together, we propose that enhanced expression of AREG in BMMC and PMBC may play a pivotal role in the pathogenesis of RA.

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Research article

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

Tomoko Toyosaki-Maeda*, Hiroshi Takano[†‡], Tetsuya Tomita[†], Yuji Tsuruta*, Miki Maeda-Tanimura*, Yasunori Shimaoka[†], Tetsu Takahashi[‡], Tsunetoshi Itoh[§], Ryuji Suzuki* and Takahiro Ochi[†]

*Department of Immunology, Shionogi Research Laboratories, Shionogi & Co Ltd, Osaka, Japan
[†]Department of Orthopedic Surgery, Osaka University Medical School, Osaka, Japan
[‡]Department of Oral Surgery, Kyushu Dental College School of Dentistry, Kokura-Kita, Kita-Kyushu, Japan
[§]Department of Immuno-Ontogeny, Tohoku University School of Medicine, Aoba-ku, Sendai, Miyagi, Japan

Correspondence: Takahiro Ochi, Department of Orthopedic Surgery, Osaka University Medical School, Yamada-Oka 2-2, Suita, Osaka 566-0871, Japan. Tel: +81 6 6879 3862; fax: +81 6 6879 3869; e-mail: ochi@ort.med.osaka-u.ac.jp

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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.

DMEM = Dulbecco's modified Eagle's medium; FCS = fetal calf serum; GM-CSF = granulocyte/macrophage-colony-stimulating factor; HLA = human major histocompatibility antigen; IL = interleukin; NLC = nurse-like cell; RA = rheumatoid arthritis; RANKL = receptor activator of nuclear-factor-κB ligand; RA-NLC = nurse-like cell derived from rheumatoid arthritis synovial tissue; TRAP = tartrate-resistant acid phosphatase.

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% CO_2. RA-NLCs were identified by their ability to support pseudoemperipolesis, 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; Myltenyi 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 κB 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 meterial) 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 morphplogically 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).

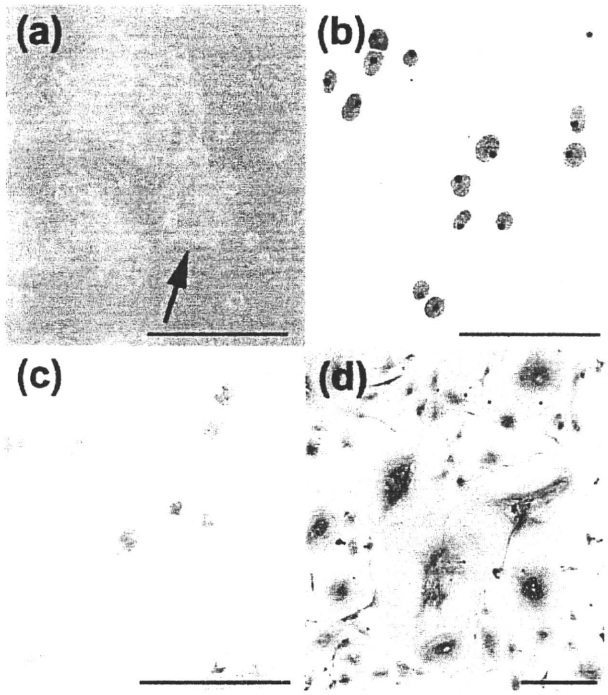
Differentiation of TRAP-positive mononuclear cells into multinucleated giant bone-resorbing cells in the absence of RA-NLCs

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

Figure 1



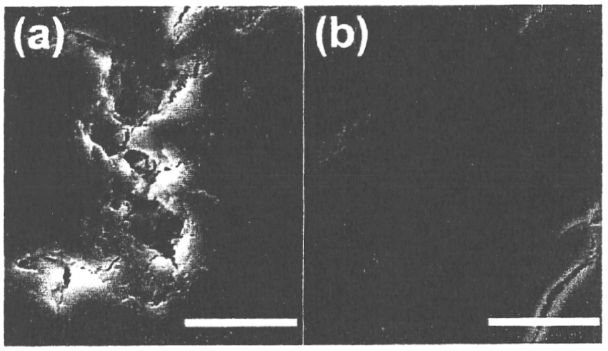
Morphology of TRAP-positive mononuclear cells induced from peripheral-blood monocytes with RA-NLCs. **(a)** Phase-contrast micrograph of monocytes co-cultured with RA-NLCs. Mononuclear cells (arrow) are growing on the RA-NLCs. **(b)** Mononuclear cells collected from the culture shown in (a). May–Grunwald–Giemsa 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 μm.

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 D_3 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 μm.

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-1α	(1 ng/ml)	1.1
IL-1β	(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

[a]TRAP-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. [b]See [15], and Supplementary material. [c]The 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-κB ligand; sIL-6R = soluble interleukin-6 receptor; TNF = tumor necrosis factor; VD_3 = 1,25-dihydroxyvitamin D_3.

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-κB) 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 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.

Acknowledgements

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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% CO_2 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 (Ancell, 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-1α was purchased from Immugenex (Los Angeles, CA). IL-1β, 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 D_3 and dexamethasone were purchased from Wako Pure Chemical Co. (Osaka, Japan). Receptor activator of nuclear-factor-κB 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% CO_2. 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 ^{125}I-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% CO_2. After formation of multinucleated giant bone-resorbing cells had been confirmed microscopically, the cells were incubated in 0.4 ml αMEM (Gibco BRL) with 0.1% bovine serum albumin and 0.2 mCi/ml of ^{125}I-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	1.18
CD11b	CR3 α chain	95.46	99.42	99.56
CD11c	CR4 α chain	99.47	99.22	99.01
CD13		99.44	96.15	93.87
CD14		95.70	99.28	99.63
CD15	Le^x	22.81	0.49	15.97
CD16	FcgR III	0.72	0.24	0.30
CD19		0.33	0.13	0.13
CD20		0.36	0.11	0.10
CD34		0.54	0.18	0.18
CD35	CR1	93.49	0.50	0.60
CD45		98.73	94.28	92.90
CD45RA		0.37	0.15	0.46
RA45RO		0.65	4.18	3.30
CD51/61	VNR[b]	0.22	0.10	0.17
CD54	ICAM-1	0.21	0.30	0.33
CD68		94.59	1.06	1.10
CD80	B7/BB1	8.19	7.41	6.52
CD83		0.37	0.10	1.42
CD86	B70/FUN-1	90.01	0.55	1.21
HLA-A, -B, -C		97.44	99.68	99.53
HLA-DR		96.65	98.45	98.16

[a]Cells were stained with monoclonal antibody specific for various antigens, and analyzed by FACScan. Data are representative of four independent analyses. [b]Vitronectin 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

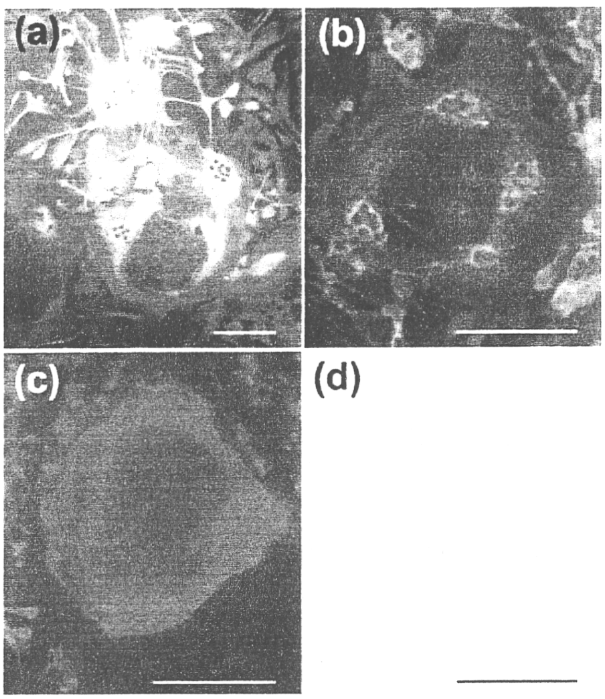
Supplementary Table 2

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

	Fusion index (%) [a]			
Antibody[b]	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

[a]Fusion 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. [b]Concentrations 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 ^{125}I-human calcitonin. Black grains mark the cells expressing calcitonin receptor. The multinucleated giant bone-resorbing cells were incubated with ^{125}I-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.

