FEBS Letters 582 (2008) 3243–3248

The role of Mac-1 (CD11b/CD18) in osteoclast differentiation induced by receptor activator of nuclear factor-κB ligand

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Received 23 June 2008; revised 5 August 2008; accepted 26 August 2008

Available online 4 September 2008

Edited by Laszlo Nagy

Abstract Multinuclear osteoclasts are derived from CD11b-positive mononuclear cells in bone marrow and in circulation. FACS sorting experiments showed impaired osteoclastogenesis in RAW264.7 cells with low CD11b expression. Neutralizing antibodies and siRNA against CD11b inhibited osteoclastogenesis induced by RANKL. Although primary cultured mouse bone marrow macrophages expressed CD11a and CD11b, osteoclastogenesis induced by M-CSF and RANKL was inhibited in the presence of anti-CD11b or anti-CD18 but not anti-CD11a antibodies. Furthermore, anti-CD11b antibodies inhibited NFATc1 expression induced by M-CSF and RANKL in BMMs. These findings suggest, at least partly, an important role of CD11b in osteoclastogenesis.
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Keywords: Osteoclastogenesis; Nuclear factor of activated T cells c1; RAW 264.7 cell line; Receptor activator of nuclear factor-κB ligand; Integrin

1. Introduction

Osteoclasts are multinucleated bone-resorbing cells formed by the fusion of mononuclear cells derived from the monocyte/macrophage lineage (osteoclast precursors (pre-OCs)). Pre-OCs in the peripheral circulation or within the bone marrow are separated from the bone surface by endothelial cells lining the blood vessels [1]. Therefore, the extravasation and the migration under osteoblasts are necessary for pre-OCs to reach the surface of the bone matrix [2]. After pre-OCs differentiate into tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells, these cells differentiate into multinucleated osteoclasts by cell fusion [3].

Adhesion molecules are necessary for leukocyte trafficking and differentiation. Integrins are one of the most important adhesion molecule family involved in trafficking of monocyte/macrophage. Integrins are non-covalentry linked αβ heterodimers. Four α subunits and β2 subunit were expressed on macrophages (αLβ2, CD11a/CD18; αMβ2, CD11b/CD18; αXβ2, CD11c/CD18; αDβ2, CD11d/CD18 integrin pairs) [4]. Among them, CD11a/CD18 integrin pair (leukocyte function-associated antigen-1, LFA-1) and CD11b/CD18 integrin pair (macrophage antigen 1, Mac-1) were expressed on pre-OCs and play important roles during osteoclastogenesis. Intercellular adhesion molecule-1 (ICAM-1, CD54) on endothelial cells is reported to play an important role in the extravasation of pre-OCs [1], and the candidates of counter receptors against ICAM-1 are LFA-1 and Mac-1, which are expressed on pre-OCs. As for the interaction between osteoblasts and pre-OCs, the involvement of ICAM-1 and LFA-1 in osteoclastogenesis has been reported [5–8]. In addition to the interactions between heterologous cells via integrin, it was also reported that direct interactions mediated by ICAM-1/LFA-1 among pre-OCs contribute to osteoclastogenesis [5,8,9]. Although one of the best markers of osteoclast precursors is Mac-1 [10,11], to our knowledge there have been no studies to examine the role of Mac-1 in osteoclastogenesis.

This study examined the possible role of Mac-1 in osteoclastogenesis using RAW264.7 cells and primary cultured bone marrow macrophages (BMMs). It is reported here that Mac-1 may play an important role in the early stage of osteoclastogenesis via the communication between pre-OCs.

