

Research article

Differentiation of monocytes into multinucleated giant bone-resorbing cells: two-step differentiation induced by nurse-like cells and cytokines

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Abstract

Bone resorption in the joints is the characteristic finding in patients with rheumatoid arthritis (RA). Osteoclast-like cells are present in the synovial tissues and invade the bone of patients with RA. The characteristics of these cells are not completely known. In the work reported here, we generated these cells from peripheral-blood monocytes from healthy individuals. The monocytes were co-cultured with nurse-like cells from synovial tissues of patients with RA (RA-NLCs). Within 5 weeks of culture, the monocytes were activated and differentiated into mononuclear cells positive for CD14 and tartrate-resistant acid phosphatase (TRAP). These mononuclear cells then differentiated into multinucleated giant bone-resorbing cells after stimulation with IL-3, IL-5, IL-7, and/or granulocyte-macrophage-colony-stimulating factor. TRAP-positive cells with similar characteristics were found in synovial fluid from patients with RA. These results indicate that multinucleated giant bone-resorbing cells are generated from monocytes in two steps: first, RA-NLCs induce monocytes to differentiate into TRAP-positive mononuclear cells, which are then induced by cytokines to differentiate into multinucleated giant bone-resorbing cells.

Keywords: monocytes, nurse cells, osteoclasts, rheumatoid arthritis, stromal cells

Our laboratory has established that nurse-like cells (NLCs) are present in the synovial tissues and bone marrow of patients with rheumatoid arthritis (RA) [1–3]. Such cells, which were first discovered in thymus, play an important role in thymocyte maturation and differentiation [4–6]. *In vitro*, they form unique complexes with thymocytes, which initially adhere to them and then

crawl beneath them [7–9]. This phenomenon, which is unique to NLCs at various tissue sites, has been called 'pseudoemperipolesis'. NLCs from RA synovial tissue (RA-NLCs) promote survival of B cells [2,3] and maintain the growth of myeloid cells of patients with RA [1], suggesting that they contribute profoundly to pathogenesis in RA.

Multinucleated cells in synovial tissues have been reported to invade the bone of patients with RA [10]. The cells' expression of tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor suggested that they are osteoclasts [11,12]. Although the presence of osteoclast-like cells in rheumatoid synovium is well understood, the mechanism by which they differentiate is not. In order to examine the effect of RA-NLCs on monocyte functions, we co-cultured peripheral-blood monocytes with RA-NLCs and looked for morphological and functional alterations of CD14- and TRAP-positive cells. We also found such cells in synovial fluid from patients with RA. These cells differentiated into multinucleated giant bone-resorbing cells in the presence of IL-3, IL-5, IL-7, and/or granulocyte/macrophage-colonystimulating factor (GM-CSF). In this way we defined the process by which bone-resorbing cells are generated from monocytic cells.

Materials and methods

Isolation of NLCs from RA synovial tissues

RA-NLCs were established from RA synovial tissues as previously described [1]. Briefly, synovial tissues were obtained from knee joints of five patients with RA who fulfilled American College of Rheumatology criteria for RA [13], after informed consent had been obtained. The cells were cultured in DMEM (Dulbecco's modified Eagle's medium [DMEM; Gibco BRL, Gaithersburg, MD, USA] supplemented with 10% fetal calf serum [FCS; Hyclone, Logan, UT, USA], 100 units/ml of penicillin [Gibco BRL], and 100 µg/ml of streptomycin [Gibco BRL] at 37°C in 7.5% CO₂. RA-NLCs were identified by their ability to support pseudoemperipolexis, seen *in vitro* in the migration of a T-cell lymphoma line, MOLT-17, beneath the NLCs, as previously described [3].

Isolation of mononuclear cells from RA synovial fluid

Synovial fluid was obtained from patients with RA who fulfilled the American College of Rheumatology criteria for RA [13]. The infiltrating cells were collected from the fluid by centrifugation at 1900 *g* and were cultured in supplemented DMEM. After 3 to 5 weeks of culture, most of the lymphocytes and granulocytes disappeared and monocyte-like cells became dominant. CD14-positive monocyte-like cells were purified from this population with a magnetic-activated cell sorter (MACS; Miltenyi Biotec GmbH, Germany) using anti-CD14 antibody conjugated to magnetic beads in accordance with the manufacturer's instructions. The purity of CD14-positive cells was analyzed using a fluorescence-activated cell sorter (FACScan™; see Supplementary material).

Isolation and culture of monocytes from peripheral blood

Peripheral-blood monocytes were collected as plastic-adherent cells, as described previously [14]. Mononuclear cells were isolated from heparinized peripheral blood from five healthy volunteers [15]. Over 97% of the adherent

cells were determined to be monocytes by morphology and CD14 expression.

