

minants (class II MHC); a sheep anti-mouse IgG<sub>1</sub> FITC conjugate (The Binding Site, Birmingham, UK); a control FITC-conjugated mouse IgG<sub>1</sub> mAb (Caltag Laboratories); BEC7, a mouse IgG<sub>1</sub> mAb against human CD157; RF3, a mouse IgG<sub>2a</sub> 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')<sub>2</sub> 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 $\gamma$  (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 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), TNF- $\alpha$ , and TNF- $\beta$  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  $\mu$ 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  $\mu$ 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 ( $\Delta$ 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 Biotex 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 (Biotex Laboratories), 5 mM DTT, 0.5 mM dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl<sub>2</sub> by using 200 U Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) per 1  $\mu$ g of total RNA. Each PCR reaction was performed with 50–100 ng of cDNA in a 100- $\mu$ l vol containing Ultraspec water (Cinna-Biotex), 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.001% gelatin (wt/vol), 200  $\mu$ M of each dNTP, 1.5 mM MgCl<sub>2</sub>, and 0.3  $\mu$ M of each primer. A lower layer of dNTPs, MgCl<sub>2</sub>, 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 <sup>32</sup>P-5' end-labeled probe, specific for PCR products amplified with a given primer pair. Hybridization was conducted in 5 $\times$  SSC, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 7% SDS, 1 $\times$  Denhardt's solution with 100  $\mu$ 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  $\times$  10<sup>5</sup>) 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  $\times$  10<sup>5</sup> 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  $\times$  10<sup>4</sup>) 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  $\alpha$ ,  $\mu$ , or  $\gamma$  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.

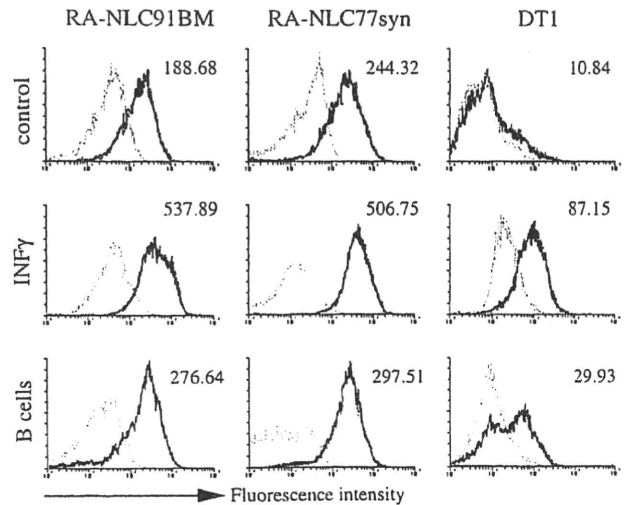
**Assay of cell proliferation.** To assay cellular proliferation, a colorimetric method was used (32). Briefly, B cells were cultured for the indicated length of time, after which 180  $\mu$ l of the supernatant was removed without disturbing the pellet. 10  $\mu$ l of a yellow tetrazolium salt, MTT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

maining pellet and incubated at 37°C for 4 h, after which 100  $\mu$ 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