2. Materials and methods

2.1. RAW264.7 cell culture and induction of osteoclast formation

The RAW 264.7 cell line used in this study was kindly provided by Dr. T. Suda (Showa University) and grown in α-MEM supplemented with 10% fetal calf serum (FCS) (growth medium) in a CO_2 incubator. RAW264.7cells were seeded in the center of the wells of a 48-well plate for spot culture (3–100 μl of 10^5 cells/ml) and incubated for 30 min. Then the medium was replaced with 0.5 ml of growth medium containing 10% FCS and 50 ng/ml of soluble RANKL (sRANKL, Oriental Yeast Co. Ltd.) and the cultures were incubated for 5 days. For uniform cell cultures, cells with different densities ($6–200 \times 10^2$ cells/ ml) were prepared, and 0.5 ml of cell suspension was seeded into the wells of a 48-well plate and incubated for 30 min. Osteoclast formation was induced by medium replacement with growth medium containing 50 ng/ml of sRANKL. Functional blocking experiments were carried out in spot culture conditions (1000 cells/ 10 μl) with 10 μg/ml of rat IgG2b, IgG2a (Functional Grade, eBioscience) or antibodies (anti-CD11a, M17/4; anti-CD11b, M1/70; or anti-CD18, M18/2, Functional Grade, eBioscience). Tartrate-resistant acid phosphatase positive ($TRAP^+$) multinuclear cells were detected using TRAP staining at 5 days after sRANKL treatment.

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doi:10.1016/j.febslet.2008.08.023

2.2. RAW264.7 cell sorting by fluorescence-activated cell-sorter (FACS)

$CD11b_{Low}$, $CD11b_{Med}$, and $CD11b_{High}$ RAW264.7 cell populations were individually isolated by FACS (FACS Aria, BD Bioscience). Briefly, RAW264.7 cells were stained with PE-labeled anti-CD11b and FITC-labeled anti-CD54 antibodies (clone M1/70, clone YN/1.7.4, eBioscience). After washing the cells three times with phosphate buffered saline (PBS) containing 2 mM EDTA, the cells were separated by the degree of CD11b expression using FACS.

2.3. Knockdown of CD11b in RAW264.7 cells

RAW264.7 cells (1.5×10^6) were cultured in a 6 cm dish (Falcon) with growth medium for 24 h. An expression plasmid encoding a short hairpin RNA against CD11b and green fluorescent protein (GFP)(SureSilencing™ shRNA plasmid for Mouse Itgam, clone 2, for control clone NC, SuperArray) was transiently transfected into the cells using Lipofectamine 2000 Reagent (Invitrogen). Two days after transfection, the cells were stained with PE-labeled anti-CD11b (clone M1/70, eBioscience) to confirm CD11b knockdown. CD11b expression of cells was analyzed and GFP positive cells were isolated using FACS.

2.4. Mouse bone marrow macrophage culture and induction of osteoclast formation

Male mice were euthanized, and femurs and tibias were obtained. Sterile bone marrow preparations were obtained by flushing the femurs and tibias with PBS using a 27-gauge needle. After washing, red blood cells were lysed using 0.83% ammonium chloride solution. The suspended cell solution was passed through a 70 μm cell strainer (BD Falcon) to remove large cell debris. The mononuclear cells obtained were seeded in a 48-well plate at a density of 10^6 cells/well and cultured with macrophage colony-stimulating factor-containing (M-CSF, 10 ng/ml, Peprotech) growth medium. Osteoclast formation was induced by medium replacement with growth medium containing M-CSF (10 ng/ml) and sRANKL (100 ng/ml) at 24 h after the start of bone marrow macrophage (BMM) culture. $TRAP^+$ multinuclear cells were detected using TRAP staining at 3 days after M-CSF and sRANKL treatment.