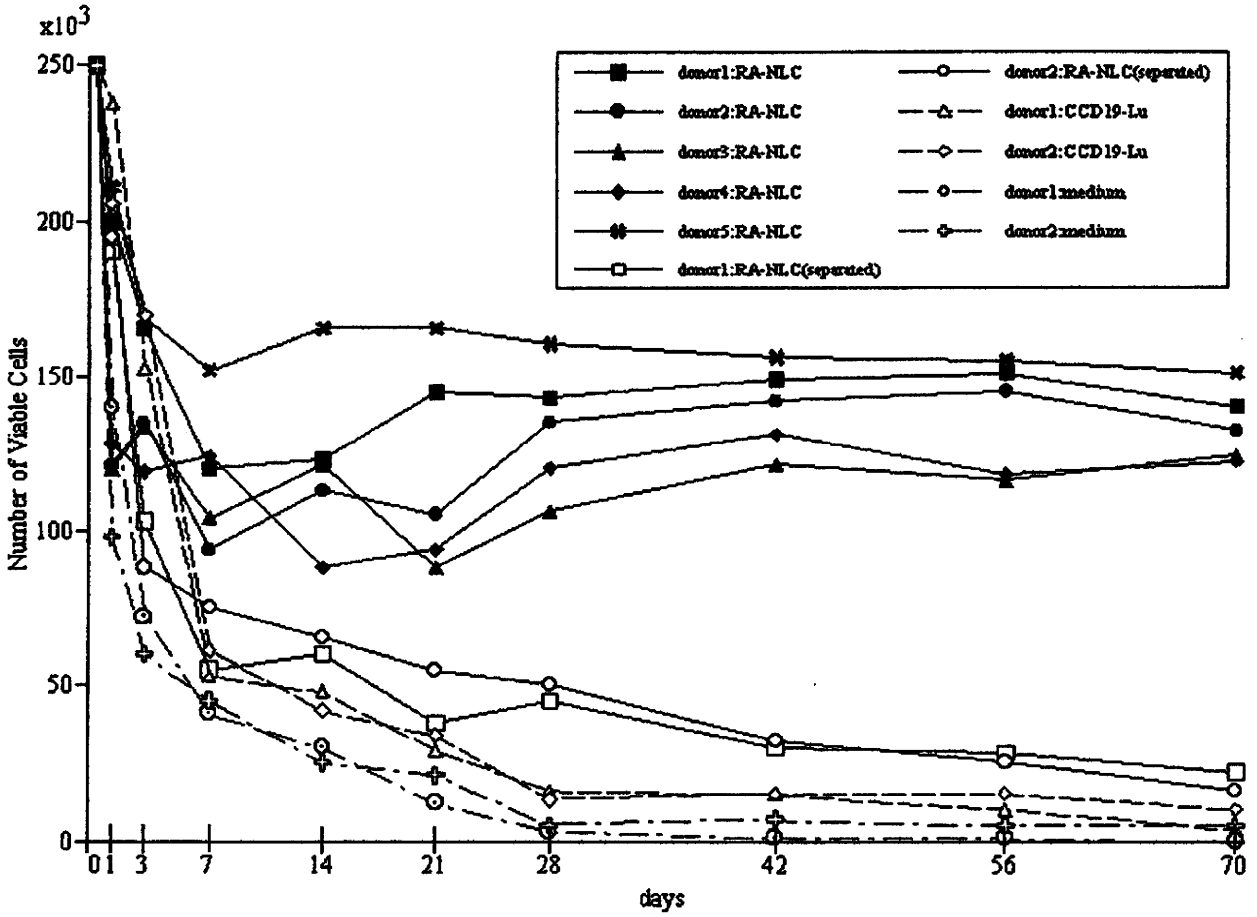
Induction of osteoclasts by IL-3, IL-5, IL-7, or GM-CSF

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.

Rheumatoid Arthritis Synovial Stromal Cells Inhibit Apoptosis and Up-Regulate Bcl-x_L Expression by B Cells in a CD49/CD29-CD106-Dependent Mechanism[1]

Kenji Hayashida,* Yasunori Shimaoka,† Takahiro Ochi,† and Peter E Lipsky[2]*

Inflammatory sites, such as rheumatoid arthritis (RA) synovial tissue, contain large numbers of activated B cells and plasma cells. However, the mechanisms maintaining B cell viability and promoting their differentiation are not known, but interactions with stromal cells may play a role. To examine this, purified human peripheral B cells were cultured with a stromal cell line (SCL) derived from RA synovial tissue, and the effects on apoptosis and expression of Bcl-2-related proteins were analyzed. As a control, B cells were also cultured with SCL from osteoarthritis synovium or skin fibroblasts. B cells cultured with medium alone underwent spontaneous apoptosis. However, B cells cultured with RA SCL cells exhibited less apoptosis and greater viability. Although SCL from osteoarthritis synovium and skin fibroblasts also rescued B cells from apoptosis, they were less effective than RA SCL. B cell expression of Bcl-x_L was markedly increased by RA SCL in a contact-dependent manner, whereas B cell expression of Bcl-2 was unaffected. Protection of B cells from apoptosis and up-regulation of Bcl-x_L by RA SCL were both blocked by mAbs to CD106 (VCAM-1), but not CD54 (ICAM-1). Furthermore, cross-linking of CD49d/CD29 (very late Ag-4) on the surface of B cells rescued them from apoptosis and up-regulated Bcl-x_L expression. These results indicate that SCL derived from RA synovial tissue play a role in promoting B cell survival by inducing Bcl-x_L expression and blocking B cell apoptosis in a CD49d/CD29-CD106-dependent manner. *The Journal of Immunology*, 2000, 164: 1110–1116.

The synovial tissue in rheumatoid arthritis (RA)[3] is characterized by infiltration with a variety of inflammatory cells, including T cells, B cells, and plasma cells (1). The lymphocyte accumulation in synovial tissue might be induced by an increased migration from peripheral blood, proliferation of these cells in situ, and/or inhibition of cell death. Increased migration into the synovium has been suggested (2, 3), whereas local proliferation appears to be minimal (4). Programmed cell death is limited in the synovium and rarely involves lymphocytes (5). Therefore, the limited programmed cell death of lymphocytes may contribute to their accumulation in the synovium. A number of mechanisms have been proposed to account for the apparently diminished apoptosis of lymphocytes, including the action of cytokines (6–17), the presence of inhibitors of apoptosis, such as soluble CD95 and Fas ligand (18, 19), and the direct impact of cell-to-cell contact (20). Among the cells that appear to be active in blocking lymphocyte apoptosis is a population of stromal cells with the characteristics of thymus and bone marrow nurse cells (21, 22). Previous studies documented that these stromal cell lines (SCL) blocked B cell apoptosis in vitro (22). The current studies examined the mechanism by which B cells were rescued from apoptosis and demonstrated that SCL derived from RA synovium induced expression of the antiapoptotic protein, Bcl-x_L, by a mechanism that involved cell-to-cell contact mediated by CD49d/CD29-CD106 interactions.