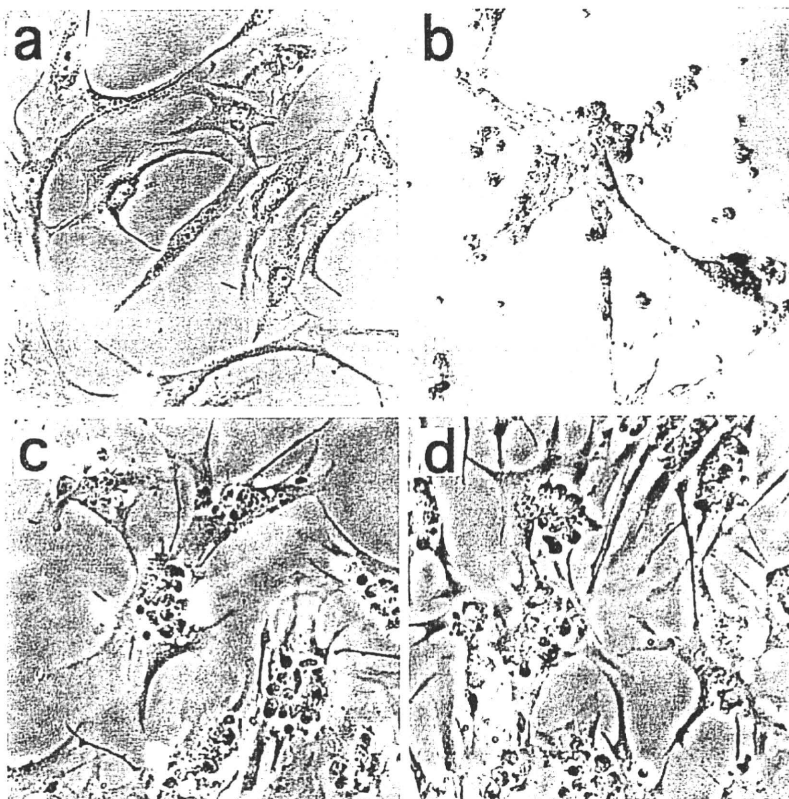
**Establishment of the RA-NLC clones.** Seven clones were established from RA-NLC parental lines from different RA patients, three from bone marrow (RA-NLC33BM, RA-NLC87BM, and RA-NLC91BM), and four from synovial tissue (RA-NLC34syn, RA-NLC45syn, RA-NLC88syn, and RA-NLC77syn). The appearance of a representative 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 $\gamma$  (100 U/ml) or with  $2 \times 10^6$  peripheral B cells for 3 d. Numbers in each histogram indicate the  $\Delta$ 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, MC/car (*d*).  $\times 200$ .