2.5. Quantification of NFATc1 mRNA by real-time RT-PCR

Total RNA was extracted from the BMMs at 24 h after the start of treatment of the cells with M-CSF and sRANKL in the presence of rat IgG or anti-CD11b antibody using TRIZOL Reagent according to the manufacturer's protocol (Invitrogen). Two micrograms of total RNA was subjected to reverse-transcription (ReveTra Ace, TOYOBO). The cDNA samples were amplified with Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). All the data were analyzed by the comparative Ct Method (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). NFATc1-specific primers were 5′- CAACGCCCTGACCACCGATAG-3′ (forward) and 5′-GGCTGCCTTCCGTCTCATAGT-3′ (reverse); GAPDH-specific primers were 5′- TACAGCAACAGGGTGGTGGAC-3′ (forward) and 5′- GTGGGTGCAGCGAACTTTATT-3′ (reverse).

2.6. Tartrate-resistant acid phosphatase (TRAP) staining

Osteoclast formation was evaluated using TRAP staining because it is well known that TRAP expression gradually increases during osteoclast differentiation. After the cells were washed with phosphate buffered saline (PBS), they were fixed with ethanol/acetone (4:1) for 1 min. The cultures were then dried and stained for TRAP by incubating them in 0.05 M sodium acetate buffer (pH 5.0) containing naphthol AS-BI phosphoric acid sodium salt (Sigma) and fast red ITR salt (Sigma) in the presence of 10 mM sodium tartrate (Sigma). TRAP-positive cells with three or more nuclei formed in the culture system were considered to be osteoclast-like multinuclear cells. The area occupied by osteoclast-like cells in low-power field (2.18 mm × 1.8 mm) was analyzed and represented by OC area.

3. Results

3.1. Differentiation of RAW264.7 cells into multinuclear osteoclasts is cell density dependent

According to a previous report, osteoclastogenesis induced by sRANKL is dependent on the cell density of the precursor cells [12]. Firstly, it was examined whether the sRANKL induced osteoclastogenesis was dependent on the cell density of RAW264.7 cells. When RAW264.7 cells were seeded in the wells of a 48-well plate at different densities, osteoclastogenesis was found to be dependent on cell-density (Fig. 1A). On the other hand, when the RAW264.7 cells were seeded in the wells at the same density but with different cell numbers, osteoclastogenesis was induced independently the number of RAW264.7 cells (Fig. 1B). These results suggest that the osteoclastogenesis induced by sRANKL is dependent on the initial cell density.

3.2. Impaired osteoclastogenesis in $CD11b_{Low}$ RAW 264.7 cells

It was hypothesized that the cell density-dependent osteoclastogenesis may be the result of impairment of cell–cell contact at low cell density at the early stage of osteoclastogenesis. Usually, cell communication is achieved by intercellular adhesion. It was reported that RAW264.7 cells and macrophages express the cell adhesion molecules ICAM-1 and Mac-1 [5,8–10,13]. Therefore, the expression of ICAM-1 and Mac-1 in RAW264.7 cells was analyzed using FACS. Although the ICAM-1 expression level was not different among RAW264.7 cells, there were heterogeneous populations with varying expression levels of CD11b (Fig. 2A). After these pop-

