

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	Fc γ R 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

^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

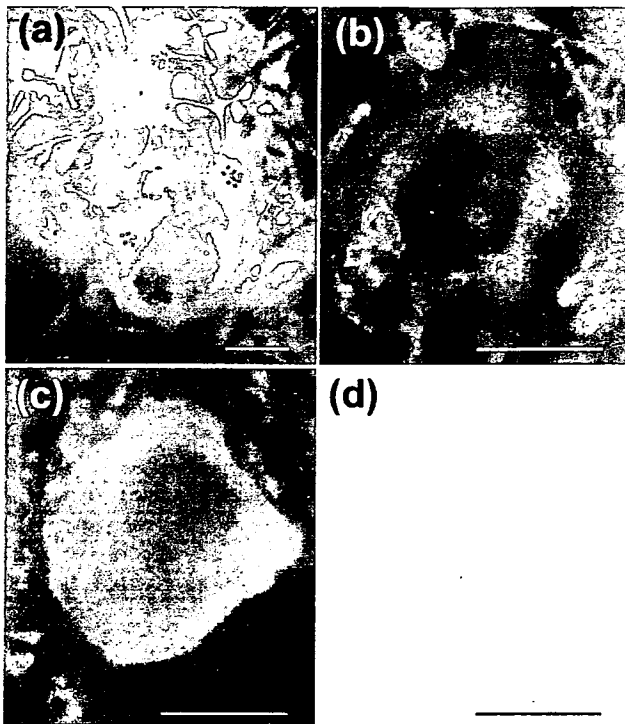
Supplementary Table 2

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.

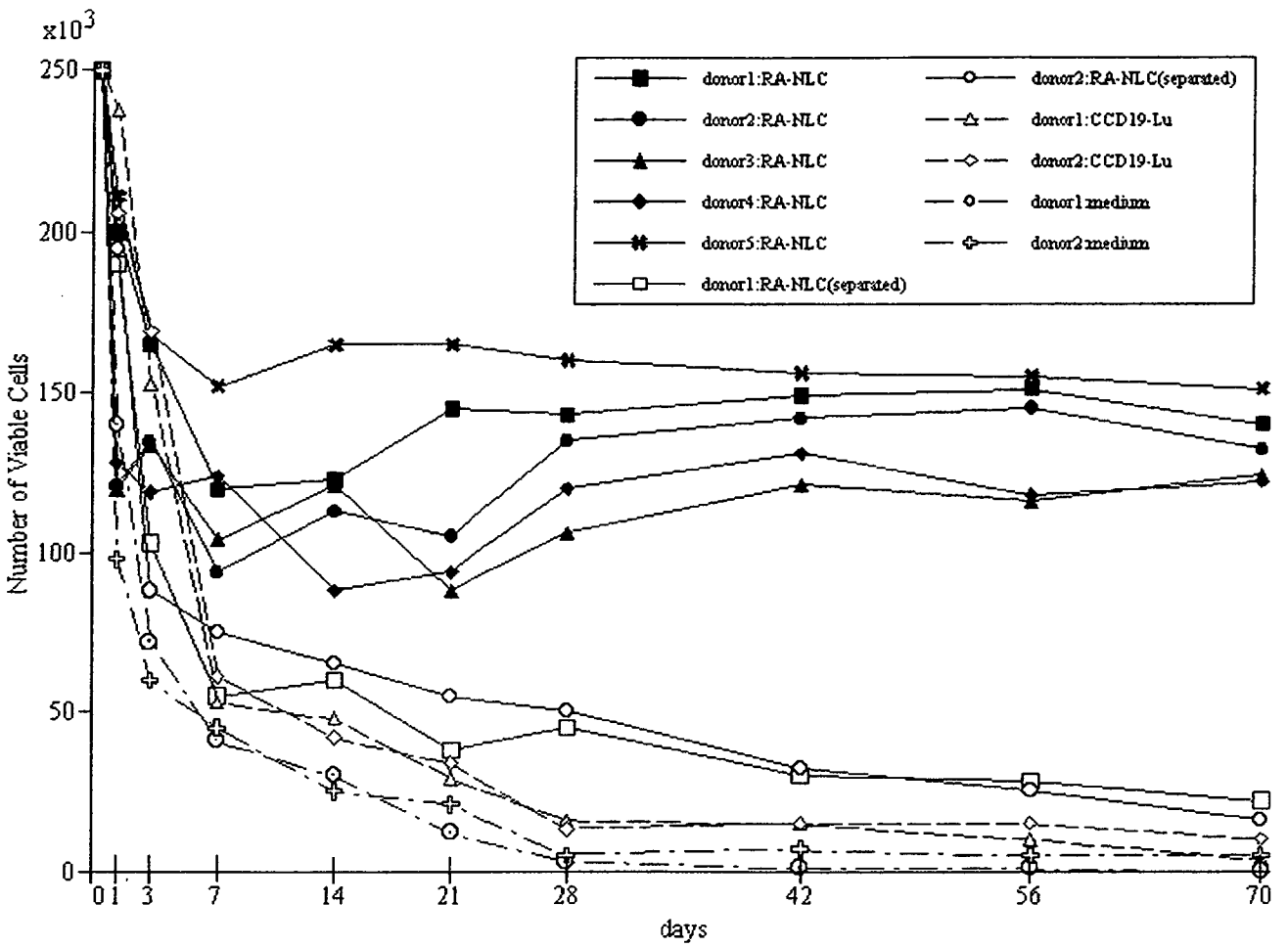
Induction of osteoclasts by IL-3, IL-5, IL-7, or GM-CSF

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.

Rheumatoid Arthritis Synovial Stromal Cells Inhibit Apoptosis and Up-Regulate Bcl-x_L Expression by B Cells in a CD49/CD29-CD106-Dependent Mechanism¹

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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_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

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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.

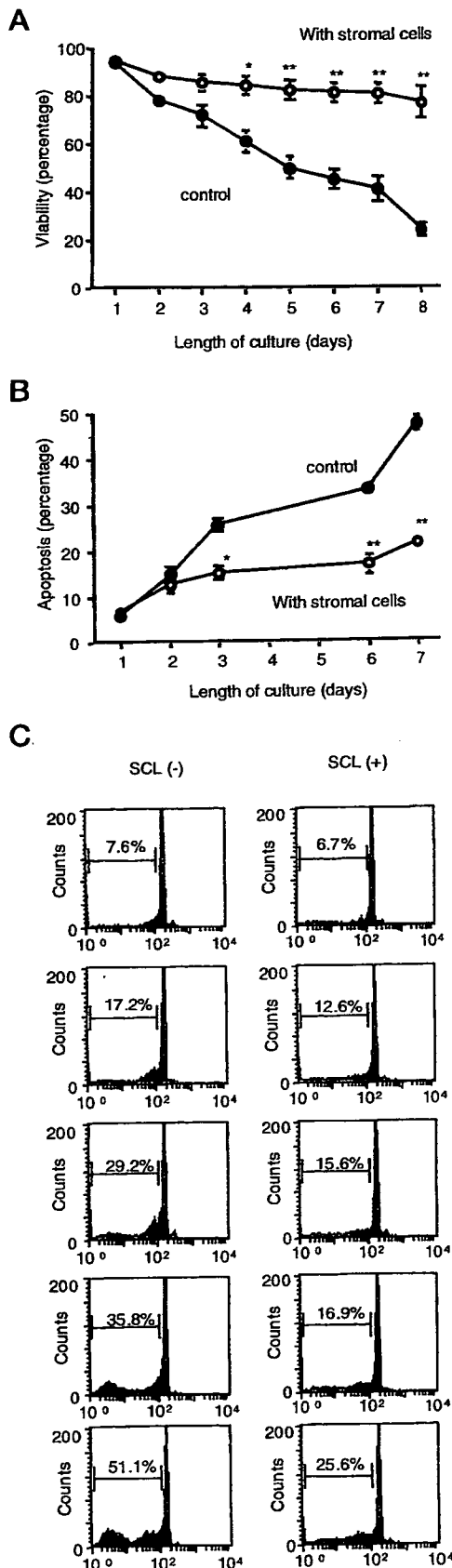


FIGURE 1. The SCL derived from RA synovial tissue rescue peripheral B cells from apoptosis. Peripheral B cells (1×10^5) were cultured with or without RA SCL (Sy77; 4×10^3) in 96-well microtiter plates. After various lengths of culture, B cells were harvested with gentle pipetting, and the percentages of viable cells (A) and apoptotic cells (B and C) were determined as described in *Materials and Methods*. Data are the mean \pm

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 CEPRA TE 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 μ g/ml gentamicin, and 0.3 mg/ml L-glutamine) in 24-well culture plates. Alternatively, B cells (1×10^5) were cultured in 200 μ l 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 μ g/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 μ g/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 μ g/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- μ m 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 μ g/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 μ g 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 μ g 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 μ g/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

SEM of five cultures from one of four independent experiments with similar results. *, $p < 0.05$; **, $p < 0.01$ (using Student's *t* test).