Materials and Methods

Antibodies and reagents

Biotinylated mouse anti-human CD19 mAb and mouse anti-human CD29 mAb (4B4) were purchased from Coulter (Miami, FL). Mouse anti-human CD20 mAb conjugated with PE, goat anti-mouse IgG conjugated with FITC, mouse anti-human CD14 mAb conjugated with FITC, goat anti-mouse IgG, and goat anti-rabbit IgG conjugated with HRP were obtained from Sigma (St. Louis, MO). Mouse anti-human CD49d mAb (HP2/1) and mouse anti-human CD106 mAb (1.G11B1) were purchased from Immunotech (Miami, FL) and Serotec (Oxford, U.K.), respectively. Mouse IgG1 (MOPC) mAb, mouse anti-human IgM heavy chain (DA4.4) conjugated with biotin, mouse anti-human CD11a mAb (TS1/22), and mouse anti-human CD18 mAb (TS1/18) were prepared from hybridoma cell lines purchased from American Type Culture Collection (Manassas, VA). Mouse anti-human CD54 mAb (R6.5) was a gift from Dr. R. Rothlein (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Rabbit anti-human Bcl-2 (C-21) and Bcl-$x_{S/L}$ (L-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Propidium iodide and streptavidin were purchased from Sigma. Triazol reagent, DNase I, and SuperScript II reverse transcriptase were obtained from Life Technologies (Frederick, MD). Taq polymerase was obtained from Promega (Madison, WI). Oligo(dT) and Ficoll/Isopaque were purchased from Pharmacia (Piscataway, NJ). DMEM with high glucose, RPMI 1640, and FBS were purchased from Life Technologies. The protein assay system, polyvinylidene difluoride membranes, and Zeta-Probe blotting membranes were purchased from Bio-Rad (Richmond, CA). ECL was purchased from Amersham (Aylesbury, U.K.).

Stromal cell lines and fibroblast lines

One RA SCL was established from synovium as previously described (22). In brief, synovial tissues of patients with RA who met American College of Rheumatology criteria (23) were obtained after obtaining informed consent and were dissociated with collagenase and trypsin. Dissociated single

*University of Texas Southwestern Medical Center, Dallas, TX 75235; and †Osaka University Medical School, Osaka, Japan

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[2] Address correspondence and reprint requests to Dr. Peter E. Lipsky, Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8884. E-mail address: peter.lipsky@email.swmed.edu

[3] Abbreviations used in this paper: RA, rheumatoid arthritis; SCL, stromal cell line; OA, osteoarthritis; VLA-4, very late Ag-4; FDC, follicular dendritic cells.

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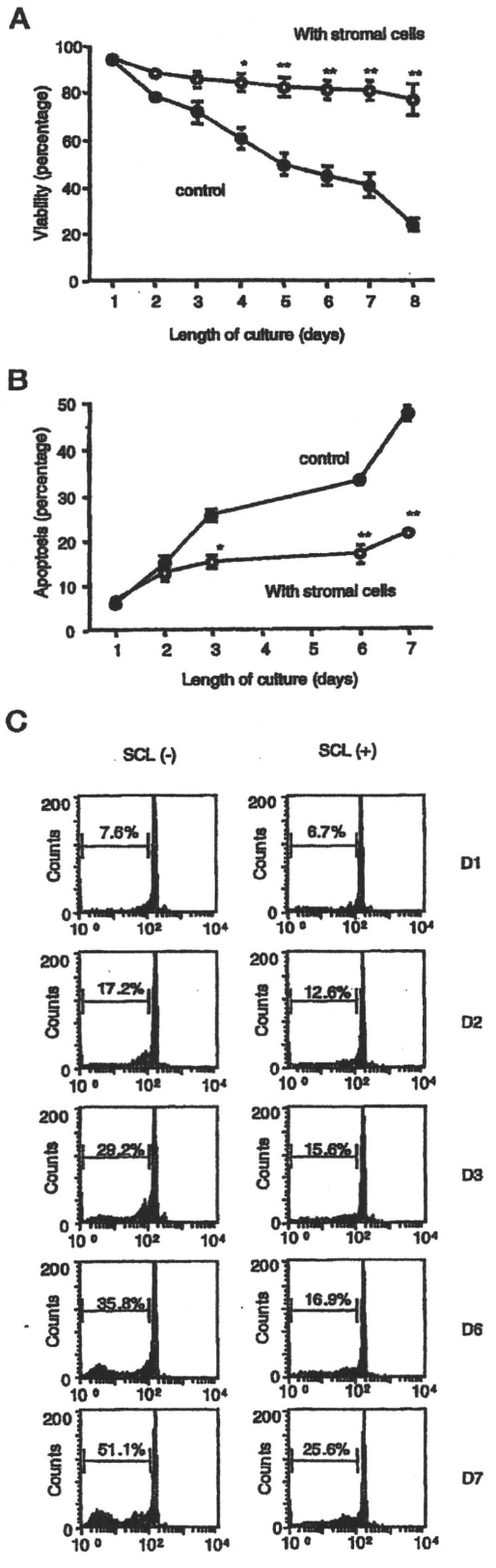

FIGURE 1. The SCL derived from RA synovial tissue rescue peripheral B cells from apoptosis. Peripheral B cells (1×10^5) were cultured with or without RA SCL (Sy77; 4×10^3) in 96-well microtiter plates. After various lengths of culture, B cells were harvested with gentle pipetting, and the percentages of viable cells (*A*) and apoptotic cells (*B* and *C*) were determined as described in *Materials and Methods*. Data are the mean ± SEM of five cultures from one of four independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$ (using Student's *t* test).

cells were cultured in DMEM supplemented with 10% FBS and 10% conditioned medium, which was prepared by incubation of PBMC from 10 healthy donors in RPMI 1640 medium with 10% FBS for 48 h. The cultures were then maintained for more than 2 mo, and the SCL were cloned by limiting dilution. Thereafter, clones were maintained and replenished with fresh DMEM with 10 or 20% FBS every 3–4 days. One clone (Sy 77) was used in the present experiments. A second RA SCL (RA6/1) was established from synovial tissue in a similar manner. After three to six passages, this RA SCL contained <2% $CD14^+$ cells. An SCL was also established from osteoarthritis synovium (OA5/26) using the same procedure. Skin fibroblast lines (DT1 and FB-HG) were provided by Dr. Heather Wisbey (University of Texas Southwestern Medical Center, Dallas, TX) and Dr. Herman Girschick (University of Texas Southwestern Medical Center).

B cell separation

The PBMC were isolated from heparinized blood of healthy adult volunteers by density sedimentation using Ficoll/Isopaque. CD19-positive B cells were purified using the CEPRATE streptavidin column (CellPro, Bothell, WA), following staining of the cells with subsaturating concentrations of biotinylated mouse anti-human CD19 mAb. The resultant population of B cells contained >95% CD20-positive B cells.

B cell culture

B cells were cultured in various ways. When cultured alone, B cells (1×10^6) were cultured in 1 ml of RPMI 1640 medium (RPMI 1640 with 10% FBS, 200 U/ml of penicillin G, 10 μg/ml gentamicin, and 0.3 mg/ml L-glutamine) in 24-well culture plates. Alternatively, B cells (1×10^5) were cultured in 200 μl of RPMI 1640 medium in 96-well flat-bottom microtiter plates. In some experiments, B cells were stimulated with anti-IgM Ab. To accomplish this, B cells were incubated with 10 μg/ml of anti-human IgM Ab (DA4.4) conjugated with biotin for 30 min at 4°C and washed twice. Afterward, B cells (1×10^6 in wells of 24-well plates) were cultured for 1 h with streptavidin (10 μg/ml) to cross-link surface IgM, harvested, washed, and used for experiments. When SCLs and fibroblasts were incubated with B cells, they were initially cultured in 24-well culture plates by incubating SCL (4×10^4) in 1 ml of DMEM (high glucose DMEM including 10% FBS, 200 U/ml of penicillin G, 10 μg/ml gentamicin, and 0.3 mg/ml L-glutamine) for 1 wk. After this period of time, they became subconfluent, and B cells (1×10^6) were added in 1 ml of RPMI 1640 culture medium. After various periods of time, B cells were harvested by gentle pipetting. Contamination of harvested B cells with SCLs and fibroblasts could be determined by scatter properties using a flow cytometer and was always <0.5%. Alternatively, SCLs and fibroblasts (4×10^3) were cultured in 96-well flat-bottom microtiter plates for 1 wk, and then B cells (1×10^5) were added, incubated, and harvested as described above.