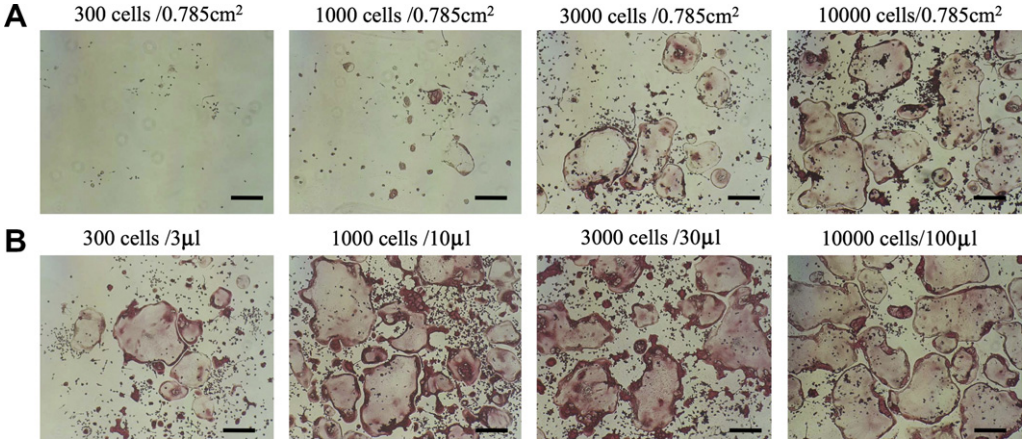

Fig. 1. Cell density dependent differentiation of RAW264.7 cells. (A) RAW264.7 cells were seeded at four different densities in equal sized culture wells (0.785 cm^2). (B) RAW264.7 cells were seeded with four different numbers of cells but the same density (100 cells/μl). Representative images of $TRAP^+$ multinuclear cells at 5 days after sRANKL (50 ng/ml) treatment. Bar = 300 μm.

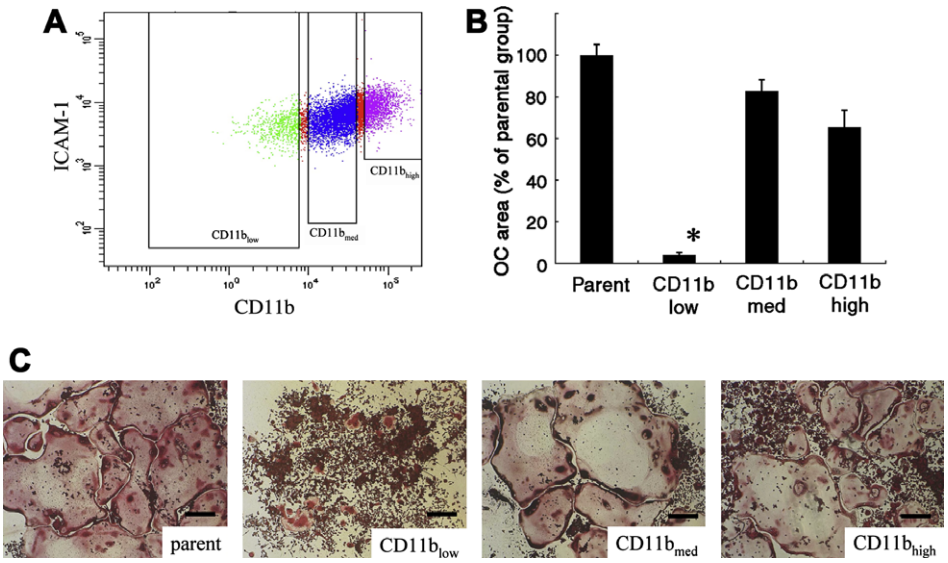

Fig. 2. The relationship Mac-1 expression and osteoclast differentiation in RANKL-stimulated RAW264.7 cells. (A) FACS analysis of RAW264.7 cells. Heterogeneity of CD11b expression was noted. Three populations ($CD11b_{Low}$, $CD11b_{Med}$, and $CD11b_{High}$) were isolated. (B) Osteoclast differentiation was induced with sRANKL (50 ng/ml) in each population. Values are presented as means ± S.E., $n = 4$, $^{*}P < 0.05$ vs parent group. Analysis of variance followed by Dunnett's test. (C) Representative images of $TRAP^{+}$ multinuclear cells at 5 days after sRANKL treatment. Bar = 300 μm.

ulations were sorted ($CD11b_{Low}$, $CD11b_{Med}$, and $CD11b_{High}$, Fig. 2A), osteoclastogenesis was induced by sRANKL. As shown in Fig. 2B, osteoclastogenesis was severely impaired in the population with low CD11b expression. This result may indicate an important role of CD11b on osteoclastogenesis in RAW264.7 cells.