Table I. RA SCL rescues B cell apoptosis^a

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

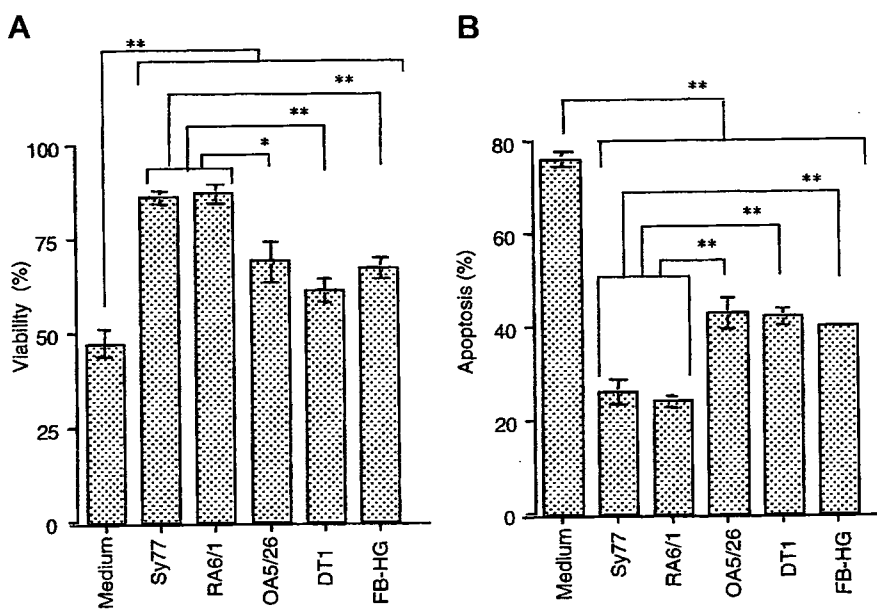
Bcl-2-related protein expression was investigated by Western blotting. B cells (2×10^6), cultured as described above, were collected and lysed with extraction buffer (PBS with 1% Triton X-100, 5 mM EDTA, 100 μg/ml PMSF, 1 μg/ml leupeptin, and 0.225 U/ml aprotinin). After 2 h at 4°C, debris was eliminated 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 $1-2 \times 10^6$ 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 MgCl₂; annealing temperature, 56°C; 30-40 cycles). The primer pair for Bcl-2 was CAGAATCCTCTGGAAGCTGAGG (5') and CTCCAGACATTCGGAGACC (3'), that of Bcl-x_L was GTGGAAGAGAACC 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).



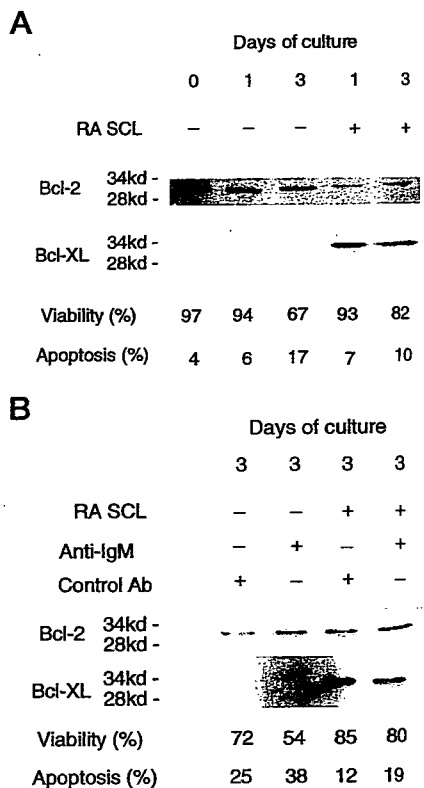


FIGURE 3. The RA SCL induce Bcl-x_L expression by B cells. *A*, After B cells (1×10^6) were cultured with RA SCL (Sy77; 4×10^4) for 1 or 3 days, expression of Bcl-2 and Bcl-x_L proteins ($40 \mu\text{g}$ of each sample) was examined with Western blotting and compared with that by fresh B cells. Percentages 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 ($40 \mu\text{g}$ 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. ³²P-labeled probes (Bcl-2, GTGACTTCGGAT 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

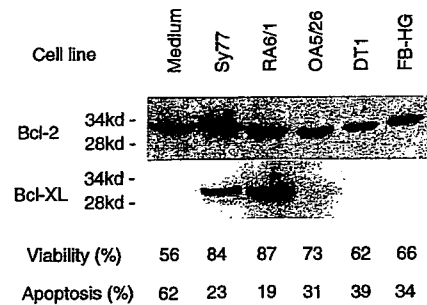


FIGURE 4. The RA SCL rescue B cell from apoptosis more effectively than other cell lines. After B cells (1×10^6) were cultured with various cell lines (4×10^4) for 3 days, expression of Bcl-2 and Bcl-x_L proteins ($40 \mu\text{g}$ of each sample) was examined with Western blotting. Percentages of viable 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; OAS/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,

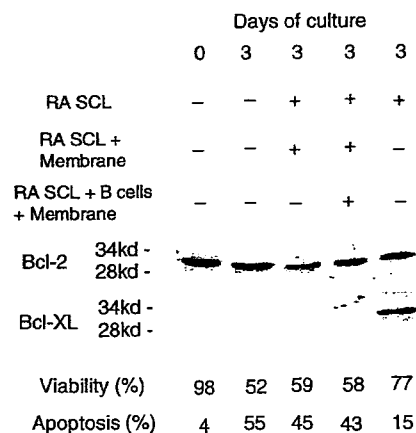


FIGURE 5. Cell contact is required to induce Bcl-x_L and rescue B cells from apoptosis. B cells (1×10^6) and SCL (4×10^4) were cultured together or separated by a $0.4\text{-}\mu\text{m}$ pore size membrane. Where indicated B cells (1×10^6) were added to the bottom chamber with SCL to examine the influence 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.

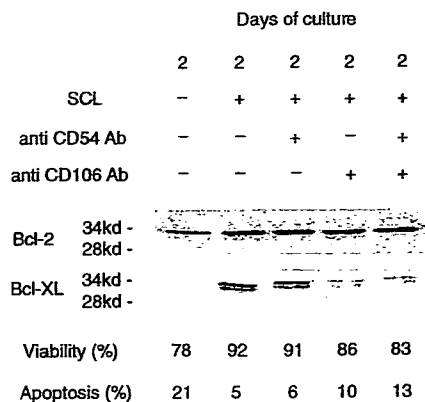


FIGURE 6. Roles of CD54 (ICAM-1) and CD106 (VCAM-1) in SCL-mediated up-regulation of Bcl-x_L and inhibition of B cell apoptosis. B cells (1×10^6) were cultured with or without SCL (4×10^4) for 2 days in the presence or the absence of mAbs to CD54 and/or CD106. Afterward, B cells were analyzed for Bcl-2 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- μ m 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

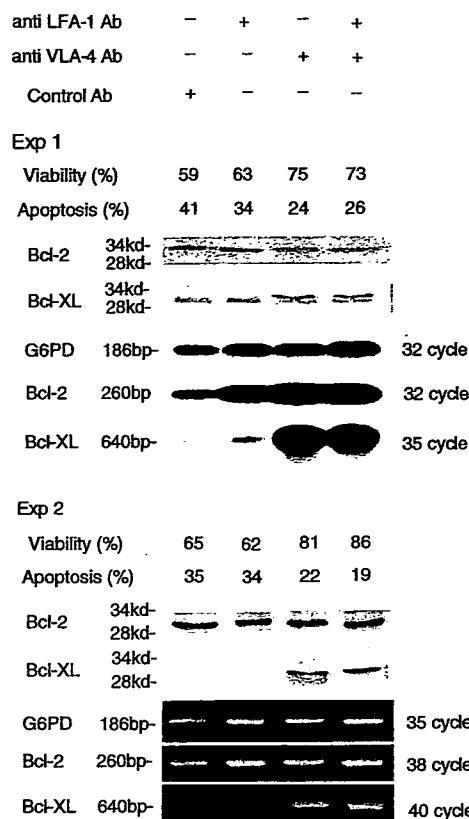


FIGURE 7. Stimulation by VLA-4 can induce Bcl-x_L protein and mRNA in B cells. B cells (1×10^6) were cultured for 3 days with mAbs to CD11a/CD18 and/or CD29/CD49d in 24-well culture plates coated with rabbit anti-mouse IgG. Afterward, the expression of Bcl-2 and Bcl-x_L protein was assessed by Western 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 findings 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