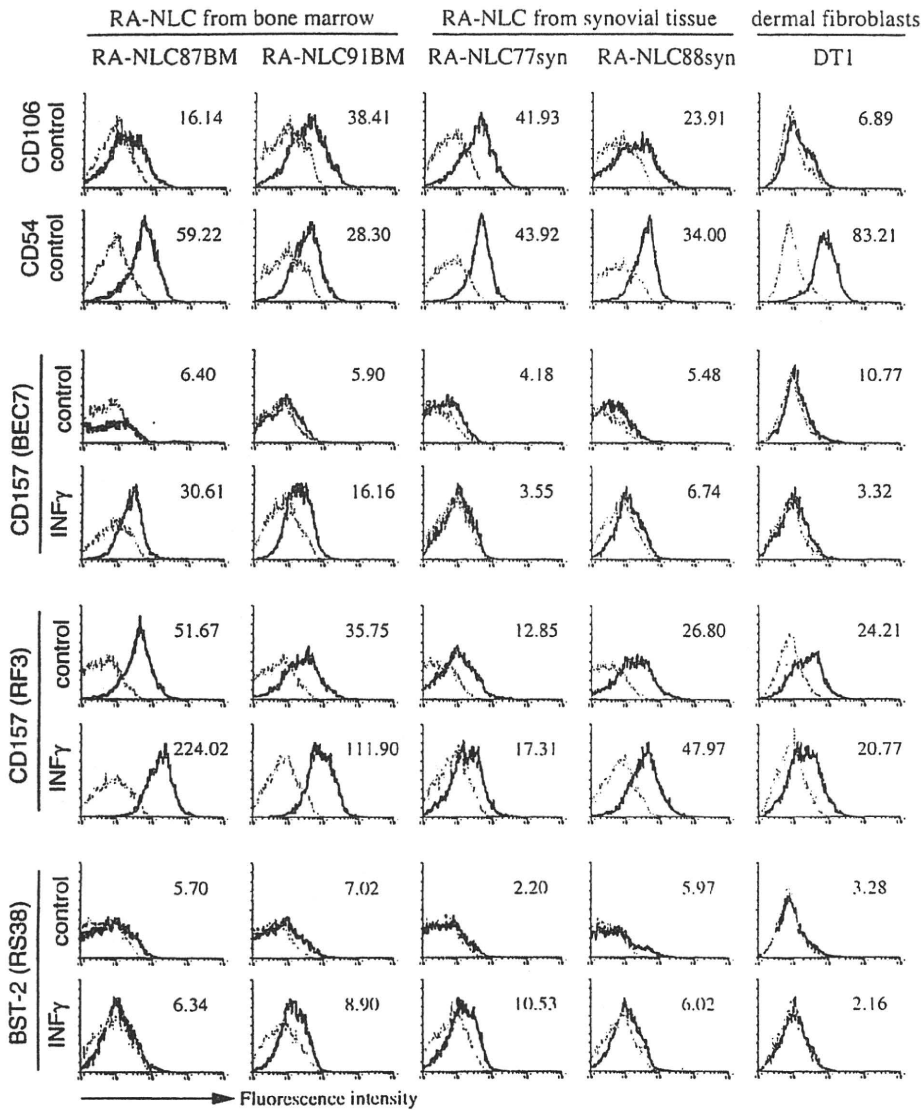


Figure 3. CD106, CD54, CD157, and BST-2 expression by RA-NLC cells and fibroblasts. Four RA-NLC cell lines or human dermal fibroblasts (DT1) were cultured in medium alone (control) or with IFN $\gamma$  (100 U/ml) for 3 d, and were detached with 0.05% trypsin-EDTA in PBS at room temperature for 5 min. 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  $\Delta$ 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 pseudoemperipolexis 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 1). Human dermal fibroblasts also expressed CD29, CD49c, CD54, and class I MHC, whereas constitutive expression of CD106 was minimal. IFN $\gamma$  (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 1).

Expression of CD106 by RA-NLCs was modestly enhanced by culture with normal peripheral B cells, and markedly enhanced by IFN $\gamma$ . In contrast, expression of CD106 by human dermal fibroblasts was much less marked after stimulation with IFN $\gamma$  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 $\gamma$	-	-	-	-	-	-
CD40	Resting	-	-	-	-	-	-
	IFN $\gamma$	+	+	+	+	+	+
CD40L	Resting	-	-	-	-	-	-
	IFN $\gamma$	-	-	-	-	-	-
CD49c	Resting	+	+	+	+	+	+
CD54	Resting	+	+	+	+	+	+
CD106	Resting	+	+	+	+	+	-/+
	IFN $\gamma$	+	+	+	+	+	+
HLA-A,B,C	Resting	+	+	+	+	+	+
HLA-DR	Resting	-	-	-	-	-	-
	IFN $\gamma$	+	+	+	+	+	+

RA-NLC or human skin fibroblasts (DT1) were cultured with medium alone (resting) or with IFN $\gamma$  (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 $\gamma$ . 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 $\gamma$ . Even after IFN $\gamma$  stimulation expression of BEC7-identified CD157 was modest. BST-2 was not expressed by any of the cells constitutively, and IFN $\gamma$  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 $\alpha$	IL-1 $\beta$	IL-2	IL-3	IL-4	IL-6	IL-7	IL-8	G-CSF	GM-CSF	TNF $\alpha$	TNF $\beta$
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.