3.3. Osteoclastogenesis was attenuated by Mac-1 blockage in RAW264.7 cells

A previous study demonstrated that the expression level of Mac-1 was dependent on the stage of osteoclast differentiation [3]. Therefore, the differentiation stages of osteoclastogenesis among the FACS sorted populations may reflect their ability for osteoclastogenesis. To exclude this possibility, the effect of neutralizing antibodies on osteoclastogenesis was examined in RAW264.7 cells. Osteoclastogenesis was not affected by the treatment of the cells with rat IgG2b or IgG2a (IgG2a, data not shown). Not only an anti-CD11b antibody but also an anti-CD18 antibody significantly inhibited the osteoclastogenesis induced by sRANKL. On the other hand, an anti-CD11a antibody did not affect the osteoclastogenesis (Fig. 3A and B). This result strongly suggests an important role of Mac-1 on osteoclastogenesis in RAW264.7 cells.

3.4. CD11b knockdown and osteoclastogenesis

To confirm the important role of Mac-1 in osteoclastogenesis, specific knockdown of CD11b by RNA interference was performed. FACS analysis showed an approximately 50% decrease in CD11b expression was achieved in GFP positive RAW264.7 cells (data not shown). Osteoclastogenesis tended to decrease in CD11b knockdown cells in comparison to control RAW264.7 cells (Fig. 4).

3.5. Role of Mac-1 in osteoclastogenesis in bone marrow macrophages

The role of Mac-1 in osteoclastogenesis in primary cultured BMMs was examined. Osteoclastogenesis was not affected by the treatment of the cells with rat IgG2b or IgG2a (IgG2a, data not shown). As shown in Fig. 5, osteoclastogenesis of BMMs induced by M-CSF and sRANKL was inhibited by treatment of the cells with antibodies against CD11b and CD18. Anti-CD11a antibody did not affect osteoclastogenesis in BMMs.

3.6. Blockage of CD11b by a neutralizing antibody inhibited mRNA expression of nuclear factor of activated T cells c1

To clarify the involvement of the inhibitory effect of the anti-CD11b antibody on osteoclastogenesis in the early stage of differentiation, the mRNA expression level of nuclear factor of activated T cells c1 (NFATc1) in BMMs was examined at 24 h after M-CSF and sRANKL treatment. More than a 3-fold increase in NFATc1 mRNA levels was detected in the cells treated with M-CSF and sRANKL. This NFATc1 up-regulation was significantly inhibited by treatment of the cells with the anti-CD11b antibody (Fig. 6). This result suggests, at least in part, an important role of Mac-1 in osteoclastogenesis in the early differentiation stage.

4. Discussion

A decade ago, the first step in the molecular elucidation of osteoclast differentiation was achieved with the discovery of RANKL (also known as OPGL, ODF, or TRANCE) [14,15]. RANKL binds to its receptor, RANK, which is expressed in pre-OCs and induces the osteoclast differentiation of pre-OCs via TNF receptor-associated factor 6 (TRAF6) activation. Genome-wide screening and gain/loss of function experiments revealed the important role of NFATc1, a master regulator of osteoclastogenesis, following induction by RANKL [16,17]. In addition to RANK/RANKL signaling, it has been revealed that immunoreceptor tyrosine-based activation motif (ITAM)-dependent co-stimulatory signals are involved in osteoclastogenesis by the elevation of cytosolic Ca^{2+}