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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 IFN γ , 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 hyperre-

activity 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

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1. *Abbreviations used in this paper:* BST-1, bone marrow stromal antigen-1; DTI, 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-cm² 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 μ l (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% CO₂/93% 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 IgG₁ mAb directed to human CD23 (Becton Dickinson, San Jose, CA); A1A5, a mouse IgG₁ mAb directed to human CD29 (integrin β 1) (Cosmo Bio, Tokyo, Japan); Ber-MAC-DRC, a mouse IgG₁ mAb directed to human CD35 (Dako Corp., Carpinteria, CA); HB-7, a FITC-conjugated mouse IgG₁ mAb directed to human CD38 (Caltag Laboratories); a control FITC-conjugated mouse IgG₁ mAb (Caltag Laboratories); G28.5, a mouse IgG₁ mAb directed to human CD40 (American Type Culture Collection); 4D9-8, a mouse IgG₁ mAb against the human CD40 ligand molecule (a kind gift of Dr. R.J. Nolle, Dartmouth Medical School); P1B5, a mouse IgG₁ mAb directed to human CD49c (Funakoshi, Tokyo, Japan); HP2/1, a mouse IgG₁ mAb directed to human CD49d (α 4 integrin) (Immunotech, Marseilles, France); 15.2, a mouse IgG₁ mAb directed to human CD54 (Cosmo Bio, Tokyo, Japan); TP/55.3.1, a FITC-conjugated mouse IgG_{2b} mAb directed to human CD69 (Caltag Laboratories); a control FITC-conjugated mouse IgG_{2b} 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 IgG₁ mAb directed to human CD95 (PharMingen); 51-10C9, a mouse IgG₁ mAb directed to human CD106 (VCAM-1) (PharMingen) for staining; 1.G11B1, a mouse IgG₁ mAb directed to human CD106 (Serotec, Oxford, England) for blocking; MOPC, a control mouse IgG₁ mAb; MB40.5 (American Type Culture Collection), a mouse IgG₁ 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-

minants (class II MHC); a sheep anti-mouse IgG₁ FITC conjugate (The Binding Site, Birmingham, UK); a control FITC-conjugated mouse IgG₁ mAb (Caltag Laboratories); BEC7, a mouse IgG₁ mAb against human CD157; RF3, a mouse IgG_{2b} mAb against human CD157; RS38, a mouse IgM mAb against human BST-2 (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan); polyclonal rabbit Ab against human CD157; control preimmune rabbit Ab. BEC7, RF3, polyclonal rabbit Ab against human CD157, and control preimmune rabbit Ab were developed and purified as described previously (16–19). FITC-conjugated sheep F(ab')₂ Ab directed to mouse IgG heavy- and light-chain (Calbiochem, La Jolla, CA) was used as secondary antibody.

Soluble forms of human recombinant CD157 and CD38 were produced and purified as described previously (19). CD157 and CD38 are ~30% homologous at the amino acid level and appear to have derived by gene duplication from an ancestral gene (20–22). Purified recombinant human IFN γ (Shionogi and Co., Ltd., Osaka, Japan) was used at 100 U/ml for stimulation of RA-NLC or human dermal fibroblasts (DT1).

Quantitation of cytokines. RA-NLCs were maintained routinely with DME supplemented with 10% FBS until they became confluent. The culture supernatant of each clone was collected, and the amount of IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), TNF- α , and TNF- β was measured with ELISA kits (Quantikine; R & D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Culture medium. B cell lines, Raji, RAMOS, and normal peripheral B cell were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 200 U/ml penicillin G, 10 μ g/ml gentamicin, 0.3 mg/ml L-glutamine, and 10% FBS (Life Technologies, Inc.). RA-NLC and fibroblast cell lines DT1, NIH3T3, and L cells, were cultured in high-glucose DME (Bio Whittaker) with 100 U/ml penicillin G, 10 μ g/ml gentamicin, 0.3 mg/ml L-glutamine, and 10% FBS (Life Technologies, Inc.).

Flow cytometric analysis. The cells were cultured for various lengths of time with various stimuli as indicated and after washing with PBS containing 2% normal human AB serum and 0.1% sodium azide, cells were reacted with saturating concentrations of various mAb at 4°C for 30 min. Analysis was performed using the FACScan (Becton Dickinson) flow cytometer. Dead cells were eliminated by propidium iodide staining. Density of staining was expressed as the change in mean fluorescence intensity (Δ MFI) for staining with the mAb of interest calculated by subtracting the MFI of staining with the control mAb.

RNA extraction, cDNA synthesis, and PCR analysis. RNA was isolated using the SNAP-O-SOL RNA/DNA isolation kit obtained from Biotecx Laboratories (Houston, TX). After quantification of isolated RNA by spectrophotometry, cDNA was synthesized by priming with 500 ng oligo dT (Pharmacia, Alameda, CA) in Ultraspec diethylpyrocarbonate water (Biotecx Laboratories), 5 mM DTT, 0.5 mM dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂ by using 200 U Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) per 1 μ g of total RNA. Each PCR reaction was performed with 50–100 ng of cDNA in a 100- μ l vol containing Ultraspec water (Cinna-Biotecx), 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.001% gelatin (wt/vol), 200 μ M of each dNTP, 1.5 mM MgCl₂, and 0.3 μ M of each primer. A lower layer of dNTPs, MgCl₂, and primers was separated from an upper layer of Taq polymerase, cDNA, and 10 \times PCR buffer (KCl, Tris-Cl, and gelatin) by an Ampli-Wax PCR Gem 100 pellet (Perkin-Elmer Corp., Norwalk, CT). The upper layer was added just before the start of amplification. After a 5-min denaturation step at 94°C, 35–40 cycles of amplification were used with the following temperature program: 1 min of denaturation at 94°C, 1.5 min at the annealing temperature for the specific primer pair, and 2 min of extension at 72°C. The primers used for the PCR analysis have been published (16, 23–29). PCR products were resolved by electrophoresis on a 1% agarose gel in 0.5 \times Tris boric acid

EDTA. After denaturation of the gel in 1.5 M NaCl and 0.5 M NaOH for 30 min, the PCR products were transferred onto a nylon membrane (Zetaprobe GT; Biorad, Hercules, CA) using a vacuum-blotting apparatus. The membrane was baked at 80°C for 30 min before screening with a ³²P-5' end-labeled probe, specific for PCR products amplified with a given primer pair. Hybridization was conducted in 5 \times SSC, 20 mM Na₂HPO₄, 7% SDS, 1 \times Denhardt's solution with 100 μ g/ml denatured salmon sperm DNA at 42°C. Blots were washed in 5 \times SSC–0.1% SDS at room temperature for 20 min and in 1 \times SSC–0.1% SDS at 42°C for 15 min and subjected to autoradiography.

Analysis of viable cells. B cells (2 \times 10⁵) were cultured in medium alone or with RA-NLC or fibroblasts in 96-well flat-bottom plates (Costar, Cambridge, MA) for several days and gently resuspended and harvested from the wells. Numbers of viable cells were counted using a hemocytometer, excluding dead cells by staining with trypan blue. For each experiment, the mean \pm SEM of the total number of cells cultured in nine wells was calculated. For time-course experiments, each experiment was carried out in duplicate, and the mean was calculated.

Flow cytometric analysis of apoptosis. Apoptosis was analyzed using the flow cytometric TUNEL method (30). Briefly, 8 \times 10⁵ cells were harvested, washed in PBS, and fixed in 1% paraformaldehyde for 20 min on ice. Cells were washed in PBS, resuspended, and stored in 70% ice-cold ethanol. For analysis, the APO-BRDU kit from Phoenix Flow (San Diego, CA) was used according to the manufacturer's protocol. Cells (1 \times 10⁴) were analyzed by flow cytometry with a FACScan (Beckon Dickinson) using a 50% logarithmic contour graph with the intensity of propidium iodide staining displayed on the horizontal axis and Br-dUTP-FITC on the vertical axis. For the negative control and to set the initial gate, B cells in medium alone, were analyzed without the addition of TdT. The percentage of apoptosis was based on the increase in TdT-mediated incorporation of Br-dUTP.