Monocytes (1×10^6) were co-cultured with RA-NLCs. After 3 to 5 weeks, TRAP-positive mononuclear cells with abundant cytoplasm became dominant. They were collected by gently washing the culture with warm supplemented DMEM and their purity was confirmed cytochemically.

Formation of multinucleated giant cells by TRAP-positive mononuclear cells

The CD14-positive and TRAP-positive mononuclear cells from the synovial fluid of patients with RA were examined for expression of surface antigen and for phagocytic activity and were stimulated with various cytokines (see Supplementary material).

TRAP-positive mononuclear cells (5×10^4) were cultured in supplemented DMEM in the presence or absence of various cytokines or in conditioned medium ([15]; and see Supplementary material) for 96–120 h. In the presence of receptor activator nuclear factor κ B ligand (RANKL), cultures were maintained for 14 days. At the end of the culture period, May–Grunwald–Giemsa (Wako Pure Chemical Co., Osaka, Japan) and TRAP staining (TRAP-staining kit; Sigma, St Louis, MO, USA) were conducted. The frequency of osteoclasts was evaluated from the fusion index, as previously described [16]. More than 1000 nuclei within TRAP-positive multinucleated cells (>4 nuclei/cell) were counted. The fusion index (%) was calculated according to the formula:

$$\frac{\text{total no. of nuclei within multinucleated cells} \times 100}{\text{total no. of nuclei counted}}$$

where 'multinucleated cells' are cells with >4 nuclei.

Examination of bone resorption

TRAP-positive mononuclear cells (5×10^4) were stimulated with various cytokines on a dentin slice for 7 days. In order to examine resorption areas with a scanning electron microscope, the differentiated cells were washed off the slices with distilled water. Then the slices were dehydrated, air-dried, and sputtered with gold.

Results

Morphological changes of peripheral-blood monocytes after co-culture with RA-NLCs

After peripheral-blood monocytes had been cultured with RA-NLCs for 3 to 4 weeks, we recovered TRAP-positive mononuclear cells (Fig. 1a–1c) with abundant cytoplasm and an off-center nucleus (Fig. 1a and 1b). These cells strongly expressed CD11b, CD11c, CD14, CD45, and human major histocompatibility antigen (HLA)-DR, sug-

gesting that they were of monocyte lineage (Table 1). However, they did not express CD11a, CD35, or CD68, which are expressed on freshly isolated monocytes (see Supplementary material).

Presence of TRAP-positive mononuclear cells in synovial fluid from patients with RA

We detected monocytic cells positive for CD14 and TRAP in synovial fluids of patients with RA. These cells also strongly expressed CD11b, CD11c, CD14, CD45, and HLA-DR (see Supplementary material) but not CD1a, CD1b, CD2, CD5, or CD86, which are expressed on dendritic cells derived from monocytes [17]. These results indicate that TRAP-positive monocytic cells present in synovial fluid and those induced *in vitro* in cultures with RA-NLCs are morphologically and phenotypically the same. These cells were obtained from the synovial fluid of all patients with RA, regardless of age and sex. CD14-positive monocyte-like cells accounted for 20–91% of the mononuclear cells freshly isolated from the synovial fluid of such patients (data not presented).

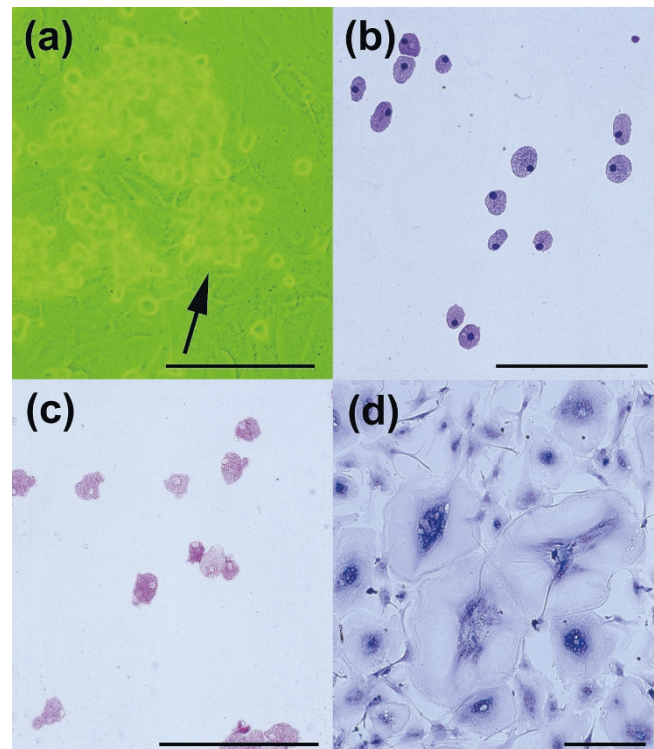
Differentiation of TRAP-positive mononuclear cells into multinucleated giant bone-resorbing cells in the absence of RA-NLCs