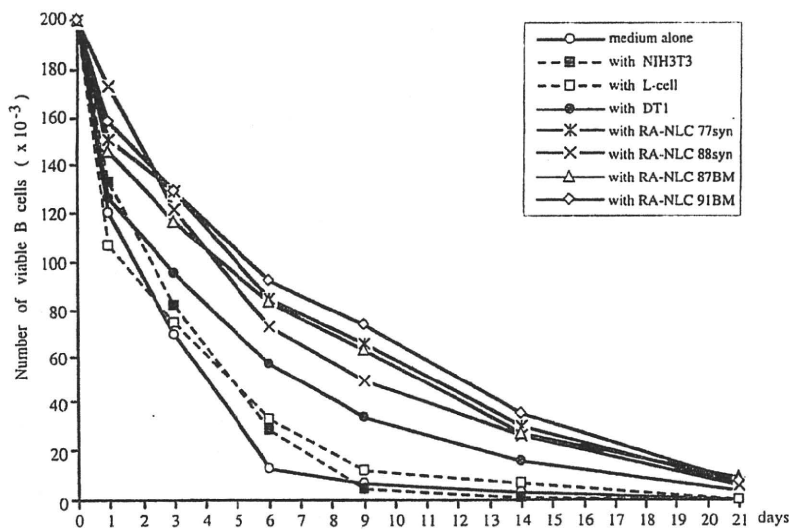


Figure 4. RA-NLC promote survival of peripheral B cells more effectively than fibroblasts. Peripheral B cells ( $2 \times 10^5$ ) were cultured in medium alone or with RA-NLC or mouse fibroblasts (NIH3T3, L cell) or human dermal fibroblasts (DT1) for 21 d, and the numbers of viable cells were counted 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 ( $2 \times 10^5$ ). 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%)	< 0.0001
+ Anti-CD157 polyclonal Ab	+	54.6 $\pm$ 6.3*	(27.3%)	
+ Control preimmune rabbit IgG	+	77.8 $\pm$ 4.5	(38.9%)	< 0.01
+ Soluble CD157	+	147.6 $\pm$ 8.3*	(73.8%)	
+ Control soluble CD38	+	79.0 $\pm$ 5.1	(39.5%)	< 0.0001

Peripheral B cells were cultured in 96-well flat-bottom culture plates ( $2 \times 10^5$ /well, 200  $\mu$ 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 (IgG<sub>1</sub>) was used as the isotype-matched control mAb for anti-CD106 mAb, P1.17 (IgG<sub>2a</sub>) 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  $\mu$ g/ml, and antibodies were used at 4  $\mu$ g/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 ( $2 \times 10^5$ ). \*P values were derived using student's *t* test.

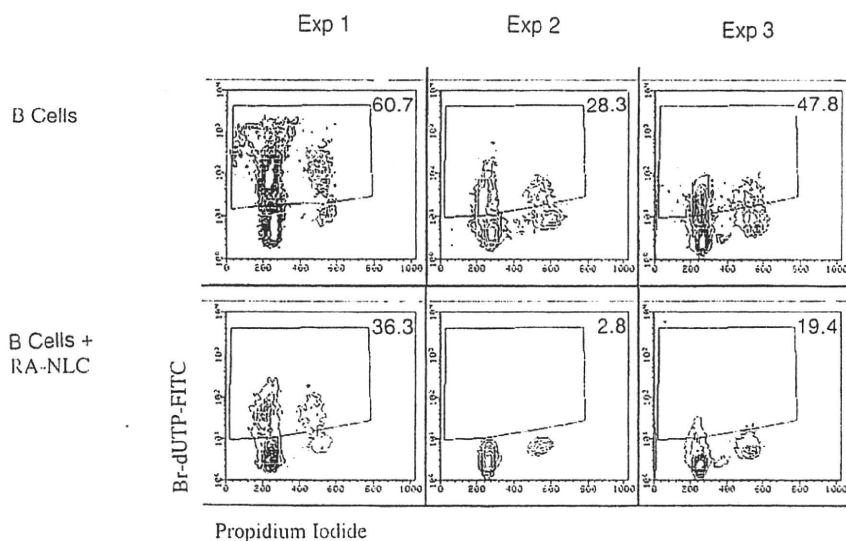


Figure 5. RA-NLC protect B cells from spontaneous apoptosis. Peripheral B cells were cultured in medium alone or with RA-NLC77syn for 3 d and assessed for the percentage of apoptotic cells by the TUNEL technique. The numbers in each box indicate the percentage of apoptotic cells.

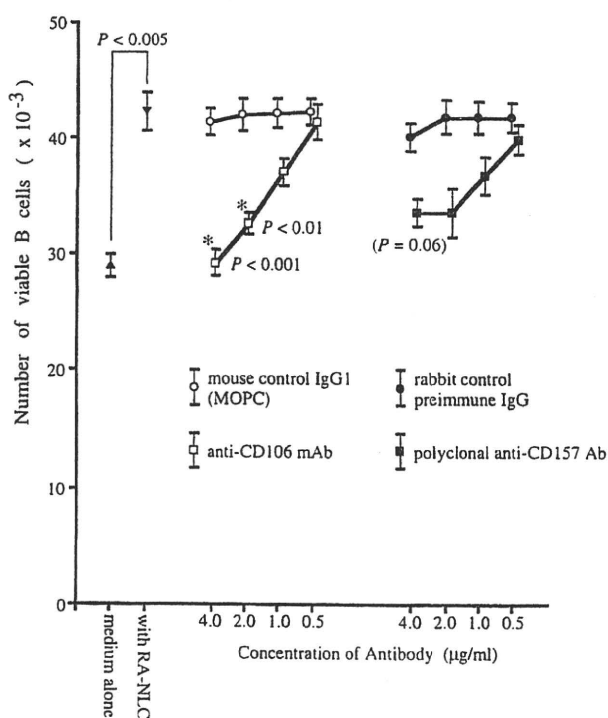


Figure 6. CD106 and CD157 are involved in the maintenance of B cell viability by RA-NLC. Peripheral B cells ( $2 \times 10^5$ ) were cultured in medium alone or with RA-NLC77syn and several concentrations of anti-CD106 mAb or anti-CD157 Ab or control Ab, and the number of viable B cells was counted at 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  $\sim 1$  in 12,500 (Fig. 9). Each of