Measurement of Ig production. At the end of 12 d of incubation, the supernatants from each culture well were collected and assayed for Ig content. Ig in the culture supernatants was quantitated by isotype-specific ELISA as previously described (31). Briefly, microtiter plates (Cooke; Dynatech Laboratories, Alexandria, VA) were coated with affinity-purified goat antibodies to human α , μ , or γ chains (Tago, Camarillo, CA) diluted in borate-buffered saline by incubating at 37°C for 2 h and kept at 4°C overnight. Plates were then washed with Tween saline and incubated with serial dilutions of standard Ig preparation (IgA and IgG; Behring Diagnostics, La Jolla, CA; IgM, Tago) or appropriately diluted culture supernatants in PBS containing 0.5% Tween 20 (Sigma Chemical Co., St. Louis, MO) at 37°C for 2 h. Bound Ig was detected with peroxidase-conjugated affinity-purified isotype-specific Abs (Tago) and o-phenylenediamine dihydrochloride (Sigma Chemical Co.) as substrate. Absorbance was determined at 490 nm on the EL312 Biokinetics Reader (Bio-Tek Instruments, Winooski, VT). The amount of Ig in the supernatants was calculated by comparison with a standard curve by using the KinetiCalc program (Bio-Tek Instruments, Inc.). Triplicates for each culture condition were performed and differed by < 10%. The sensitivities of the specific assays were 12 ng/ml for IgA and IgG, and 24 ng/ml for IgM.

Limited dilution analysis of B cell outgrowth. To estimate the number of precursors of EBV-transformed B cells in peripheral blood, limiting dilution frequency analysis was performed. Various numbers of peripheral B cells from four different donors were cultured with RA-NLC91BM for 4 wk in 96-well flat-bottomed plates. Nine replicates of each cell dilution were carried out and the percentage of wells yielding B cell growth was calculated.

Assay of cell proliferation. To assay cellular proliferation, a colorimetric method was used (32). Briefly, B cells were cultured for the indicated length of time, after which 180 μ l of the supernatant was removed without disturbing the pellet. 10 μ l of a yellow tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.), and 100 μ l of PBS were added to the re-

maining pellet and incubated at 37°C for 4 h, after which 100 μ l of 0.1 N HCl/isopropanol alcohol was added to terminate the reaction and dissolve the formazan product. The contents of the well were then pipetted gently until mixed, and the absorbance was read at 570 and 630 nm on the EL312 Biokinetics Reader.

Results

Establishment of the RA-NLC clones. Seven clones were established from RA-NLC parental lines from different RA patients, three from bone marrow (RA-NLC33BM, RA-NLC87BM, and RA-NLC91BM), and four from synovial tissue (RA-NLC34syn, RA-NLC45syn, RA-NLC88syn, and RA-NLC77syn). The appearance of a representative clone, RA-NLC34syn, is shown in Fig. 1 *a*. RA-NLCs were large adherent cells with an irregular polygonal shape, long dendritic processes, and rich cytoplasm. They did not exhibit either a typical fibroblastic (slender and elongated) or macrophage-like appearance (irregular with many membrane ruffles). Each of the clones was similar in appearance to NLC lines derived from bone marrow and synovium and previously described (9–11; Takeuchi, E., T. Tomita, T. Toyosaki-Maeda, H. Hashimoto, M. Kaneko, H. Takano, K. Sugamoto, R. Suzuki, and T. Ochi, manuscript submitted for publication; 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).

All RA-NLC clones were screened for the ability to promote pseudoemperipolesis. RA-NLCs were incubated with the human T cell lymphoma line, MOLT-17, and their ability to induce MOLT-17 cells to migrate beneath them was exam-

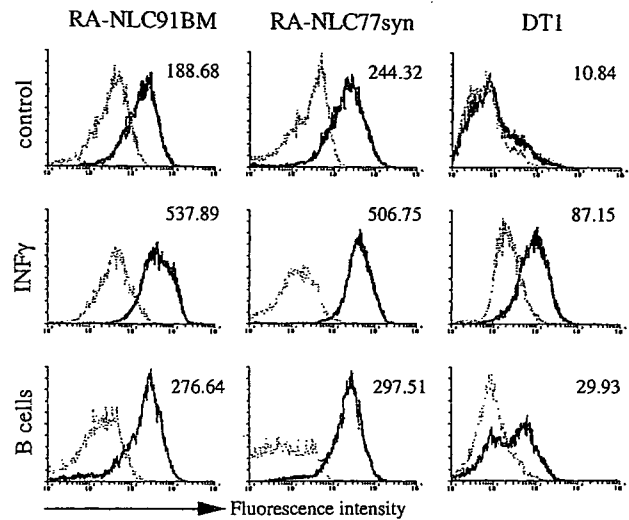


Figure 2. Expression of CD106 by RA-NLC and fibroblasts. Two RA-NLC cell lines and DT1 were cultured in medium alone or with IFN γ (100 U/ml) or with 2×10^6 peripheral B cells for 3 d. Numbers in each histogram indicate the Δ MFI for staining with anti-CD106 mAb calculated by subtracting the MFI of staining with an isotype-matched control mAb.

ined by phase-contrast microscopy (Fig. 1 *c*). As previously reported for thymic nurse cells and skin-derived NLC (6, 9), and noted for RA-derived nurse-like cell lines (10, 11), MOLT-17 cells were induced to undergo pseudoemperipolesis by the hu-

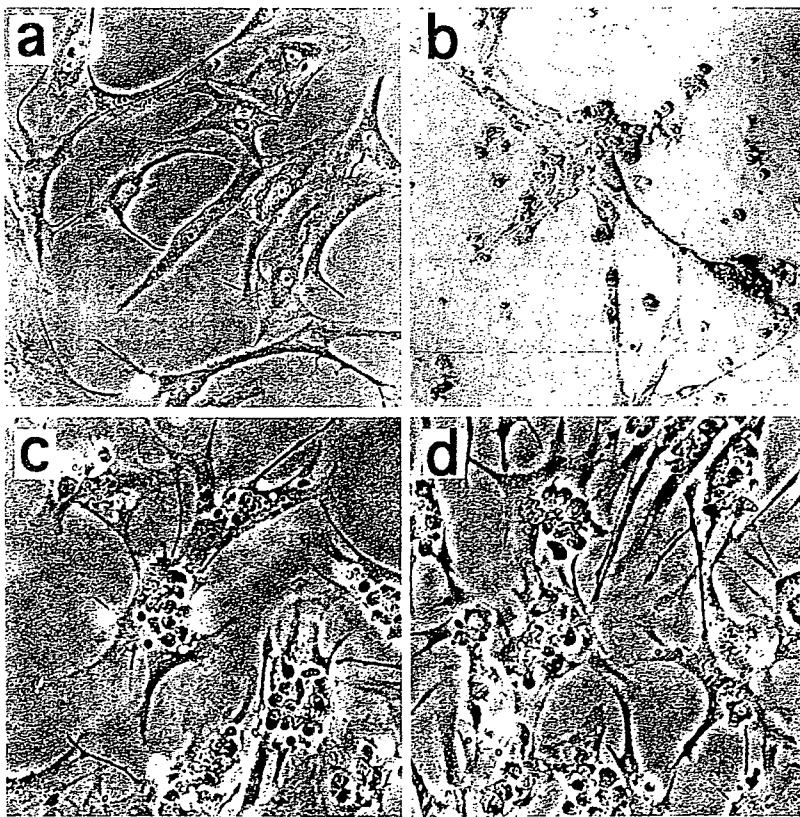


Figure 1. RA-NLC induce pseudoemperipolesis of B and T lymphoma cells and bind peripheral B cells. The morphologic appearance of RA-NLC34syn is shown by phase-contrast microscopy when cultured alone (*a*), with normal peripheral B cells for 4 d (*b*), or with the T cell lymphoma line, MOLT-17 (*c*), or the B cell lymphoma line, MCLcar (*d*). $\times 200$.