The TRAP-positive cells induced *in vitro* and those isolated from synovial fluid both differentiated into multinucleated cells after being cultured for 72 to 96 h with the conditioned medium (Fig. 1d). These multinucleated cells still possessed TRAP activity (data not shown) and formed resorption areas on dentin slices (Fig. 2), suggesting that they had bone-resorbing activity as osteoclasts.

Induction of multinucleated cells by IL-3, IL-5, IL-7 or GM-CSF

The cytokines IL-3, IL-5, IL-7, and GM-CSF induced differentiation of TRAP-positive cells induced *in vitro* or those isolated from synovial fluid into osteoclasts (Table 1). Regardless of which cytokine was used to stimulate differentiation of the osteoclasts, they were all positive for TRAP and formed resorption pits on dentin slices, suggesting that they were all identical to the cells induced by conditioned medium (data not shown). The fusion index of osteoclasts induced by a mixture of cytokines was higher than those stimulated with a single cytokine. The cytokines IL-6 and IL-8, which are produced by RA-NLCs [2], did not induce osteoclast formation. RANKL was recently reported to induce osteoclasts from human peripheral blood [18,19]; however, a mixture of macrophage-colony-stimulating factor and RANKL exhibited only weak activity for induction of osteoclasts from the TRAP-positive mononuclear cells (Table 1). Phytohemagglutinin, which was contained in the conditioned medium, did not induce differentiation. TRAP-positive cells from the synovial fluid of patients with RA have a fusion index similar to that found for the TRAP-positive cells obtained experimentally

Figure 1



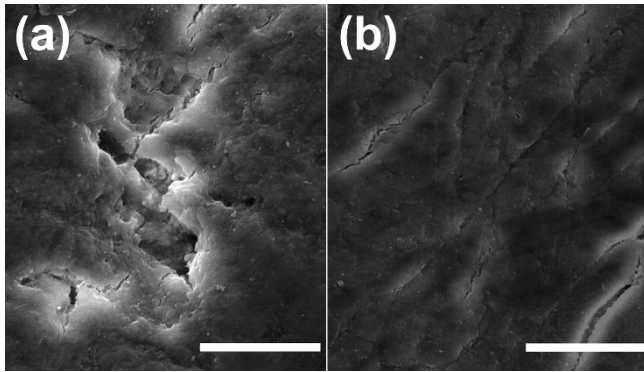
Morphology of TRAP-positive mononuclear cells induced from peripheral-blood monocytes with RA-NLCs. (a) Phase-contrast micrograph of monocytes co-cultured with RA-NLCs. Mononuclear cells (arrow) are growing on the RA-NLCs. (b) Mononuclear cells collected from the culture shown in (a). May-Grunwald-Giemsa staining. (c) Detection of TRAP expressed by the mononuclear cells (TRAP-positive cells were stained red with their cytoplasm). (d) Differentiated mononuclear cells. The cells shown here are multinucleated giant cells. May-Grunwald-Giemsa staining. Scale lines = 100 μ m.

from monocytes (data not shown). Induction of the osteoclasts was completely neutralized by the antibody to each cytokine (see Supplementary material).

Discussion

We have shown that the novel ability of RA-NLCs may contribute to the pathogenesis of RA by encouraging the generation of TRAP-positive mononuclear cells, which are osteoclast precursors. The TRAP-positive precursor cells have phagocytic activity and are negative for CD83, suggesting that they are different from peripheral-blood monocytes and dendritic cells [14,17].