Direct cell-to-cell contact between B cells and SCL was blocked with a 0.4-μm pore size membrane (Falcon, Becton Dickinson, Franklin Lakes, NJ). For these experiments, SCL (4×10^4) were cultured as described above, and B cells (1×10^6) were added to culture contained in the membrane chamber. In the experiments examining the effect of blocking mAb, SCL (4×10^4) were cultured with 5 μg/ml of mAb for 60 min before addition of B cells as described above. In some experiments, B cells were stimulated with anti-CD11a/CD18 and/or CD49d/CD29 mAbs. For these experiments, 5 μg of rabbit anti-mouse IgG mAb were coated in the wells of 24-well culture plates by incubating them in Tris-HCl at pH 9.8 overnight. B cells (1×10^6) were incubated with 2 μg of mAbs for 30 min, washed once, added to the 24-well plates precoated with rabbit anti-mouse IgG, and cultured in RPMI medium as described above.

Analysis of viable cells

The numbers of viable cells were assessed microscopically after staining with trypan blue.

Detection of apoptosis

The hypotonic propidium iodide staining method was used to detect apoptosis of cells by flow cytometry (24). Harvested B cells were resuspended in hypotonic propidium iodide reagent (0.1% sodium citrate containing 50 μg/ml of propidium iodide and 0.1% Triton X-100). Within 60–120 min, the stained cells were analyzed by flow cytometry using the FACScan

Table I. *RA SCL rescues B cell apoptosis*[a]

		D1	D3	D6
Viability (%)				
Expt. 1 (n = 6)	Medium	92.0 ± 0.8	47.0 ± 4.1	24.7 ± 3.9
	+ RA SCL	92.3 ± 1.0	82.0 ± 3.3*	71.0 ± 2.1**
Expt. 2 (n = 5)	Medium	95.3 ± 0.2	77.0 ± 0.5	56.0 ± 2.1
	+ RA SCL	95.7 ± 0.9	89.0 ± 2.8	82.0 ± 0.6**
Expt. 3 (n = 5)	Medium	95.0 ± 0.4	56.1 ± 1.6	28.5 ± 1.1
	+ RA SCL	94.7 ± 0.9	84.9 ± 1.4**	70.5 ± 0.2**
Apoptosis (%)				
Expt. 1 (n = 6)	Medium	7.3 ± 1.3	55.0 ± 6.5	61.7 ± 5.1
	+ RA SCL	6.7 ± 1.2	18.7 ± 2.0	21.3 ± 1.9**
Expt. 2 (n = 5)	Medium	4.3 ± 0.5	25.0 ± 1.0	46.0 ± 1.9
	+ RA SCL	4.7 ± 0.2	7.7 ± 0.9**	17.7 ± 0.9**
Expt. 3 (n = 5)	Medium	5.7 ± 0.5	62.3 ± 1.4	86.5 ± 0.2
	+ RA SCL	5.1 ± 0.7	21.3 ± 1.7**	30.0 ± 0.7**

[a] Peripheral blood B cells (1×10^5) were cultured with or without RA SCL (Sy77: 1×10^4) in 96-well culture plates. Percent viability was measured after trypan blue staining. Apoptosis was measured by flow cytometry after hypotonic propidium iodide staining. Data indicates mean ± SEM from the number of replicates indicated (n). Table shows data from three of seven similar experiments.

*, $p < 0.05$; **, $p < 0.01$: statistical difference between B cells with medium alone and B cells with RA SCL using Student's t test.

(Becton Dickinson), and the percentage of hypodiploid cells was determined.

Western blotting

Bcl-2-related protein expression was investigated by Western blotting. B cells (2×10^6), cultured as described above, were collected and lysed with extraction buffer (PBS with 1% Triton X-100, 5 mM EDTA, 100 μg/ml PMSF, 1 μg/ml leupeptin, and 0.225 U/ml aprotinin). After 2 h at 4°C, debris was eliminated by centrifugation at 15,000 rpm for 10 min, and the supernatant was collected. After measurement of protein concentration with a protein assay kit (Bio-Rad), each sample was adjusted to 4 μg/μl, then mixed with 2× sample loading buffer containing 2-ME (1.5 M) and SDS (4%), and stored at 4°C until analysis. Before analysis, the protein concentration of each sample was adjusted to 2 μg/μl. Forty micrograms of protein (20 μl of sample) was separated by 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blocked with 0.1% Tween and 5% skim milk overnight. The immunoblots were incubated with anti-Bcl-2 rabbit polyclonal Ab (1/50) or anti-Bcl-x rabbit polyclonal Ab (1/100) in PBS with 1% BSA for 1 h. Goat IgG $F(ab')_2$ anti-rabbit IgG conjugated with HRP was used as a second Ab. All immunoblots were detected by enhanced chemiluminescence.

RNA isolation and RT-PCR

RNA was extracted from 1–2 × 10^6 B cells using Triazol reagent in accordance with the company's instructions. One microgram of the extracted RNA was treated with DNase I to eliminate DNA and reverse transcribed by SuperScript II reverse transcriptase at 42°C for 70 min using oligo(dT). The PCR was conducted with Taq polymerase using 1–3 μl of cDNA (1.5 mM MgCl; annealing temperature, 56°C; 30–40 cycles). The primer pair for Bcl-2 was CAGAATCCTCTGGAACTTGAGG (5′) and CTTCCAGA CATTCGGAGACC (3′), that of Bcl-$x_{s/L}$ was GTGGAAGAGAACG GGGCTGAGG (5′) and ATGTGGTGGAGCAGAGAAGG (3′), and that of G6PD was TGACCTCAGCTGCACATTCC (5′) and CAGTGGC CAATAAGCTCTGG (3′). The PCR products were resolved by electrophoresis on 1.5% agarose gels and identified with ethidium bromide staining. In some cases, PCR-Southern hybridization was conducted. After denaturation of the gel in 1.5 M NaCl and 0.5 M NaOH for 30 min, the PCR

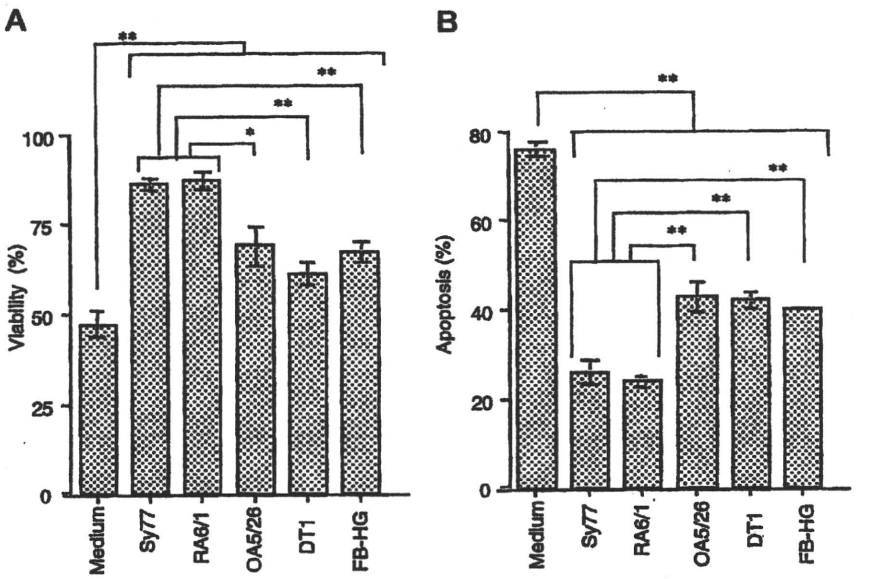

FIGURE 2. The RA SCL rescue B cells from apoptosis more effectively than other cell lines. Peripheral B cells (1×10^5) were cultured with various SCLs or fibroblasts (4×10^3) in 96-well microtiter plates. After 4 days of culture, B cells were harvested with gentle pipetting, and the percentages of viable cells (*A*) and apoptotic cells (*B*) were determined as described in *Materials and Methods*. Data are the mean ± SEM of five cultures from one of two independent experiments with similar results. Sy77, RA synovial stromal cell clone; RA6/1, RA synovial cell line; OA5/26, OA synovial cell line; DT1 and FB-HG, fibroblast cell line from human skin. *, $p < 0.05$; **, $p < 0.01$ (using Student's t test).