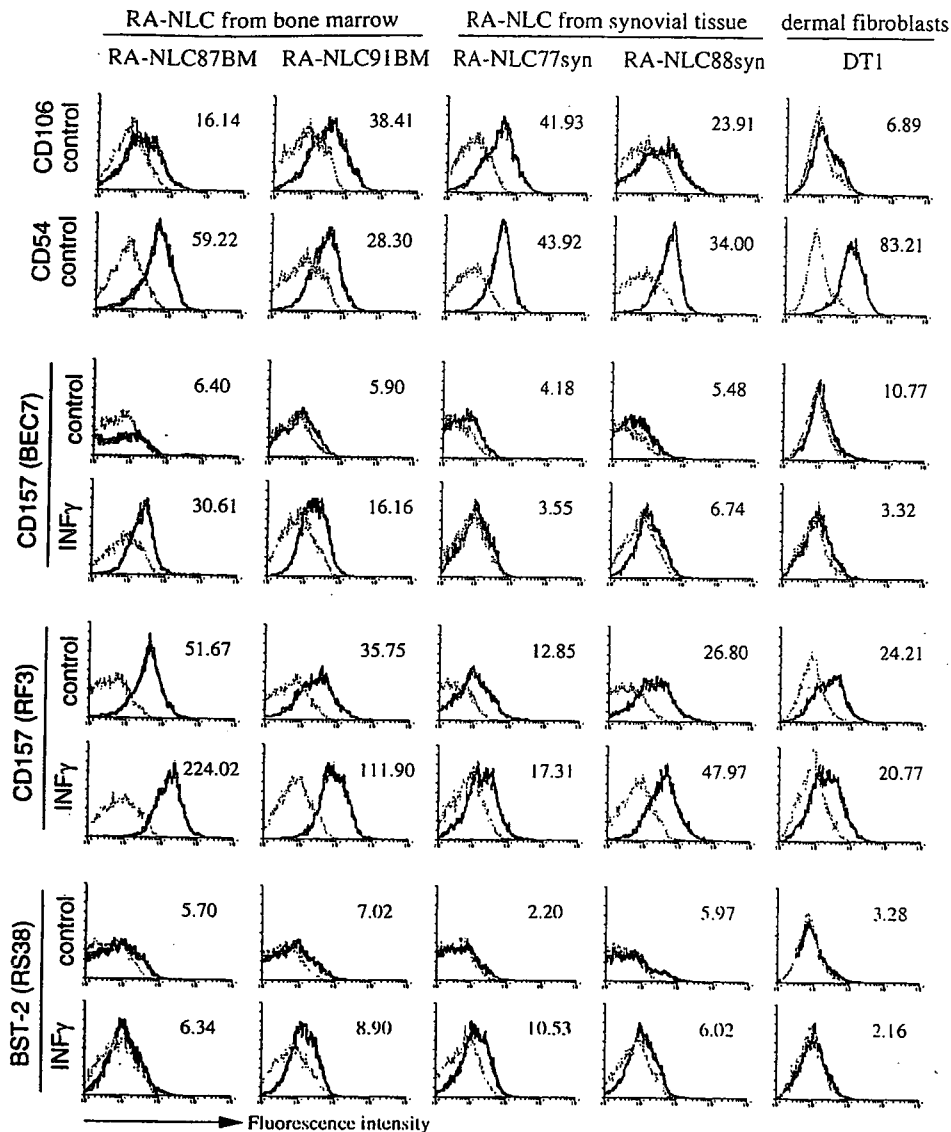


Figure 3. CD106, CD54, CD157, and BST-2 expression by RA-NLC cells and fibroblasts. Four RA-NLC cell lines or human dermal fibroblasts (DT1) were cultured in medium alone (control) or with IFN γ (100 U/ml) for 3 d, and were detached with 0.05% trypsin-EDTA in PBS at room temperature for 5 min. They were then stained with various mAbs, and analyzed by FACScan. For the staining of CD157, two different mAbs, BEC7 and RF3, were used. Numbers in each histogram indicate the Δ MFI for staining with anti-CD106, CD54, CD157 (BST-1), or BST-2 mAb calculated by subtracting the MFI of staining with an isotype-matched control mAb. Controls are RA-NLC or DT1 cultured in medium alone.

man RA-NLC clones, forming typical clusters and, thereby, permitting the RA-NLC clones to be classified functionally as nurse cells and not fibroblasts (9–11; Takeuchi, E., T. Tomita, T. Toyosaki-Maeda, H. Hashimoto, M. Kaneko, H. Takano, K. Sugamoto, R. Suzuki, and T. Ochi, manuscript submitted for publication; 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). The B cell lymphoma line, MC/car also migrated under RA-NLCs (Fig. 1 d). Of note, peripheral B cells from normal donors bound to RA-NLCs (Fig. 1 b) but did not migrate beneath them. Peripheral B cells were 14- to 20-fold smaller than RA-NLCs B cells, and primarily bound to the processes of RA-NLC. Despite the binding of peripheral B cells to RA-NLCs, there was no obvious pseudoemperipolesis by these cells. All of the RA-NLC clones interacted with MOLT-17, Mc/car, and peripheral B cells comparably.

Surface phenotype of RA-NLC. RA-NLC clones from bone marrow and synovial tissue expressed CD29, CD49c, CD54, CD106, and HLA-A,B,C (class I MHC) constitutively, but did not express CD35, CD40, or CD40L (Figs. 2 and 3; Table I). Human dermal fibroblasts also expressed CD29, CD49c, CD54, and class I MHC, whereas constitutive expression of CD106 was minimal. IFN γ (100 U/ml) stimulation of RA-NLC induced expression of CD40 and HLA-DR (class II MHC), but not expression of CD35 or CD154/CD40L (Table I).

Expression of CD106 by RA-NLCs was modestly enhanced by culture with normal peripheral B cells, and markedly enhanced by IFN γ . In contrast, expression of CD106 by human dermal fibroblasts was much less marked after stimulation with IFN γ or by culture with peripheral B cells (Fig. 2).

Expression of CD157(BST-1) and BST-2, which are molecules that can support the growth of murine pre-B cell lines (16–18), was also examined (Fig. 3). Using mAb RF3, expres-

Table I. Surface Phenotype of RA-NLC

Surface determinant		RA-NLC					Human skin fibroblasts (DT1)
		Bone marrow		Synovial tissue			
		87BM	91BM	77syn	88syn	45syn	
CD29	Resting	+	+	+	+	+	+
CD35	Resting	-	-	-	-	-	-
	IFN γ	-	-	-	-	-	-
CD40	Resting	-	-	-	-	-	-
	IFN γ	+	+	+	+	+	+
CD40L	Resting	-	-	-	-	-	-
	IFN γ	-	-	-	-	-	-
CD49c	Resting	+	+	+	+	+	+
CD54	Resting	+	+	+	+	+	+
CD106	Resting	+	+	+	+	+	-/+
	IFN γ	+	+	+	+	+	+
HLA-A,B,C	Resting	+	+	+	+	+	+
HLA-DR	Resting	-	-	-	-	-	-
	IFN γ	+	+	+	+	+	+

RA-NLC or human skin fibroblasts (DT1) were cultured with medium alone (resting) or with IFN γ (100 U/ml) for 3 d, and were detached with 0.05% trypsin-EDTA in PBS at room temperature for 5 min. They were stained with anti-human mAb, and analyzed by FACScan.

sion of CD157 was detected on all RA-NLCs, as well as on human dermal fibroblasts (DT1). Expression of CD157 by RA-NLCs, but not dermal fibroblasts, was enhanced by IFN γ . This was much more marked with bone marrow-derived compared with synovium-derived RA-NLC. Using mAb BEC7, only RA-NLC87BM and RA-NLC91BM were found to express CD157 and then only after stimulation with IFN γ . Even after IFN γ stimulation expression of BEC7-identified CD157 was modest. BST-2 was not expressed by any of the cells constitutively, and IFN γ enhanced expression minimally and only by some clones. It should be noted that expression of CD106 and CD157 mRNA was found in all RA-NLC clones by reverse transcription (RT)-PCR (data not shown). To assure that RA-NLCs had not been transformed by EBV, mRNA for the EBV-encoded gene, BamW was also examined. All RA-NLCs and DT1 were negative for mRNA for this EBV gene product (data not shown).

Cytokine production by RA-NLC. The capacity of RA-NLC to produce various cytokines was studied. Table II shows cytokines produced by RA-NLC33BM from bone marrow and

RA-NLC34syn from synovial tissue. RA-NLC from bone marrow produced detectable levels of IL-6, IL-7, IL-8, and GM-CSF, and the production of IL-8 was very great. RA-NLC from synovial tissue produced detectable levels of IL-6, IL-8, G-CSF, and GM-CSF proteins, and the production of IL-6 and IL-8 was very great. Bone marrow-derived, but not synovial RA-NLC produced IL-7, whereas synovial RA-NLC produced G-CSF and greater amounts of IL-6.