Fujikawa *et al* reported that synovial macrophages differentiated into osteoclasts after incubation in the presence of a rat osteoblast-like cell line [20]. The fibroblasts isolated from RA synovia induced differentiation of monocytes into multinucleated cells in the presence of 1,25-dihydroxyvitamin D₃ and macrophage-colony-

Figure 2

Scanning electron micrographs of dentin slices, showing (a) resorption areas formed on dentin by TRAP-positive multinucleated giant cells derived from monocytes and stimulated with granulocyte-macrophage-colony-stimulating factor for 96 h and (b) a control slice incubated with the TRAP-positive mononuclear cells in the absence of cytokines. Scale lines = 50 μ m.

stimulating factor [21]. Further study will be required to determine the identity of those monocytic cells and our cells. Fibroblastic cells in synovial fluid from patients with RA have been reported to support pseudoemperipolesis, which was considered to be the unique feature of the nurse cells, in the presence of IL-4 [22]. Shigeyama *et al* recently reported that RA synovial fluid may promote osteoclastogenesis from monocytes by expressing osteoclast differentiation factor [23]. It is likely that RA fibroblasts and RA-NLCs share several roles in the pathogenesis of RA, including activation of monocytes. However, the molecules required in our study for osteoclastogenesis from monocytes were different from those in the study of Shigeyama *et al* [23]. These findings suggest that multiple pathways for osteoclastogenesis in RA synovia may cause severe joint destruction.

There may be two steps for generation of the osteoclasts in the joints of patients with RA: first, differentiation of monocytes into TRAP-positive mononuclear cells induced and maintained by RA-NLCs, followed by cytokine-induced differentiation of these mononuclear cells into osteoclasts. The interaction between monocytes and RA-NLCs required adhesion molecules, but RANK (receptor activator of nuclear-factor- κ B) and RANKL were not necessary to induce the TRAP-positive cells in preliminary studies in our laboratory (unpublished observation). The molecules required in the interaction are under investigation. In addition, we found the presence of TRAP-positive mononuclear cells which differentiated into osteoclasts in synovial fluids of patients with RA *in vitro*. Monocytes may infiltrate the affected joints and differentiate into TRAP-positive mononuclear cells under the influence of RA-NLCs. This conclusion is consistent with previous findings

Table 1

Differentiation of human TRAP-positive mononuclear cells derived from peripheral-blood monocytes into multinucleated cells after stimulation with IL-3, IL-5, IL-7, and GM-CSF

Stimulator	Concentration	Fusion index (%) ^a
None		1.8
Conditioned medium ^b	(10% v/v)	86.1
IL-1 α	(1 ng/ml)	1.1
IL-1 β	(1 ng/ml)	1.3
IL-2	(250 U/ml)	7.7
IL-3	(5 ng/ml)	64.8
IL-4	(100 U/ml)	1.3
IL-5	(1 ng/ml)	66.1
IL-6	(20 ng/ml)	5.6
IL-6 + sIL-6R	(sIL-6R: 200 ng/ml)	5.3
IL-7	(20 ng/ml)	72.4
IL-8	(20 ng/ml)	2.1
IFN- γ	(100 U/ml)	7.1
GM-CSF	(1 ng/ml)	73.8
M-CSF	(25 ng/ml)	5.8
TNF- α	(1 ng/ml)	6.9
VD ₃	(10 ⁻⁷ mol/l)	3.4
Dexamethasone	(10 ⁻⁸ mol/l)	0.8
M-CSF + IL-4	(M-CSF, 25 ng/ml; IL-4, 100 U/ml)	0.1
IL-3 + IL-7		75.4
IL-7 + GM-CSF		77.3
IL-3 + IL-7 + GM-CSF		78.7
Phytohemagglutinin	(1% v/v)	2.0
RANKL ^c	(100 ng/ml)	2.6
RANKL + M-CSF ^c	(RANKL/ODF, 100 ng/ml; M-CSF, 25 ng/ml)	38.1

^aTRAP-positive mononuclear cells were stimulated with various cytokines for 96–120 h. Fusion indices were calculated using the formula given in the text and previously [16]. Data are representative of three independent experiments using TRAP-positive cells induced from monocytes. ^bSee [15], and Supplementary material. ^cThe culture was maintained for 14 days. GM-CSF = granulocyte macrophage-colony-stimulating factor; IFN = interferon; IL = interleukin; M-CSF = macrophage-colony-stimulating factor; ODF = osteoclast differentiation factor; RANKL = receptor activator of nuclear-factor- κ B ligand; sIL-6R = soluble interleukin-6 receptor; TNF = tumor necrosis factor; VD₃ = 1,25-dihydroxyvitamin D₃.

of TRAP-positive multinucleated giant cells in the synovial tissue of patients with RA [10–12]. Further studies are required to characterize these osteoclasts derived from the TRAP-positive mononuclear cells and to delineate the unique course of differentiation into bone-resorbing cells promoted by RA-NLCs.