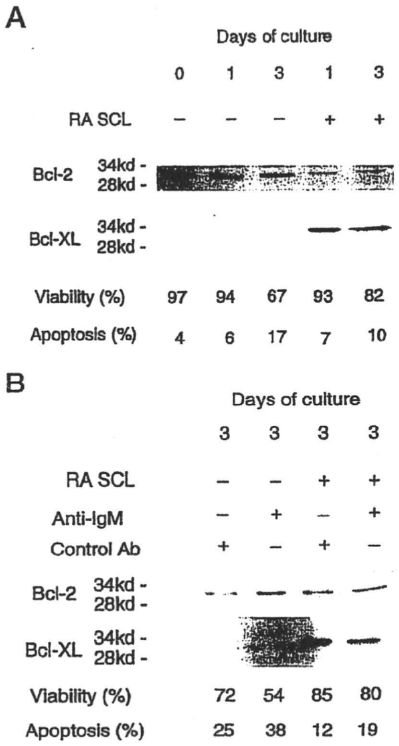

FIGURE 3. The RA SCL induce Bcl-x_L expression by B cells. *A*, After B cells (1×10^6) were cultured with RA SCL (Sy77; 4×10^4) for 1 or 3 days, expression of Bcl-2 and Bcl-x_L proteins (40 μg of each sample) was examined with Western blotting and compared with that by fresh B cells. Percentages of viable and apoptotic cells were assessed at the same time as described in *Materials and Methods*. Representative data from one of five similar experiments with similar results are shown. *B*, After B cells (1×10^6) stimulated by cross-linking IgM Ab or control Ab were cultured with RA SCL (Sy77; 4×10^4) for 3 days, expression of Bcl-2 and Bcl-x_L proteins (40 μg of each sample) was examined with Western blotting. Percentages of viable and apoptotic cells were assessed at the same time as described in *Materials and Methods*. Representative data from one of two experiments with similar results are shown.

products were transferred onto Zeta-Probe blotting membranes using a vacuum blotting apparatus. ^{32}P-labeled probes (Bcl-2, GTGACTTCCGAT CAGGAAGG; Bcl-x_L, GGTATTGGTGAGTCGGATCG; GAPDH, CCTCCAGACCCTGCCTGAGC) specific for PCR products were used to detect PCR products by Southern hybridization. To adjust the amount of cDNA of each sample precisely, G6PD expression was examined first using 32–35 cycles of RT-PCR to amplify 1, 2, and 3 μl of cDNA. After resolving the PCR products on agarose gels and identifying the relevant bands with ethidium bromide, the optimal amounts of cDNA were determined. Bcl-2 and Bcl-x_L expression in this amount of cDNA was examined using 32, 35, 38, and 40 cycles of PCR amplification, and the results in the linear part of the amplification curve are reported in the figures.

Results

The SCL from RA synovium can rescue B cells from apoptosis

When cultured alone, B cells spontaneously underwent apoptosis and died (Fig. 1). However, when B cells were cultured with RA SCL, apoptosis was blocked, and B cell viability was preserved. In each experiment (Table I) significant differences in viability and apoptosis between B cells cultured with medium alone and those with RA SCL were noted after 6 days of incubation. However, in some experiments (Expt. 2), but not others (Expt. 1 and 3), differences in viability were not seen on day 3. Similarly, significant differences between apoptosis noted in cultures with and without

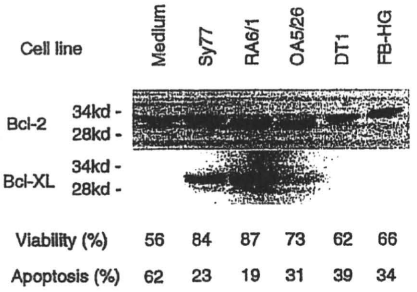

FIGURE 4. The RA SCL rescue B cell from apoptosis more effectively than other cell lines. After B cells (1×10^6) were cultured with various cell lines (4×10^4) for 3 days, expression of Bcl-2 and Bcl-x_L proteins (40 μg of each sample) was examined with Western blotting. Percentages of viable and apoptotic cells were assessed at the same time as described in *Materials and Methods*. Representative data from one of two experiments with similar results are shown. Sy77, RA synovial stromal cell clone; RA6/1, RA synovial cell line; OA5/26, OA synovial cell line; DT1 and FB-HG, fibroblast lines from human skin.

SCL were not always noted on day 3. To determine whether RA SCL were unique in their ability to promote the viability of B cells, the activities of a variety of other cell lines were tested (Fig. 2). All the cell lines rescued B cells from apoptosis to varying degrees, but the viability-promoting activity of RA SCL was significantly better than those of the other cell lines.

Bcl-X is up-regulated by B cells cultured with RA SCL

To determine whether rescue from apoptosis reflected up-regulation of antiapoptotic proteins, expression of Bcl-2 related proteins was investigated before severe apoptosis (Fig. 3*A*). B cell expression of Bcl-2 protein was unchanged as a result of culture with or without SCL. In contrast, Bcl-x_L protein was not detected in fresh B cells, but was markedly up-regulated within 1 day of culture with SCL, but not when B cells were cultured alone. Increased B cell viability and decreased apoptosis were again observed upon coculture with SCL on day 3, but the changes were less marked because of the short length of the incubation. RA-SCL also rescued anti-IgM-stimulated B cells from apoptosis (Fig. 3*B*). Moreover,

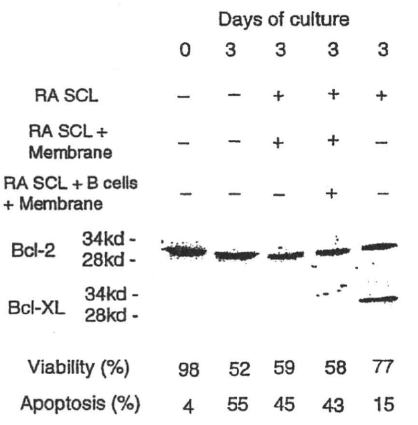

FIGURE 5. Cell contact is required to induce Bcl-x_L and rescue B cells from apoptosis. B cells (1×10^6) and SCL (4×10^4) were cultured together or separated by a 0.4-μm pore size membrane. Where indicated B cells (1×10^6) were added to the bottom chamber with SCL to examine the influence of soluble factor induced after mixed culture. After a 3-day culture, B cells were analyzed for expression of Bcl-2 and Bcl-x_L protein and were assessed for viability and apoptosis. Representative data from one of four similar experiments with similar results are shown.

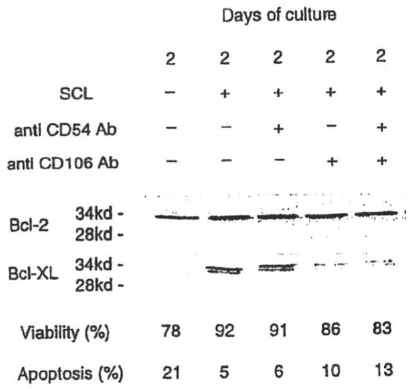

FIGURE 6. Roles of CD54 (ICAM-1) and CD106 (VCAM-1) in SCL-mediated up-regulation of Bcl-x_L and inhibition of B cell apoptosis. B cells (1×10^6) were cultured with or without SCL (4×10^4) for 2 days in the presence or the absence of mAbs to CD54 and/or CD106. Afterward, B cells were analyzed for Bcl-2 and Bcl-x_L expression and for viability and apoptosis. Representative data from one of three experiments with similar results are shown.

RA-SCL up-regulated Bcl-x_L, but not Bcl-2, expression by anti-IgM-stimulated B cells. Finally, the impact of coculture with other cell lines on B cell expression of Bcl-x_L was examined (Fig. 4). Bcl-x_L expression was more markedly up-regulated by coculture with RA-SCL. These results show that RA SCL can induce Bcl-x_L protein expression in resting and stimulated B cells more effectively than in other cell lines tested and, in addition, can rescue B cells from apoptosis more effectively.