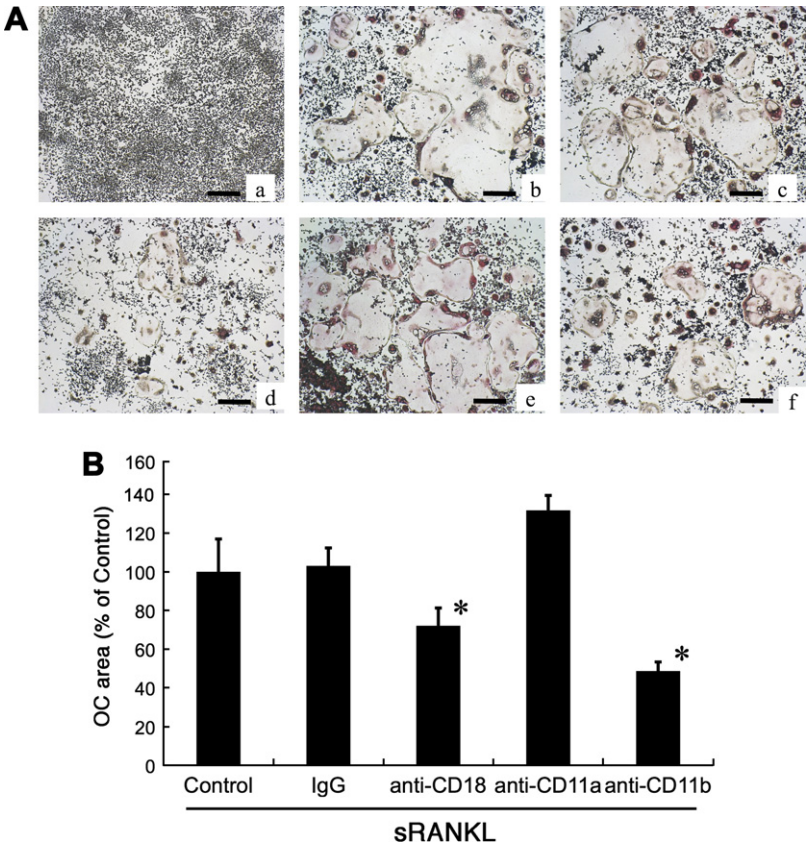

Fig. 3. Effect of antibodies on osteoclast differentiation in RAW264.7 cells. (A) Representative images of $TRAP^+$ multinuclear cells at 5 days after sRANKL treatment. (a) sRANKL (−); (b) sRANKL+ (control); (c) sRANKL+IgG (IgG2b); (d) sRANKL+anti-CD18 Ab (anti-CD18); (e) sRANKL+ anti-CD11a Ab (anti-CD11a); and (f) sRANKL+ anti-CD11b Ab (anti-CD11b). Bar = 300 μm. (B) Quantification of $TRAP^+$ osteoclast differentiation at 5 days after treatment with sRANKL (50 ng/ml) and antibodies (10 μg/ml). Values are presented as means ± S.E., $n = 4$, $^*P < 0.05$ vs IgG group. Analysis of variance followed by Dunnett's test.

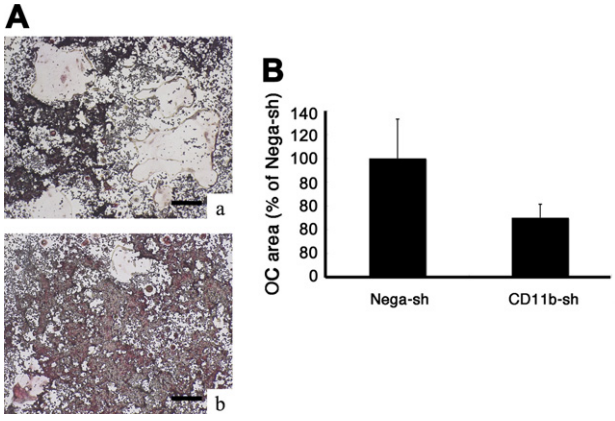

Fig. 4. Effect of CD11b knockdown by shRNA on RAW264.7 cell differentiation. (A) Representative images of $TRAP^+$ multinuclear cells at 5 days after sRANKL treatment. (a) negative-shRNA (GFP^+) and (b) CD11b-shRNA (GFP^+). Bar = 300 μm. (B) Quantification of $TRAP^+$ osteoclast differentiation at 5 days after treatment with sRANKL (50 ng/ml). Values are presented as means ± S.E., $n = 4$.

for the nuclear transport of NFATc1 [18,19]. It has been shown that mice lacking ITAM-harboring adaptors, Fc receptor γ subunit (FcRγ) and DNAX-activating protein (DAP) 12, exhibit severe osteopetrosis owing to impaired osteoclast differentiation. It was supposed that the phosphorylation of ITAM results in the recruitment of spleen tyrosine kinase (Syk), and subsequently Syk activates calcium signaling via phospholipase C (PLC).