Conclusion

In order to elucidate the role of RA-NLCs, monocytes were co-cultured with RA-NLCs. Monocytes differentiated into TRAP-positive mononuclear cells, the precursor cells of osteoclasts. Osteoclasts were generated from TRAP-positive mononuclear cells in the presence of IL-3, IL-5, IL-7, and GM-CSF. TRAP-positive cells were also present in synovial fluids of patients with RA. RA-NLCs may play a significant role in the activation of monocytes and long-term maintenance of differentiated monocytes (osteoclast precursors). The present study suggests that monocytes may differentiate into osteoclast precursor cells in the affected joints of patients with RA.

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Supplementary material

Supplementary materials and methods

Cell lines

Human lung fibroblasts CCD-19Lu were obtained from American Type Culture Collection (Rockville, MD, USA). Human T cell line MOLT-17 was a generous gift from Dr J Minowada (Fujisaki Cell Center, Okayama, Japan). These cell lines were cultured as recommended by the providers.

Examination of pseudoemperipolesis

Pseudoemperipolesis was measured as previously described [3]. RA synovial stromal cells (3×10^4) were incubated in supplemented DMEM in 24-well culture

plates overnight. The next day, MOLT-17 cells (1×10^6) were added to the RA-NLC culture. Stromal cells with more than three MOLT-17 cells beneath them after 6 h of incubation were considered NLCs.

Long-term maintenance of monocytes by RA-NLCs

Monocytes (2.5×10^5) were co-cultured with RA-NLCs or CCD-19Lu with or without culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) in supplemented DMEM in 48-well culture plates at 37°C in 7.5% CO₂ for up to 70 days. Half of the medium was changed once a week. The TRAP-positive mononuclear cells were collected from the culture, stained with trypan blue, and counted for viability under a microscope.

Antibodies and staining of cells

The cell-surface antigens on freshly isolated monocytes and TRAP-positive mononuclear cells were examined by staining with monoclonal antibodies specific for CD1a, CD4, CD5, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD20, CD34, CD45, CD45RA, CD45RO, CD54, HLA-DR (Becton Dickinson), CD1b (Nichirei, Tokyo, Japan), CD2, CD3 (Ortho Diagnostics, Raritan, NJ, USA), CD35, CD68 (DAKO Japan, Kyoto, Japan), CD51/61, CD83, HLA-A, B and C (Pharmingen, San Diego, CA, USA), CD80, and CD86 (Ansell, Bayport, MN, USA). Antigen-expression was analyzed with a FACScan flow cytometer (Becton Dickinson). Multinucleated giant bone-resorbing cells differentiated from TRAP-positive mononuclear cells were fixed with cold acetone and stained immunohistochemically with rabbit polyclonal antibodies specific for actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), carbonic anhydrase II (Rockland, Gilbertsville, PA, USA), or vitronectin receptor (Chemicon International, Inc., Temecula, CA, USA). TRAP activity in TRAP-positive mononuclear cells and multinucleated giant bone-resorbing cells was examined using a TRAP-staining kit (Sigma, St Louis, MO, USA). Neutralizing antibodies to human IL-3, IL-5, IL-7, and GM-CSF were purchased from Genzyme (Cambridge, MA, USA).

Cytokines and reagents

Conditioned media were prepared as previously reported [15]. Briefly, a mixture of peripheral-blood mononuclear cells from 10 healthy donors was stimulated with phytohemagglutinin at 37°C for 72 h. Culture supernatant fluids were collected, filtered, and used as conditioned media. IL-1 α was purchased from Immugenex (Los Angeles, CA). IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, soluble IL-6 receptor (sIL-6R), interferon gamma (IFN- γ), granulocyte/macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor, and tumor necrosis factor (TNF)- α were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). IL-7 and IL-8 were purchased from Genzyme. 1,25-dihydroxyvitamin D₃ and dexamethasone were purchased from Wako Pure Chemical Co. (Osaka, Japan). Receptor

activator of nuclear-factor- κ B ligand (RANKL) was obtained from Peprotech (London, UK).

Assessment of phagocytic activity

Phagocytic activity of TRAP-positive mononuclear cells was assessed from their ingestion of heat-killed yeast. TRAP-positive mononuclear cells (1×10^6) were incubated with 2×10^7 yeast cells in phosphate-buffered saline supplemented with 10% fresh human serum, type AB, at 37°C for 45 min. The cells were washed and stained with fuchsin (Wako Pure Chemical Co.), and the cells with ingested yeast were counted under a microscope.