Direct contact is necessary for RA SCL-mediated rescue of B cell from apoptosis and induction of Bcl-x_L in B cells

As shown in Fig. 5, both up-regulation of Bcl-x_L and rescue of B cells from apoptosis were inhibited when direct contact between RA SCL and B cells was blocked by interposition of a 0.4-μm pore size membrane. Importantly, B cells in the upper chamber could not be rescued from apoptosis by soluble factors even when they were generated by RA SCL cultured with B cells in the lower chamber. It should be noted that the effect of the membrane separation appeared to be somewhat greater for up-regulation of Bcl-x_L than for rescue from apoptosis, in that there was minimal up-regulation of Bcl-x_L when the cells were separated, but there was some rescue from apoptosis. These results indicate that direct cell-to-cell contact between B cells and SCL cells is importantly involved in both up-regulation of Bcl-x_L by B cells and rescue from apoptosis, with the former more dependent than the latter.

Interactions mediated by CD106 (VCAM-1) and CD49d/CD29 (VLA-4) play a significant role in the rescue of B cells from apoptosis and induction of Bcl-x_L

The RA SCL expresses both CD54 and CD106 (22). Blocking CD54-mediated interactions with an mAb that blocks binding of both CD11a/CD18 and CD11b/CD18, had no effect on the ability of SCL to up-regulate B cell Bcl-x_L and rescue B cells from apoptosis (Fig. 6). By contrast, an mAb to CD106 inhibited both SCL-induced up-regulation of Bcl-x_L and rescue of B cells from apoptosis, but not completely.

Cross-linking CD49/CD29 rescues B cells from apoptosis and induces Bcl-x mRNA and protein

Cross-linking CD49d/CD29 with mAb up-regulated Bcl-x_L mRNA and protein in the absence of RA SCL and also rescued B

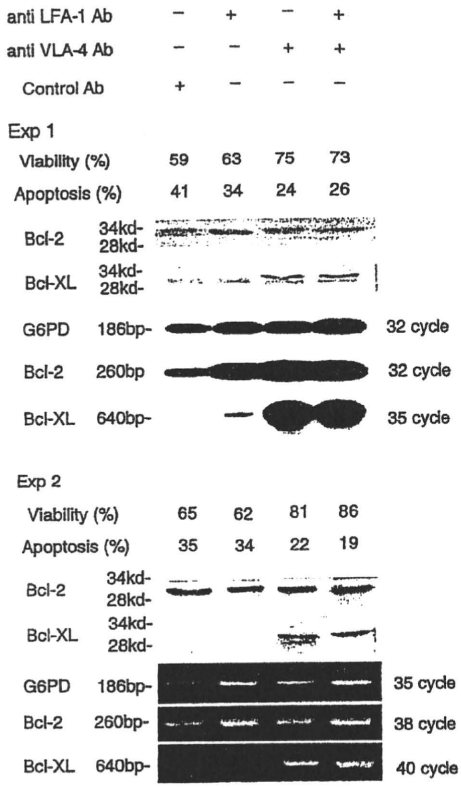

FIGURE 7. Stimulation by VLA-4 can induce Bcl-x_L protein and mRNA in B cells. B cells (1×10^6) were cultured for 3 days with mAbs to CD11a/CD18 and/or CD29/CD49d in 24-well culture plates coated with rabbit anti-mouse IgG. Afterward, the expression of Bcl-2 and Bcl-x_L protein was assessed by Western blotting, and the percentages of viable and apoptotic cells were measured as described in *Materials and Methods*, and mRNA for Bcl-2, Bcl-x_L, and G6PD were assessed by RT-PCR as described in *Materials and Methods*. The results of Southern blotting are shown in Expt. 1, and the expression of PCR products by ethidium bromide staining is shown in Expt. 2. One to three micrograms of cDNA was used for each amplification. The number of PCR cycles was modified to ensure that the PCR products obtained were from the linear phase of amplification. The cycle number of each PCR is shown in the figure. Representative data from two of five experiments with similar results are shown.

cells from apoptosis. Cross-linking CD11a/CD18 modestly rescued B cells from apoptosis, but had no impact on expression of Bcl-x_L (Fig. 7). Because of the smaller amount of Bcl-x_L induced by cross-linking CD49d/CD29, it was only detected with a more prolonged exposure. In this circumstance, some Bcl-x_L expression could also be detected in B cells cultured with medium alone (Expt. 1). It should be noted that mRNA and protein expressions were regulated in a qualitatively similar manner, although the quantitative effects were markedly different, with a much greater impact on mRNA levels noted. This suggests that there may be translational or post-translational regulation of Bcl-x_L expression in these cells that is controlled differently from the regulation of mRNA levels.

Discussion

The results of this study indicate that SCL derived from RA synovial tissue can induce Bcl-x_L and rescue peripheral blood B cells from apoptosis, and this ability of RA SCL is greater than that of OA SCL and skin fibroblasts. The data also indicate that a direct physical interaction between RA SCL and B cells mediated by the

coreceptor pair VCAM-1 and VLA-4 provides signals that induce Bcl-x_L and plays a role in the rescue of B cells from programmed cell death. A similar phenomenon in the rheumatoid synovium may contribute to the local accumulation and activation of B cells at this inflammatory site.

It has become apparent that maintenance of the viability of resting B cells is a dynamic process, involving the active countering of proapoptotic mechanisms. One of the major mechanisms to maintain the viability of lymphocytes involves the activity of the Bcl-2 family of molecules that inhibit programmed cell death following mitochondrial disruption (25). Previous results had indicated that a population of SCL isolated from bone marrow or rheumatoid synovium could prevent apoptosis of resting B cells and also stimulate their differentiation into Ab-forming cells (22). These SCL constitutively expressed VCAM-1 (22). The current studies demonstrate that engagement of B cell VLA-4 by VCAM-1 expressed on SCL up-regulates Bcl-x_L, an antiapoptotic Bcl-2 family member, and protects resting B cells from programmed cell death. These results are the first demonstration that engagement of VLA-4 on B cells can induce Bcl-x_L and also the first mechanistic explanation of the means by which interaction with SCL can protect B cells from apoptosis.

In RA synovial tissue, minimal apoptosis of lymphocytes has been noted despite intensive infiltration (5). Previous reports have suggested that soluble factors produced by RA synovial fibroblasts might promote the viability of synovial T cells (26). However, the previous studies clearly showed that the viability of resting B cells could be maintained not by fibroblast cell lines, but, rather, by a specific population of synovial SCL (22). Moreover, the maintenance of B cell viability could not be explained by soluble factors produced by SCL. In the current studies, soluble factors produced by RA SCL could maintain B cell viability modestly, although the major contribution of RA SCL to the rescue of B cells from apoptosis involved direct cell-to-cell contact mediated by VCAM-1.

The mechanism, by which B cells are rescued from apoptosis, has been extensively investigated in secondary lymphoid tissue (20, 27–29). In germinal centers, B cell expression of Bcl-x_L is increased (20, 30). Various combinations of surface molecules and cytokines have been reported to induce Bcl-x_L in B cells or B cell lines. In peripheral B cells, CD40 ligand (CD154) and IL-13 can induce Bcl-x_L (31). CD40 ligand and IgM cross-linking can also induce Bcl-x_L on tonsil B cells and/or WEHI-231 cells (27, 32), whereas the combination of the polyclonal activator, SAC, and IL-10 can also induce Bcl-x_L on tonsil B cells (29). Finally, anti-IgM stimulation induced Bcl-x_L protein and rescued murine A20 cells from apoptosis (33). Besides interactions with Ag and activated T cells, direct contact with follicular dendritic cells (FDC) could contribute to B cell survival. In this regard, FDC and SCL have certain similarities, including the constitutive expression of the adhesion molecules, ICAM-1 and VCAM-1, and their involvement in spontaneous interactions with B cells (22). Because SCL do not express CD40 ligand (22), the possibility that adhesion molecules play a role in maintaining the viability of resting B cells was considered. The results of the mAb blocking experiments indicated that direct cell contact with SCL through VCAM-1-VLA-4 interactions is important in rescuing resting B cells from apoptosis. In view of the central importance of VCAM-1-VLA-4 interactions between B cells and FDC (34–36), a similar process could be involved in up-regulating Bcl-x_L and protecting against apoptosis in the germinal center. We have previously noted the functional and phenotypic similarities between SCL and FDC. However, there are differences between these cell types, including the observation that SCLs do not express CD21 and CD35 on their surface.

