproteins, we found that each mAb *per se* had a profound inhibitory effect on $TRAP^+$ osteoclast formation in two separate *in vitro* culture systems. Genetic deletion of CD47 had a similar negative effect on the formation of $TRAP^+$ osteoclasts *in vitro*. In strong support of the *in vitro* data, we also found a significantly reduced number of $TRAP^+$ osteoclasts in femoral bones of CD47-deficient mice.

Our finding that the CD47/SIRPα-interaction was playing a significant role in the formation of osteoclasts *in vitro* is in agreement with previous reports on the role of the CD47/SIRPα-interaction in rat macrophage fusion and formation of multinucleated giant cells in the presence of human serum [14,15]. In our system, where osteoclast differentiation and fusion was induced by stimulating the osteoclast-specific receptor RANK, blocking the CD47/SIRPα-interaction did not seem to affect the size of the formed osteoclasts or the number of nuclei per osteoclast, despite inducing a strong reduction in osteoclast numbers, which would argue against a predominant role of this receptor interaction in fusion of preosteoclasts. However, since osteoclasts can be derived *in vitro* using a number of well-established experimental protocols with slight differences in culture conditions, it is still possible that the importance of the CD47/SIRPα-interaction in regulating cell fusion and the morphology of osteoclasts may be more or less evident depending on the exact culture condition.

Since the formation and life span of osteoclasts is regulated by proliferation, differentiation, fusion and osteoclast apoptosis [23], CD47 and SIRPα, by interacting or *per se*, may also affect osteoclast formation by increasing the osteoclast apoptosis rate. However, CD47 has only been shown to induce apoptosis, either *per se* [24,25] or in facilitating Fas-induced apoptosis [26], further suggesting that absence of CD47 or its blockade would not accelerate osteoclast apoptosis. Thus, the exact nature of the CD47/SIRPα-interaction and the signaling pathways involved in regulation of preosteoclast differentiation, osteoclast formation, and possibly also osteoclast survival, needs to be further investigated. Still, the biological importance of CD47 in formation and/or maintenance of osteoclasts in bone were clearly shown in the present study where $CD47^{-/-}$ mice were found to have a significant reduction in femoral bone osteoclasts.

In conclusion, we show that CD47 is of major importance for osteoclast formation, both *in vitro* and *in vivo*. Further knowledge on the exact mechanisms by which CD47 and its association with other membrane proteins, either in *cis* or in *trans*, are involved in osteoclastogenesis could result in novel ways of treatment in diseases such as osteoporosis, rheumatoid arthritis, periodontitis and metastatic cancers.

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