Inhibition of the generation of multinucleated giant cells from TRAP-positive mononuclear cells by neutralizing antibodies

Neutralizing antibodies specific for IL-3, IL-5, IL-7, and GM-CSF were used for inhibition of the generation of multinucleated giant bone-resorbing cells. Irrelevant polyclonal mouse IgG from Jackson ImmunoResearch (West Grove, PA, USA) was used as a control. The TRAP-positive mononuclear cells (5×10^4) were pre-incubated with each antibody in DMEM containing 10% FCS in microtubes at 37°C for 1 h. The cells were cultured in 4-well chamber slides, and stimulated with a cytokine for 96–120 h at 37°C in 7.5% CO₂. At the end of the culture period, the cells were stained for TRAP and the fusion index was calculated as described in the main paper.

Detection of calcitonin receptors

Calcitonin receptors on the multinucleated giant bone-resorbing cells were detected *in situ* using ¹²⁵I-human calcitonin were performed as described elsewhere [23]. TRAP-positive mononuclear cells (5×10^4) were stimulated with IL-3, IL-5, IL-7, or GM-CSF at the optimal concentrations in 4-well chamber slides (Nalge Nunc International, Rochester, NY, USA) for 96–120 h at 37°C in 7.5% CO₂. After formation of multinucleated giant bone-resorbing cells had been confirmed microscopically, the cells were incubated in 0.4 ml α MEM (Gibco BRL) with 0.1% bovine serum albumin and 0.2 mCi/ml of ¹²⁵I-labeled human calcitonin (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 h at 22°C. Nonspecific binding was assessed on each slide in the presence of an excess amount of unlabeled human calcitonin. Then the cells were washed three times with phosphate-buffered saline solution and fixed with 2.5% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The slides were washed and dried as previously described [S1]. Air-dried slides were dipped in photographic emulsion (Kodak NTB3; Eastman Kodak, Rochester, NY, USA), drained, and dried for 2 h and were kept in a light-proof container with desiccant at 4°C for 10 days. The slides were developed in accordance with the manufacturer's instructions.

Supplementary Table 1

Expression of surface antigen by TRAP-positive mononuclear cells generated by co-culture with RA-NLC

Antigen	% Positive ^a		
	Monocytes from peripheral blood	TRAP-positive cells induced from monocytes	TRAP-positive cells from RA-SF
CD1a	1.24	0.11	0.24
CD1b	0.22	0.13	0.12
CD2	2.20	0.18	1.01
CD3	0.82	0.85	0.82
CD4	0.28	0.61	0.35
CD5	0.23	0.44	0.29
CD11a	LFA-1	94.72	1.16
CD11b	CR3 α chain	95.46	99.42
CD11c	CR4 α chain	99.47	99.22
CD13		99.44	96.15
CD14		95.70	99.28
CD15	Le ^x	22.81	0.49
CD16	Fc γ R III	0.72	0.24
CD19		0.33	0.13
CD20		0.36	0.11
CD34		0.54	0.18
CD35	CR1	93.49	0.50
CD45		98.73	94.28
CD45RA		0.37	0.15
RA45RO		0.65	4.18
CD51/61	VNR ^b	0.22	0.10
CD54	ICAM-1	0.21	0.30
CD68		94.59	1.06
CD80	B7/BB1	8.19	7.41
CD83		0.37	0.10
CD86	B70/FUN-1	90.01	0.55
HLA-A, -B, -C		97.44	99.68
HLA-DR		96.65	98.45

^aCells were stained with monoclonal antibody specific for various antigens, and analyzed by FACScan. Data are representative of four independent analyses. ^bVitronectin receptor. RA-SF = synovial fluid from patients with rheumatoid arthritis.

Supplementary results

Phenotypic characterization of the TRAP-positive mononuclear cells induced by NLCs

The TRAP-positive mononuclear cells, whether induced from monocytes or collected from the synovial fluid of patients with RA, strongly expressed CD11b, CD11c, CD14, CD45, HLA-A, HLA-B, HLA-C, and HLA-DR but did not express CD1a, CD1b, CD2, CD4, CD5, CD16, CD19, CD20, or CD83. These observations suggest that

these cells belong to the monocyte/macrophage lineage (Supplementary Table 1). However, the cells did not express CD11a, CD35, or CD68, which are expressed on freshly isolated monocytes from peripheral blood (Supplementary Table 2). The TRAP-positive mononuclear cells had strong phagocytic activity against heat-killed yeast (data not presented). In addition, the cells were positive for carbonic anhydrase II, actin, and vitronectin receptor (Supplementary Fig. 1), and calcitonin receptors were also