It should be noted that the finding that VCAM-1-mediated interactions could not block B cell apoptosis completely suggested that other mechanisms could be involved. SCL are known to produce IL-6, IL-8, GM-CSF, G-CSF, and hyaluronic acid and might also express surface molecules such as CD157 that could contribute to B cell survival (22, 37). Despite the possible contributions of these other molecules, it is clear that VCAM-1-VLA-4 interactions play a central role in the SCL-dependent rescue of resting B cells from apoptosis.

Bcl-x_L expression is largely regulated at the level of gene transcription (25). In this regard, it has been reported that leukemia inhibitory factor induced Bcl-x_L via the gp130 and STAT1 signaling pathway in cardiac myocytes (38). In addition, an erythropoietin-stimulated Jak2 kinase-initiated signal pathway has been reported to inhibit radiation-induced apoptosis and induce Bcl-x_L in the DA3 murine myeloid cell line (39). However, there were no previous reports of a relationship between Bcl-x_L expression and VLA-4 stimulation. VLA-4 stimulation has been reported to result in phosphorylation of focal adhesion kinase, which can then activate phosphatidylinositol 3-kinase and the generation of PIP3 (40–42). Subsequently, Akt (activated form serine/threonine kinase) can be phosphorylated and activated, and provide a cell survival signal (43). Besides this putative pathway of activation, the promoter region of human and mouse Bcl-x_L contains several transcription binding sites, including sites for Ets-1, AP-4, NF-E2, Lyf-1, AP-1, Oct-1, GATA-1, and Evi-1, which are candidates to be involved in up-regulation of transcription (44). Current studies are focusing on the mechanism of Bcl-x_L up-regulation following VLA-4 engagement.

Synovial stromal cells in RA have many potential functions. These cells induce B cell activation and Ig production and also secrete many cytokines and chemokines related to inflammation (22, 37, 45–48). Additionally, they can protect naive B cells from apoptosis by the VLA-4-VCAM-1-dependent mechanism, as described here. These finding suggest that synovial stromal cells may play a central role in propagating the inflammatory response characteristic of RA, especially the accumulation of B cells and their activation to produce Ig locally within the synovium.

Acknowledgments

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Nurse-like Cells from Bone Marrow and Synovium of Patients with Rheumatoid Arthritis Promote Survival and Enhance Function of Human B Cells

Yasunori Shimaoka,* Jeanne F. Attrep,‡ Toshio Hirano,§ Katsuhiko Ishihara,§ Ryuji Suzuki,‖ Tomoko Toyosaki,‖ Takahiro Ochi,* and Peter E. Lipsky‡

Department of Orthopedic Surgery, Osaka University Medical School, 2-2, Yamada-oka, Suita, Osaka 565, Japan; ‡Harold C. Simmons Arthritis Research Center, Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235; §Division of Molecular Oncology, Department of Oncology, Biomedical Research Center, Osaka University Medical School, 2-2, Yamada-oka, Suita, Osaka 565, Japan; ‖Department of Immunology, Shionogi Research Laboratories, 5-12-4, Sagisu, Fukushima-ku, Osaka 553, Japan

Abstract

Thymic nurse cells are known to interact with T cells and play a role in their functional maturation. However, the role of nurse cells in B cell maturation and differentiation is less well established, especially at extralymphoid sites. To address this issue, nurse-like cell clones from bone marrow and synovial tissue of patients with RA (RA-NLC) were established and characterized. RA-NLC constitutively expressed CD29, CD49c, CD54 (ICAM-1), CD106 (VCAM-1), CD157 (BST-1), and class I MHC molecules, and secreted IL-6, IL-7, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). Bone marrow–derived and synovial RA-NLC differed in that the former secreted IL-7 and expressed a greater density of CD157 constitutively and after stimulation with IFNγ, whereas the latter secreted G-CSF and more IL-6. Stimulation of both bone marrow and synovial RA-NLC induced expression of CD40 and class II MHC, but not CD154 (CD40L) or CD35. RA-NLC rescued peripheral B cells from spontaneous apoptosis and promoted survival of B cells for > 4 wk. B cell survival was blocked by antibodies to CD106 or CD157. RA-NLC also increased Ig production from B cells. After long-term culture (4–6 wk) with RA-NLC, but not alone or with fibroblasts, outgrowth of B cells was observed. All B cell lines derived from these cultures had been transformed by EBV, although the RA-NLC themselves were not infected with EBV. Precursor frequency analysis indicated that ~ 1 in 12,500 peripheral B cells could give rise to these EBV-transformed B cell lines upon coculture with RA-NLC. These results indicate that RA-NLC from bone marrow and synovium have the capacity to rescue B cells from spontaneous apoptosis, facilitate Ig production, and promote the outgrowth of EBV-transformed B lymphoblastoid cells. These findings suggest that RA-NLC may play a role in the local and systemic hyperreactivity of B cells characteristic of rheumatoid arthritis. (*J. Clin. Invest.* 1998. 102:606–618.) Key words: RA • B cells • apoptosis • stromal cells

Introduction

Nurse cells were first recognized in cell suspensions from dissociated thymus (1, 2). Thymic nurse cells form unique complexes with bone marrow–derived T cell precursors, and play an important role in thymocyte maturation and differentiation, eventually leading to the generation of positively (3, 4) or negatively (5) selected T cells that subsequently migrate to the T cell-dependent areas of secondary lymphoid organs. After initial adhesion to thymic nurse cells, thymocytes crawl beneath them in vitro (6, 7). This phenomenon, known as pseudoemperipolesis, is peculiar to nurse cells (8), and has been used to identify nurse-like cells (NLC)[1] at various tissue sites. Previous studies have characterized NLC clones from human dermal tissue (9), and nurse-like stromal cell lines have been established from bone marrow of RA patients (10; Takeuchi, E., T. Tomita, T. Toyosaki-Maeda, H. Hashimoto, M. Kaneko, H. Takano, K. Sugamoto, R. Suzuki, and T. Ochi, manuscript submitted for publication). Moreover, NLC have been established from the synovium of RA patients (11; Tomita, T., E. Takeuchi, T. Toyosaki-Maeda, H. Oku, M. Kaneko, H. Takano, K. Sugamoto, K. Ohzono, R. Suzuki, and T. Ochi, manuscript submitted for publication). A common feature of all NLC was their capacity to support pseudoemperipolesis, similarly to thymic nurse cells. Thus, nurse cells may play an important role in extralymphoid immune responsiveness, as well as promoting maturation of T cells in the thymus.

In addition to their association with T cells, nurse cells have also been noted to interact physically with B cell lymphoma lines (9). This finding suggests that NLC may also promote B cell function, but the role of NLC in B cell maturation and differentiation has not been delineated. Furthermore, the functional potential of extrathymic nurse cells is not known.

RA is characterized by chronic inflammation with infiltration of a variety of inflammatory cells, including those of myeloid origin as well as T and B lymphocytes into the affected synovium. One feature of rheumatoid inflammation is local B cell activation, resulting in the production of large amounts of

Address correspondence to Peter E. Lipsky, M.D., Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-8884.

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1. *Abbreviations used in this paper:* BST-1, bone marrow stromal antigen-1; DT1, human dermal fibroblast cell line; FDC, follicular dendritic cells; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; MFI, mean fluorescence intensity; NLC, nurse-like cells; PE, phycoerythrin; RA-NLC, rheumatoid arthritis NLC; VCAM, vascular cell adhesion molecule.