In this study, it was found that functional impairment of Mac-1 resulted in inhibition of osteoclast differentiation in both RAW264.7 cells and BMMs using neutralizing antibodies and a gene silencing technique. The essential role of Syk in integrin-mediated leukocyte activation and Syk co-localization with CD18 have been reported [20–25]; therefore, the inhibition of osteoclastogenesis might be the result of the attenuation of Syk signaling by the functional blockage of Mac-1. Interestingly, Mócsai et al. reported that the activation of Syk by integrins required DAP12 and FcγR [26]. Conversely, the osteopetrotic phenotype of double knockout mice ($DAP12^{-/-}$ and $Fc\gamma R^{-/-}$) may be partly due to a lack of integrin signaling in these mice. In this study, RANKL-induced increases in NFATc1 mRNA levels were inhibited by the treatment of BMMs with a neutralizing antibody against CD11b. This result suggests that integrin signaling plays an important role as a co-stimulatory molecule in the early osteoclast differentiation of BMMs induced by M-CSF and sRANKL.

The counter receptors for Mac-1 were thought to be ICAM-1 and -2, which are expressed in pre-OCs and macrophages [5,8,9,27]. BMMs expressed both ICAM-1 and -2, and

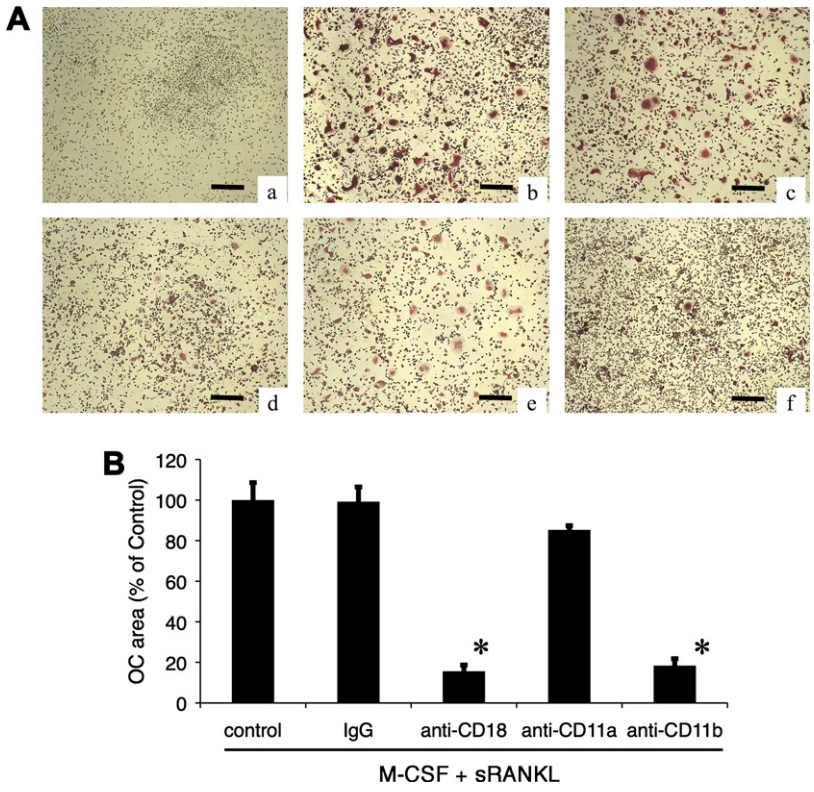

Fig. 5. Effect of antibodies on osteoclast differentiation in BMMs. (A) Representative images of $TRAP^+$ multinuclear cells at 3 days after M-CSF (10 ng/ml) and sRANKL (100 ng/ml) treatment. (a) M-CSF; (b) M-CSF + sRANKL (control); (c) M-CSF+ sRANKL+IgG (IgG2b); (d) M-CSF + sRANKL + anti-CD18 Ab (anti-CD18); (e) M-CSF + sRANKL + anti-CD11a Ab (anti-CD11a); (f) M-CSF + sRANKL + anti-CD11b Ab (anti-CD11b). Bar = 300 μm. (B) Quantification of $TRAP^+$ osteoclast differentiation at 3 days after treatment with sRANKL (100 ng/ml), M-CSF (10 ng/ml), and antibodies (10 μg/ml). Values are presented as means ± S.E., $n = 6$, $^*P < 0.05$ vs IgG group. Analysis of variance followed by Dunnett's test.

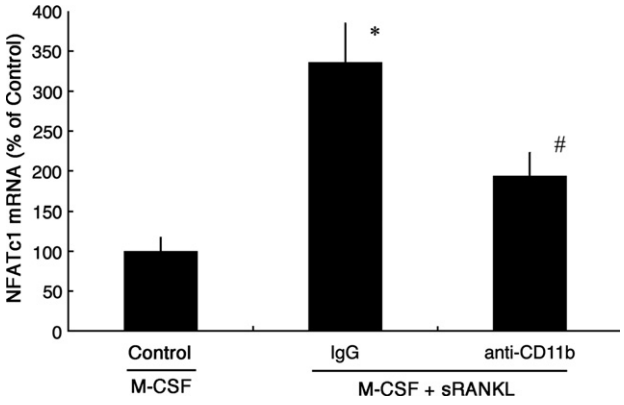