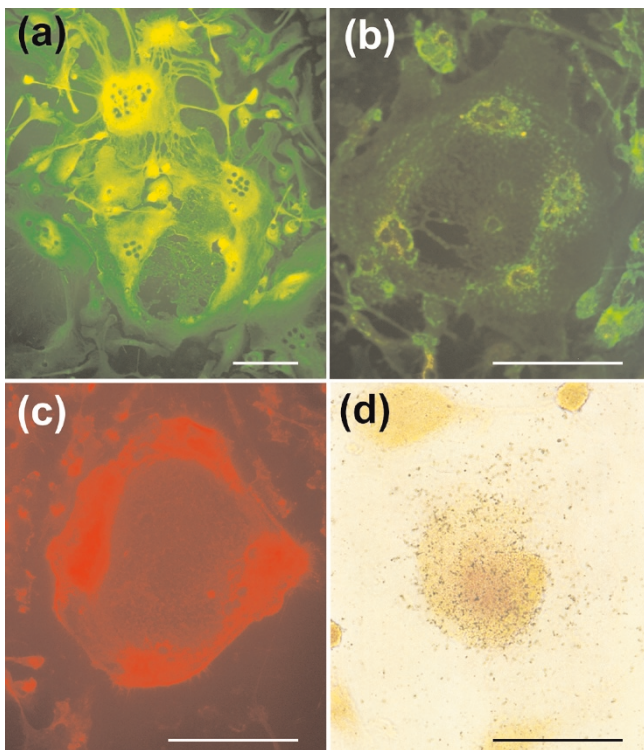
Supplementary Table 2

Inhibition of formation of multinucleated giant cells by neutralizing antibodies specific for IL-3, IL-5, IL-7, and GM-CSF

Antibody ^b	Fusion index (%) ^a			
	IL-3 (5 ng/ml)	IL-5 (1 ng/ml)	IL-7 (20 ng/ml)	GM-CSF (1 ng/ml)
Polyclonal mouse IgG	63.8 ± 1.2	62.3 ± 3.6	66.2 ± 5.4	67.7 ± 6.0
Anti-IL-3	7.3 ± 1.6	–	–	–
Anti-IL-5	–	3.8 ± 1.8	–	–
Anti-IL-7	–	–	5.5 ± 5.8	–
Anti-GM-CSF	–	–	–	4.0 ± 1.3

^aFusion indices were calculated using the formula given in the Methods section. Each value is the mean ± SD of three independent experiments using TRAP-positive cells induced from monocytes of three individuals. ^bConcentrations of cytokines: polyclonal mouse IgG, 10 mg/ml; anti-IL-3, 10 mg/ml; anti-IL-5, 5 mg/ml; anti-IL-7, 10 mg/ml; anti-GM-CSF, 2 mg/ml. – = not examined; GM-CSF = granulocyte/macrophage-colony-stimulating factor; IL = interleukin.

Supplementary Figure 1



Immunohistochemical staining of multinucleated giant bone-resorbing cells. The cells were stained green for (a) carbonic anhydrase II and (b) vitronectin receptor. The cells were also positive for (c) actin, which showed red, ring-form staining. (d) *In situ* detection of calcitonin receptor using ¹²⁵I-human calcitonin. Black grains mark the cells expressing calcitonin receptor. The multinucleated giant bone-resorbing cells were incubated with ¹²⁵I-human calcitonin for 1 h. The cells were washed, fixed, and dried as described in the Supplementary materials and methods section. Air-dried slides were exposed at 4°C for 10 days. Scale lines = 100 μm.

detected (Supplementary Fig. 1); these four receptors are considered characteristic of osteoclasts [10,23].

Long-term maintenance of monocytes by RA-NLCs

Monocytes obtained from all five healthy individuals and co-cultured with RA-NLCs grew and differentiated into TRAP-positive mononuclear cells (Supplementary Fig. 2). We also used five RA-NLC lines, each from a different patient with RA. All five cell lines induced the generation of TRAP-positive mononuclear cells from human peripheral blood monocytes (data not presented). The generation of these TRAP-positive cells required direct contact with RA-NLCs.