RA-NLC support the survival of peripheral B cells by blocking spontaneous apoptosis. Peripheral B cells were cultured in medium alone or with RA-NLC or fibroblasts, and the number of viable B cells was estimated by trypan blue exclusion (Fig. 4). B cells cultured in medium alone rapidly died (6.2% viable at day 6, 3.4% viable at day 9, 1.4% viable at day 14, and 0 viable at day 21). Coculture of B cells with NIH3T3 or L cells had little effect on viability. By contrast, culture of B cells with RA-NLC markedly increased their viability (41.9% viable at day 6, 31.4% viable at day 9, 14.9% viable at day 14, and 3.7% viable at day 21). DT1 also supported B cell viability, but less effectively than RA-NLC. The loss of viability of B

Table II. Cytokines Secreted by RA-NLC

	IL-1 α	IL-1 β	IL-2	IL-3	IL-4	IL-6	IL-7	IL-8	G-CSF	GM-CSF	TNF α	TNF β
RA-NLC from bone marrow (RA-NLC33BM)	< 0.5	< 10.0	< 40.0	< 7.0	< 0.5	17.0	14.5	7876.0	< 10.0	3.0	< 0.5	< 0.5
RA-NLC from synovial tissue (RA-NLC34syn)	< 0.5	< 10.0	< 40.0	< 7.0	< 0.5	1365.0	< 0.5	1395.5	113.5	5.5	< 0.5	< 0.5

RA-NLC were cultured in 24-well plates with DME plus 10% FBS to confluence. After changing the medium, RA-NLC were cultured for 7 d and supernatant was obtained. Cytokines in supernatant were measured with ELISA and expressed as picograms per milliliter. Data are shown from one of two experiments with similar results.

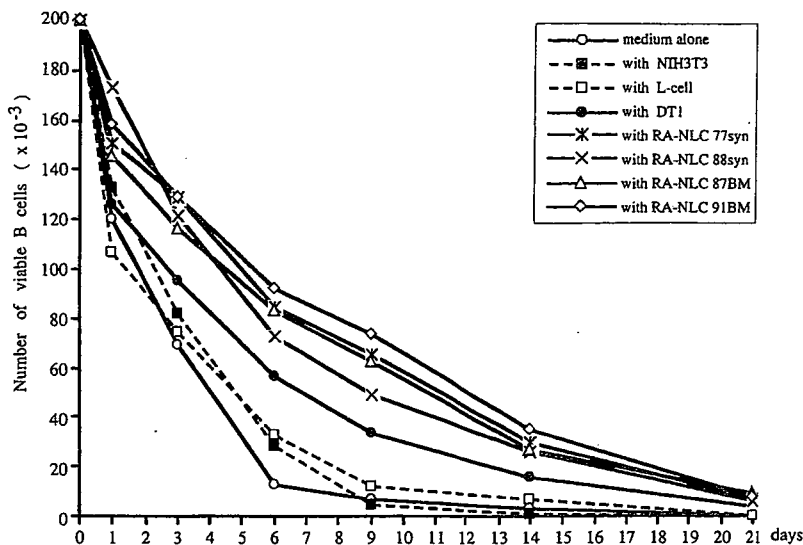


Figure 4. RA-NLC promote survival of peripheral B cells more effectively than fibroblasts. Peripheral B cells (2×10^5) were cultured in medium alone or with RA-NLC or mouse fibroblasts (NIH3T3, L cell) or human dermal fibroblasts (DT1) for 21 d, and the numbers of viable cells were counted at days 1, 3, 6, 9, 14, and 21 by trypan blue exclusion. Each experiment was carried out in duplicate, and the graph shows mean of the number of viable cells. The percentage of viable cells was calculated by dividing the number of viable cells on each day by the initial cell number (2×10^5). The percentages at day 6 with RA-NLC, $41.9 \pm 2.0\%$ (mean \pm SEM); DT1, 28.4%; medium alone, 6.2%, and at day 9, RA-NLC, $31.4 \pm 2.5\%$; DT1, 16.8%; medium alone 3.4%, and at day 14, RA-NLC, $14.9 \pm 1.0\%$; DT1, 7.8%; medium alone 1.4%, and at day 21, RA-NLC, $3.7 \pm 0.3\%$; DT1, 1.8%; medium alone, none.

cells cultured alone related to the induction of apoptosis, as Fig. 5 shows. Coculture of B cells with RA-NLC substantially blocked spontaneous apoptosis of peripheral B cells.

Effect of CD106 and CD157 expressed by RA-NLC on the survival of peripheral B cells. The effect of CD157 on the survival of peripheral B cells was examined using a soluble recombinant form of CD157 (17). Since CD157 has an $\sim 30\%$ amino acid sequence homology with CD38, and both CD157 and CD38 have ADP-ribosyl cyclase and cADPR hydrolase activities (18, 20–23, 33–35), a soluble recombinant form of CD38 was used as a control. As Table III shows, only 17.6% of B cells cultured in medium alone survived in culture for 5 d, and neither soluble CD157 alone (16.9%) nor soluble CD38

(15.8%) affected peripheral B cell survival. By contrast, peripheral B cells cultured with RA-NLC91BM, which express both CD106 and CD157, exhibited significantly greater survival (45.2% , $P < 0.0001$). B cell survival promoted by RA-NLC91BM was reduced by a blocking anti-CD106 mAb (18.2% , $P < 0.0001$) to the same level of B cells cultured in medium alone (17.6%). B cell survival promoted by RA-NLC was also reduced by a blocking anti-CD157 polyclonal Ab (27.3% , $P < 0.01$), but not by the anti-CD157 mAb (RF3) used for staining. Soluble CD157, but not soluble CD38, together with RA-NLC further increased survival of B cells (73.8% , $P < 0.0001$).

The effect of anti-CD106 mAb or anti-CD157 on the pro-

Table III. Effect of CD106 and CD157 on the Survival of Peripheral B Cells

	RA-NLC	Viable cells (mean \pm SEM [$\times 10^3$])		*P
B cells with medium alone	–	35.2 \pm 5.7	(17.6%)	< 0.0001
+ Soluble CD157	–	33.9 \pm 4.9	(16.9%)	
+ Control soluble CD38	–	31.6 \pm 4.2	(15.8%)	
B cells with RA-NLC cells	+	90.5 \pm 5.6*	(45.2%)	< 0.0001
+ Anti-CD106 mAb (1.G11B1)	+	36.4 \pm 5.5*	(18.2%)	
+ Control IgG1 mAb (MOPC)	+	78.8 \pm 6.0	(39.4%)	< 0.0001
+ Anti-CD157 mAb (RF3)	+	82.4 \pm 5.8	(41.2%)	
+ Control IgG2a mAb (P1.17)	+	81.8 \pm 4.9	(40.9%)	
+ Anti-CD157 polyclonal Ab	+	54.6 \pm 6.3*	(27.3%)	
+ Control preimmune rabbit IgG	+	77.8 \pm 4.5	(38.9%)	
+ Soluble CD157	+	147.6 \pm 8.3*	(73.8%)	< 0.01
+ Control soluble CD38	+	79.0 \pm 5.1	(39.5%)	

Peripheral B cells were cultured in 96-well flat-bottom culture plates (2×10^5 /well, $200 \mu\text{l}$) in medium alone or with soluble CD157 or with control soluble CD38 for 5 d, or with RA-NLC91BM and with several antibodies or soluble molecules indicated. MOPC (IgG₁) was used as the isotype-matched control mAb for anti-CD106 mAb, P1.17 (IgG_{2a}) was used as the isotype-matched control mAb for anti-CD157 mAb (RF3), and preimmune rabbit IgG was used as the control for polyclonal antibody to CD157. Soluble CD157 and soluble CD38 were used at $10 \mu\text{g}/\text{ml}$, and antibodies were used at $4 \mu\text{g}/\text{ml}$. The number of viable B cells was quantitated at day 5 after staining with trypan blue. Each experiment was carried out in replicate, and mean \pm SEM of the total number of cells cultured in nine wells was calculated. The percentage of viable cells (shown in parentheses) was calculated by dividing the number of viable cells by the initial cell number (2×10^5). * P values were derived using student's t test.