Ig and autoantibodies. The features of the synovium that promote local B cell differentiation are not completely delineated, although previous studies have suggested that extralymphoid B cell activation in the rheumatoid synovium may be promoted by fibroblast-like synoviocytes. Thus, B lymphocytes, some of which expressed proliferating cell nuclear antigen, were noted to be in intimate contact with synoviocytes in the subintimal layer of the rheumatoid synovium (12), suggesting a role for this interaction in facilitating B cell responses locally. In addition, synoviocytes have been noted to support the terminal differentiation of activated B cells into immunoglobulin-secreting plasma cells (13). These results suggest a role for synoviocytes in facilitating local B cell responses in RA synovium. Whether the capacity to facilitate B cell activation is a general property of synoviocytes or, alternatively, reflects the activity of a small number of contaminating NLC is currently unknown.

These studies were undertaken to examine the possibility that NLC promote B cell as well as T cell activation and differentiation. To address these issues, NLC clones from bone marrow and synovial tissue of patients with RA (RA-NLC) were established and their capacity to promote B cell survival and activation was explored. RA-NLC enhanced B cell viability and inhibited spontaneous apoptosis by a mechanism that was dependent on their expression of vascular cell adhesion molecule-1 (VCAM-1, CD106) and bone marrow stromal cell antigen-1 (BST-1, CD157). RA-NLC also enhanced Ig production and promoted the outgrowth of B lymphoblastoid cell lines. These findings are consistent with the conclusion that NLC in bone marrow or in synovial tissue may play a role in the local and systemic stimulation of B cell activity characteristic of RA.

Methods

Isolation of NLC clones from RA bone marrow and synovial tissue. NLC lines were established from RA bone marrow and synovial tissue as previously described (9–11) and clones of NLC were established from these lines. In brief, heparinized bone marrow from the iliac crest or synovial tissue from knee joints of patients with RA who met American College of Rheumatology criteria (14) was obtained after informed consent. Synovial tissue was dissociated by collagenase and trypsin. Isolation of nurse-like cell lines was accomplished as described previously (9–11), by culturing dissociated single cells in DME (Bio Whittaker, Walkersville, MD) supplemented with 10% FBS (Life Technologies, Inc., Grand Island, NY) and 10% conditioned medium. Conditioned medium for the initial culture was prepared by incubation of PBMC from 10 healthy donors (1×10^6 cells/ml) in a 75-cm^2 culture flask (Becton Dickinson, Mountain View, CA) in RPMI 1640 (Life Technologies, Inc.) with 10% FBS for 48 h as described (9). The conditioned medium was added to the cultures twice weekly until stable cell lines were obtained. The cultures were then maintained for > 2 mo, with repeated weekly cell transfers. Afterward, the NLC lines were cloned by limiting dilution. To accomplish this, the NLC lines were adjusted to 20 cells/ml in DME with 10% FBS, and 25 μl (0.5 cells) of the cell suspension was added to individual wells of a 96-well flat-bottom microtiter plate (Becton Dickinson), and incubated at 37°C in 7% CO_2/93% air. The growing cells in individual wells were transferred to a 24-well flat-bottom culture plate (Becton Dickinson) containing DME with 10% FBS. Thereafter, clones were maintained and replenished with fresh DME medium with 10% FBS every 3–4 d.

Several NLC clones were obtained from the RA patients. RA-NLC33BM, RA-NLC87BM, and RA-NLC91BM were from RA bone marrow, whereas RA-NLC34syn, RA-NLC45syn, RA-NLC88syn, and RA-NLC77syn were from RA synovial tissue. All these RA-NLCs were identified by the capacity to support pseudoemperipolesis, as indicated by the migration of T cells beneath them observed by phase-contrast microscopy in vitro (Fig. 1). The T cell lymphoma line, MOLT-17 (American Type Culture Collection, Rockville, MD) was used to document the capacity of the various nurse cell clones to support pseudoemperipolesis.

Other cell lines. Mouse fibroblast cell lines NIH3T3 and L cells, the human B lymphoblastoid cell line, Raji, the human B cell lymphoma lines MC/car and RAMOS, and the human T cell lymphoma line, MOLT-17 were obtained from American Type Culture Collection. The EBV-transformed B cell lymphoblastoid cell line, ML-1, was a kind gift of Dr. Ton Logtenberg (University of Utrecht, The Netherlands). Human dermal fibroblasts (DT1) were a gift from Dr. Heather Wisbey (University of Texas Southwestern Medical Center, Dallas, Texas).

B cell separation. PBMC were isolated from heparinized blood of healthy adult volunteers by centrifugation over sodium diatrizoate/Ficoll gradients (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). $CD19^+$ B cells were purified by using the CEPRATE streptavidin column (CellPro, Bothell, WA), after staining of the cells with subsaturating concentrations of biotinylated mouse anti–human CD19 mAb (Coulter Corp., Miami, FL). The resultant population of B cells contained > 97% $CD20^+$ B cells.

$CD4^+$ T cell separation. $CD4^+$ T cells were prepared from B cell-depleted PBMC by negative selection, using a panning technique (15) to deplete contaminating HLA-DR^+ cells and $CD8^+$ T cells. Cells were reacted with saturating concentrations of the anti-HLA-DR mAb, L243, and the anti-CD8mAb, OKT8 (both from American Type Culture Collection). After being washed, the cells were incubated on goat anti–mouse Ig (GaMIg) (Cappel Laboratories, Inc., Cochranville, PA) coated panning dishes. After a 70-min incubation at 4°C, the nonadherent cells were gently aspirated and were panned a second time on another GaMIg-coated panning dish, after which the nonadherent cells were aspirated. The $CD4^+$ T cell population obtained in this manner contained < 0.1% esterase-positive cells, < 1% $CD8^+$ T cells, and > 96% $CD4^+$ T cells.

Antibodies, cytokines, and reagents. Various antibodies were used, including a goat $F(ab')_2$ Ab directed to human IgD (Caltag Laboratories, Burlingame, CA); a control goat $F(ab')_2$ Ab (Caltag Laboratories); Leu-20, a phycoerythrin (PE)-conjugated mouse IgG_1 mAb directed to human CD23 (Becton Dickinson, San Jose, CA); A1A5, a mouse IgG_1 mAb directed to human CD29 (integrin β1) (Cosmo Bio, Tokyo, Japan); Ber-MAC-DRC, a mouse IgG_1 mAb directed to human CD35 (Dako Corp., Carpinteria, CA); HB-7, a FITC-conjugated mouse IgG_1 mAb directed to human CD38 (Caltag Laboratories); a control FITC-conjugated mouse IgG_1 mAb (Caltag Laboratories); G28.5, a mouse IgG_1 mAb directed to human CD40 (American Type Culture Collection); 4D9-8, a mouse IgG_1 mAb against the human CD40 ligand molecule (a kind gift of Dr. R.J. Noelle, Dartmouth Medical School); P1B5, a mouse IgG_1 mAb directed to human CD49c (Funakoshi, Tokyo, Japan); HP2/1, a mouse IgG_1 mAb directed to human CD49d (α4 integrin) (Immunotech, Marseilles, France); 15.2, a mouse IgG_1 mAb directed to human CD54 (Cosmo Bio, Tokyo, Japan); TP/55.3.1, a FITC-conjugated mouse IgG_{2a} mAb directed to human CD69 (Caltag Laboratories); a control FITC-conjugated mouse IgG_{2a} mAb (Caltag Laboratories); 38-13, a rat IgM mAb directed to human CD77 (Cosmo Bio); B70, a PE-conjugated mouse IgG_{2b} mAb directed to human CD86 (PharMingen, San Diego, CA); a control PE-conjugated mouse IgG_{2b} mAb (PharMingen); DX2, a mouse IgG_1 mAb directed to human CD95 (PharMingen); 51-10C9, a mouse IgG_1 mAb directed to human CD106 (VCAM-1) (PharMingen) for staining; 1.G11B1, a mouse IgG_1 mAb directed to human CD106 (Serotec, Oxford, England) for blocking; MOPC, a control mouse IgG_1 mAb; MB40.5 (American Type Culture Collection), a mouse IgG_1 mAb directed to human HLA-A,B,C (class I MHC); L243 (American Type Culture Collection), a mouse IgG_{2a} mAb directed to monomorphic HLA-DR deter-