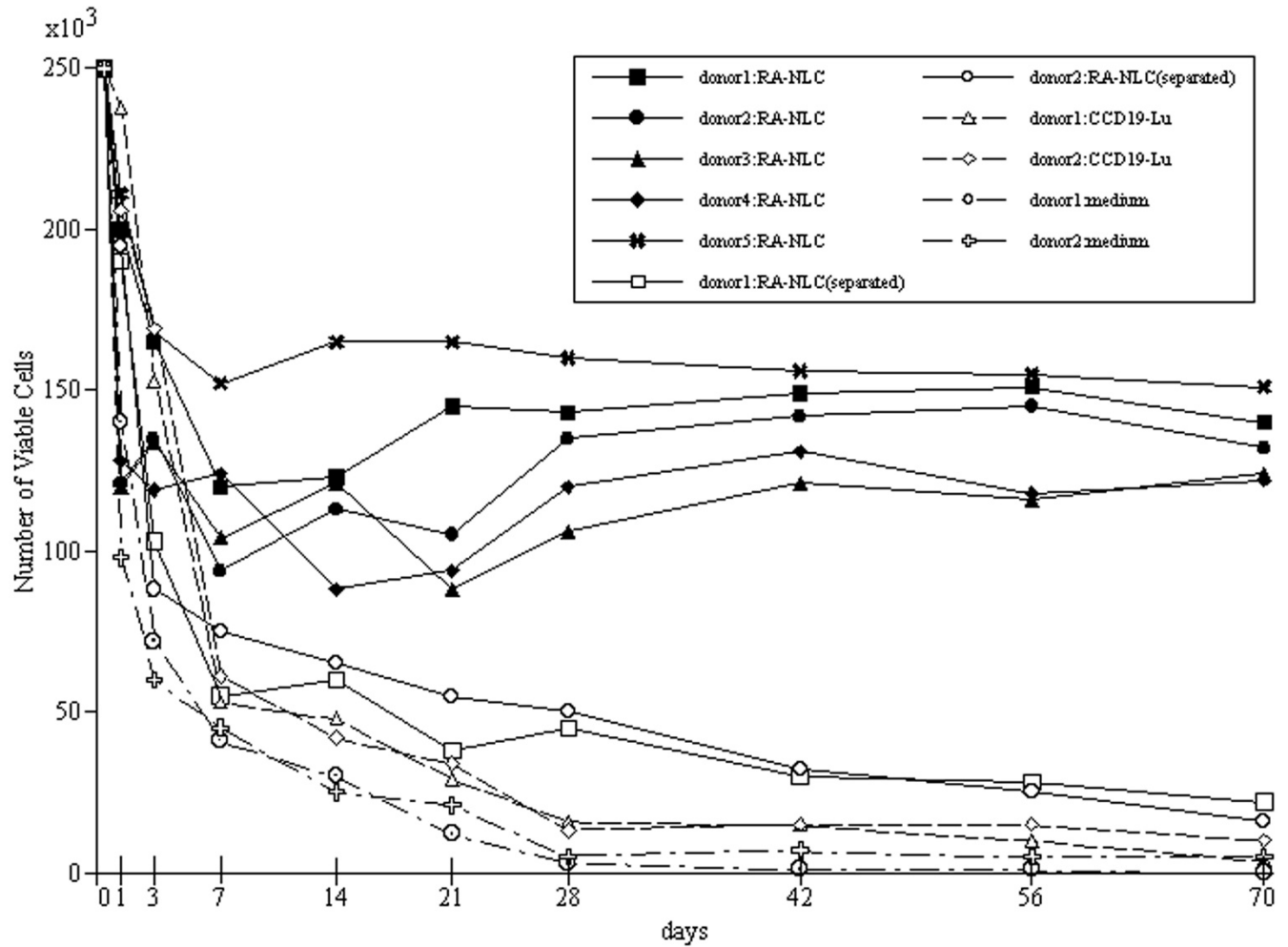
Induction of osteoclasts by IL-3, IL-5, IL-7, or GM-CSF

The conditioned medium contains several cytokines. In order to determine which cytokines induce TRAP-positive mononuclear cells to differentiate into multinucleated giant bone-resorbing cells, *in vitro* induced TRAP-positive cells and those isolated from synovial fluid of patients with RA were collected and stimulated with various cytokines in the absence of RA-NLCs. In a preliminary study, a high-performance liquid chromatography fraction of the conditioned medium, which was used to induce TRAP-positive mononuclear cells to differentiate into multinucleated giant bone-resorbing cells, contained proteins with a molecular weight of approximately 20 kDa (data not shown). Therefore, we mainly examined cytokines with approximately that molecular weight. The activities of IL-3, IL-5, IL-7, and GM-CSF for inducing multinucleated giant bone-resorbing cells were completely neutralized by the antibody to the respective cytokine (Supplementary Table 2).

Supplementary reference

S1. Nicholson GC, Moseley JM, Sexton PM, Mendelsohn FAO, Martin TJ: **Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterization.** *J Clin Invest* 1986, **78**:355-360.

Supplementary Figure 2



Effects of culture conditions on survival of monocytes from five donors with RA-NLCs or CCD-19Lu, in the presence (separated) or absence (no indication) of culture inserts. Each culture was maintained for up to 70 days. The resulting mononuclear cells were collected, stained with trypan blue, and counted under a microscope. Each experiment was conducted in duplicate, and each point represents the mean number of viable cells in two cultures.