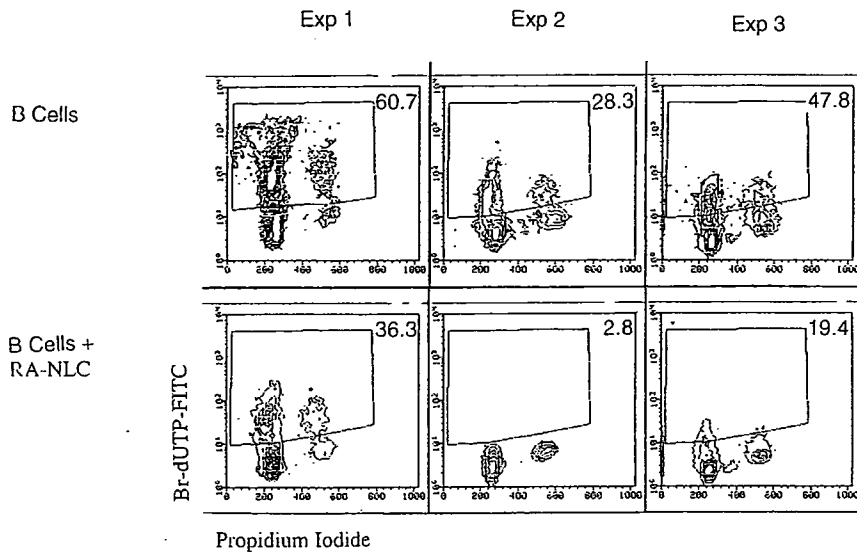


Figure 5. RA-NLC protect B cells from spontaneous apoptosis. Peripheral B cells were cultured in medium alone or with RA-NLC77syn for 3 d and assessed for the percentage of apoptotic cells by the TUNEL technique. The numbers in each box indicate the percentage of apoptotic cells.

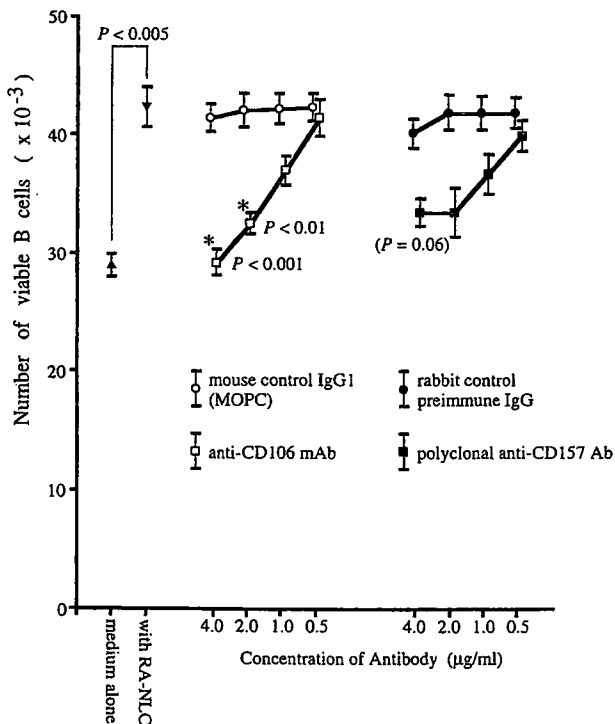


Figure 6. CD106 and CD157 are involved in the maintenance of B cell viability by RA-NLC. Peripheral B cells (2×10^5) were cultured in medium alone or with RA-NLC77syn and several concentrations of anti-CD106 mAb or anti-CD157 Ab or control Ab, and the number of viable B cells was counted at day 7 after staining with trypan blue. MOPC was used as the isotype-matched control mAb for anti-CD106 mAb, P1.17 was used as the isotype-matched control mAb for anti-CD157 mAb (RF3), and preimmune rabbit IgG was used as the control for polyclonal antibody to CD157. The graph shows mean \pm SEM of the total number of cells cultured in nine wells. Significance between control Ab and anti-CD106 mAb or anti-CD157 Ab was derived using the student's *t* test.

motion of survival by synovial tissue-derived RA-NLC was also examined (Fig. 6). RA-NLC77syn promoted B cell survival that was significantly blocked by an mAb to CD106 in a concentration-dependent manner. At higher concentrations, anti-CD106 mAb completely blocked B cell survival promoted by RA-NLC77syn ($P < 0.01$) to the same level as B cells cultured in medium alone. Although anti-CD157 Ab appeared to block the survival of B cells partially, this effect was not statistically significant ($P = 0.06$). This may relate to the markedly diminished expression of CD157 by RA-NLC77syn (Fig. 3). Expression of a number of B cell surface antigens was increased by coculture with RA-NLC. These included CD38, CD95, CD23, CD69, and CD86 (data not shown). Of these, each was also increased by culture with control dermal fibroblasts (DT1 cells) except CD95, whose expression was increased on B cells by culture with RA-NLC, but not with DT1 cells (data not shown).

Secretion of Ig from peripheral B cells. Coculture with RA-NLC markedly increased secretion of Ig by peripheral blood B cells in the absence of stimulation by a polyclonal activator (Fig. 7). In contrast, a variety of control cells, including L cells, NIH3T3 cells, and DT1 exerted a considerably more modest impact on Ig production by B cells. In addition, RA-NLC enhanced IgG and IgA production by B cells stimulated with anti-CD3-activated T cells (data not shown). The effect of RA-NLC was significantly greater than that of human fibroblasts (DT1). The impact of RA-NLC on proliferation of peripheral blood B cells and a variety of B cell lines was examined. Despite the positive influence on Ig production previously noted, RA-NLC had no effect on initial proliferation of either peripheral blood B cells or a variety of B cell lines (data not shown).

Support of the outgrowth of B cell lines by RA-NLC. Despite the lack of an impact on initial cellular proliferation, clusters of growing cells were observed after a 7-wk incubation of B cells with RA-NLC, but not with DT1 cells (Fig. 8). By limiting dilution analysis, the precursor frequency of peripheral B cells generating outgrowth was ~ 1 in 12,500 (Fig. 9). Each of

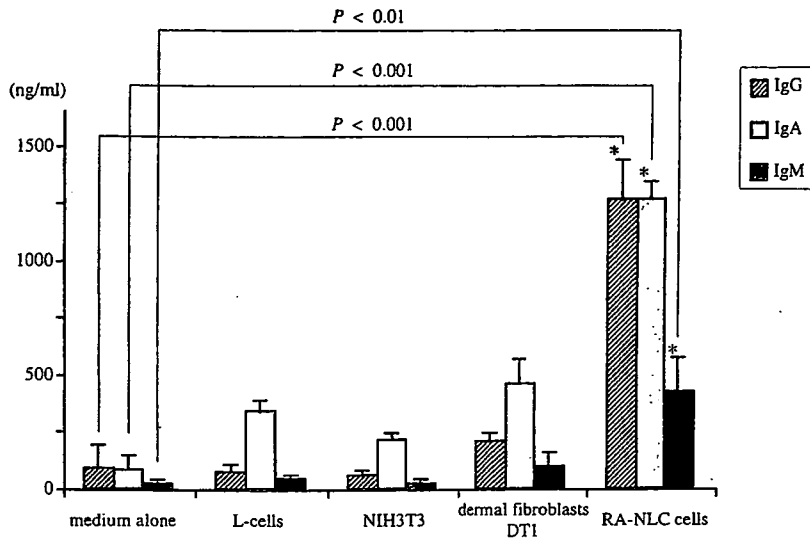


Figure 7. RA-NLC induce secretion of immunoglobulin from peripheral B cells. Peripheral B cells (5×10^4) were cultured in medium alone or with mouse fibroblasts (L-cells, NIH3T3 cells), human dermal fibroblasts (DT1), or with RA-NLC33BM with no other stimulation. The supernatants were harvested after 12 d of culture and Ig content was analyzed by isotype-specific ELISA. Data from three experiments are shown and expressed as mean \pm SEM. *P* values were derived using the student's *t* test.

the B cell lines produced by culture of peripheral B cells with RA-NLC expressed EBV-encoded mRNA (data not shown). As noted previously, however, RA-NLC did not express EBV-encoded gene products.

Discussion

Rheumatoid synovitis is characterized by the local activation of B cells and the production of large amounts of Ig (36, 37). It has been suggested that synoviocytes may contribute to the stimulation of B cell differentiation within the synovium (12, 13). Recently, cells with nurse cell characteristics have also been found in rheumatoid synovium and bone marrow (10, 11; Takeuchi, E., T. Tomita, T. Toyosaki-Maeda, H. Hashimoto, M. Kaneko, H. Takano, K. Sugamoto, R. Suzuki, and T. Ochi, manuscript submitted for publication; Tomita, T., E. Takeuchi, T. Toyosaki-Maeda, H. Oku, M. Kaneko, H. Takano, K. Suga-

moto, K. Ohzono, R. Suzuki, and T. Ochi, manuscript submitted for publication). To examine the possibility that NLC might contribute to systemic and local activation of B cells within the synovium, RA-NLC clones were established from both sites and their capacity to promote B cell responses analyzed. The RA-NLC clones were identified as nurse cells because they supported pseudoemperipolesis by a T cell lymphoma line, a characteristic that is considered to be specific for nurse cells and to distinguish them from other cell types, including fibroblasts (6-9). The RA-NLC clones also supported pseudoemperipolesis of B lymphoma cells as was recently reported for human skin-derived nurse cell clones (9) as well as bone marrow and synovial derived NLC (Takeuchi, E., T. Tomita, T. Toyosaki-Maeda, H. Hashimoto, M. Kaneko, H. Takano, R. Sugamoto, R. Suzuki, and T. Ochi, manuscript submitted for publication; Tomita, T., E. Takeuchi, T. Toyosaki-Maeda, H. Oku, M. Kaneko, H. Takano, K. Sugamoto, K.