Fig. 6. Effect of anti-CD11b antibody on NFATc1 expression in BMMs. Quantification of NFATc1 mRNA expression at 24 h after treatment of sRANKL (100 ng/ml), M-CSF (10 ng/ml), and antibody (10 μg/ml). Control, M-CSF; IgG, M-CSF + sRANKL + IgG2b, anti-CD11b, M-CSF + sRANKL + anti-CD11b Ab. Values are presented as means ± S.E., $n = 7$, $^*P < 0.05$ vs control group. $^{\#}P < 0.05$ vs IgG group. Analysis of variance followed by Tukey–Kramer multiple comparison test.

RAW264.7 cells expressed ICAM-1 (data not shown). In the current experiment, RANKL-induced osteoclastogenesis was dependent on the cell density of pre-OCs but not on the number of pre-OCs. In addition to RANKL signaling, Mac-1 occupancy with ICAMs may trigger the differentiation of pre-OCs via ITAM-harboring adaptors as mentioned above. This hypothesis may explain the advantage of cell–cell communication in the differentiation of pre-OCs, because pre-OCs must recognize the existence of other pre-OCs to fuse with each other before the beginning of osteoclast differentiation. A recent report showed that the phosphorylation of CD11b was required for integrin activation to bind to ICAMs [28], but the molecule that triggers the phosphorylation of CD11b remains to be determined.

In conclusion, this study showed the importance of the density of pre-OCs in osteoclast differentiation induced by sRANKL. This observation led to the hypothesis that communication among pre-OCs plays an important role in the beginning of osteoclast differentiation. Integrin blockage experiments revealed the involvement of Mac-1 in osteoclast differentiation induced by sRANKL. Moreover, the expression of NFATc1 mRNA induced by RANKL was, at least in part, dependent on integrin occupancy. Collectively, the current findings demonstrate the possible role of integrin in early osteoclast differentiation. These results will contribute to the molecular understanding of osteoclastogenesis.

Acknowledgements: This work was supported in part by research Grants 20390470, 20659306, and 20390463 from the Japan Society for the Promotion of Science, Tokyo, Japan.

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