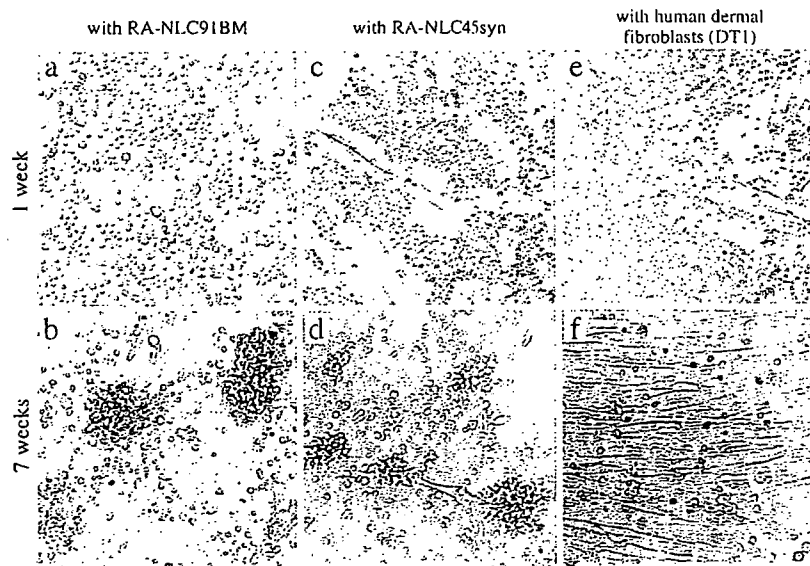


Figure 8. Outgrowth of B cells with RA-NLC. Long-term culture of normal peripheral B cells with RA-NLC (a-d) or human dermal fibroblasts (e and f) was performed. B cells were observed by phase-contrast microscopy after a culture of 1 (a, c, and e) and 7 wk (b, d, and f), respectively. At 1 wk, approximately one-third of B cells were alive in all conditions (a, d, and g). After 7 wk of culture with RA-NLC, B cell aggregations and blast cells were observed, whereas all of the B cells were dead in culture with DT1. $\times 60$.

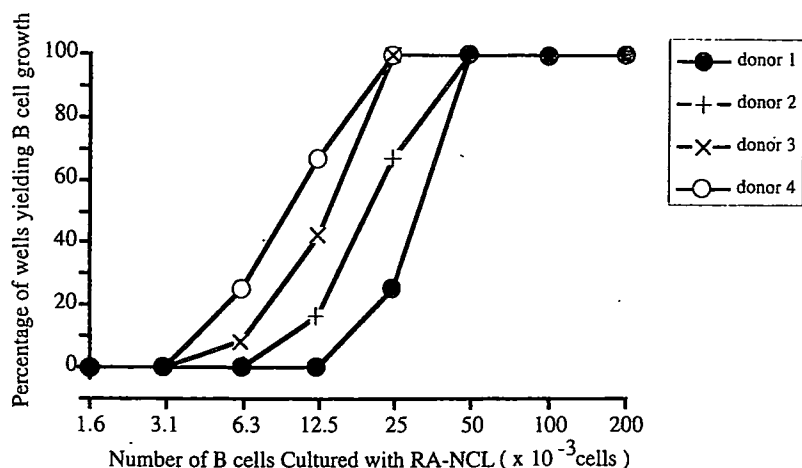


Figure 9. RA-NLC promote outgrowth of a small population of B cells. Several densities of peripheral B cells from four different donors were cultured with RA-NLC91BM for 4 wk in 96-well flat-bottomed plates. The vertical axis shows percentage of wells yielding B cell growth, and horizontal axis shows number of B cells initially cultured in each well.

Ohzono, R. Suzuki, and T. Ochi, manuscript submitted for publication). Although peripheral B cells bound to RA-NLC, they did not migrate beneath them. However, RA-NLC induced a number of B cell functional activities, presumably as a result of signals transmitted during physical interactions between the cell types.

Nurse cells are thought to be of mesenchymal origin and are considered to represent a unique cell lineage. Despite extensive characterization, however, including analysis of surface phenotype and cytokine production, the most unique characteristic of these cells remains their capacity to support pseudoemperipolesis by thymocytes and some lymphoma cells in vitro (6–9). The nurse cell clones derived from RA bone marrow and synovium share characteristics with nurse cells derived from other sites (6–9), including their morphologic appearance as large, adherent cells with an irregular shape and multiple dendritic processes. As with other nurse cell populations, their appearance was distinctly different from that of monocyte/macrophages or of fibroblasts. Moreover, the RA-NLC were CD68 negative (data not shown) and proliferated in culture, making it very unlikely that they were members of the myeloid lineage. Furthermore, they constitutively expressed CD106 (VCAM-1) after long-term culture in the absence of cytokine stimulation. Although synovial fibroblasts express CD106 in situ (12, 38, 39), in vitro culture in the absence of cytokines results in the loss of expression of this adhesion molecule (12, 40, 41). Thus, constitutive expression of CD106 (VCAM-1) appears to be a characteristic of nurse cell lines, permitting them to be distinguished from fibroblasts. Of note, constitutive expression of CD106 by RA-NLC appears to be greater than that seen in human skin-derived nurse cells (9), although each of these populations is clearly positive. Moreover, constitutive expression of CD106 appears to be of importance in nurse cell function, as it has been shown to play a central role in pseudoemperipolesis (7). In summary, NLC can be derived from RA synovium and bone marrow and they appear to play an important role in promoting B cell viability by blocking spontaneous apoptosis and also facilitating differentiation of B cells into antibody-secreting cells.

Of note, RA-NLC also expressed CD157 or BST-1, a glycosyl-phosphatidylinositol (GPI)-anchored protein, which has been reported to facilitate the growth of murine pre-B cell

lines in vitro (16–23, 33–35). CD157 is known to be expressed by bone marrow stromal cell lines, T and B lymphocyte progenitor cells, mature myeloid cells, and the reticular cells in splenic white pulp, lymph nodes, and Peyer's patches; it is also known to be expressed in the gut (16, 42–46). Although CD157 expressed by bone marrow stromal cells can promote the growth of a pre-B cell line in vitro (16), engagement of CD157 itself also costimulated proliferation of anti-CD3-activated pre-T cells, promoted the generation of $\alpha\beta$ T cell receptor expressing T cells in fetal thymic organ culture (45), and also induced tyrosine phosphorylation in myeloid cells (47). Thus, CD157 may function both as a signaling molecule on RA-NLC and as a receptor for an as yet unidentified ligand that provides activation signals to pre-B cells and perhaps other cell types. The wide distribution of BST-1 in the reticular cells in spleen and lymph node (44) suggests that it might have a broader range of functional activities.

RA-NLC produced a variety of cytokines that could contribute to inflammation and propagate immune responses. In general, the array of cytokines produced by RA-NLC was similar to that produced by skin-derived nurse cells (9). One major difference between synovial- and bone marrow-derived RA-NLC related to the production of IL-7 that was secreted by bone marrow-derived, but not synovial RA-NLC. This could be an important difference because IL-7 is a stromal cell-derived factor that stimulates B-lineage cell growth and maturation (48). Additional RA-NLC clones will need to be screened to confirm this difference, however, as only some skin-derived nurse cell lines, but no skin-derived nurse cell clones produced IL-7 (9).

RA-NLC promoted survival of peripheral blood B cells by blocking spontaneous apoptosis. The activity of RA-NLC was much greater than the nonspecific effect of human or mouse fibroblasts and could not be explained by the activity of secreted factors. Previous reports have shown that synoviocytes could promote the survival of T cells (49) and B cells (13). An adhesion molecule-mediated mechanism has been suggested to account for T cell survival (49), but the mechanism by which synoviocytes promote T cell survival has not been further delineated. Moreover, the possible contamination of the synoviocytes used in these studies with nurse-like cells has not been evaluated. In these studies, RA-NLC were found to be