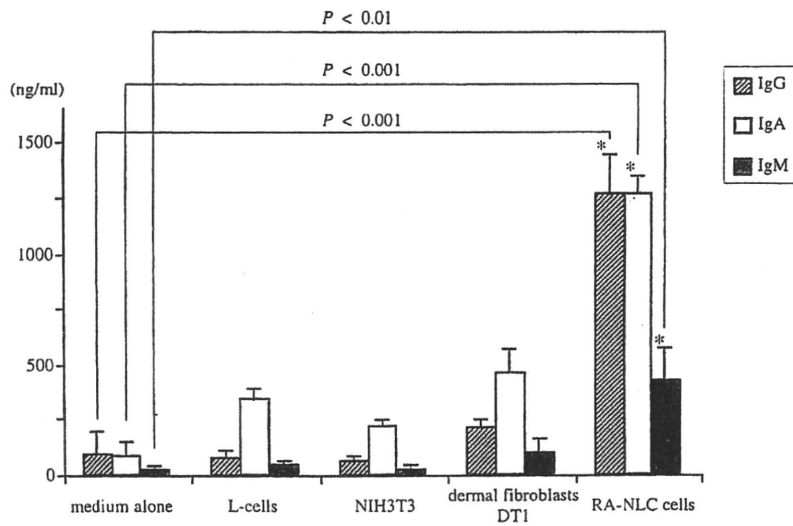


Figure 7. RA-NLC induce secretion of immunoglobulin from peripheral B cells. Peripheral B cells ( $5 \times 10^4$ ) were cultured in medium alone or with mouse fibroblasts (L-cells, NIH3T3 cells), human dermal fibroblasts (DT1), or with RA-NLC33BM with no other stimulation. The supernatants were harvested 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 synovial cells 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. Suga-

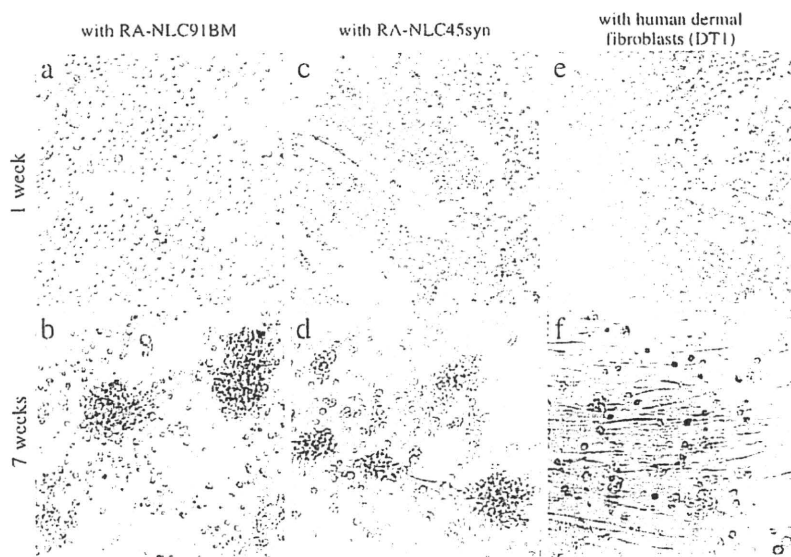


Figure 8. Outgrowth of B cells with RA-NLC. Long-term culture of normal peripheral B cells with RA-NLC (a-d) or human dermal fibroblasts (e and f) was performed. B cells were observed by phase-contrast microscopy after a culture of 1 (a, c, and e) and 7 wk (b, d, and f), respectively. At 1 wk, approximately 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.  $\times 60$ .

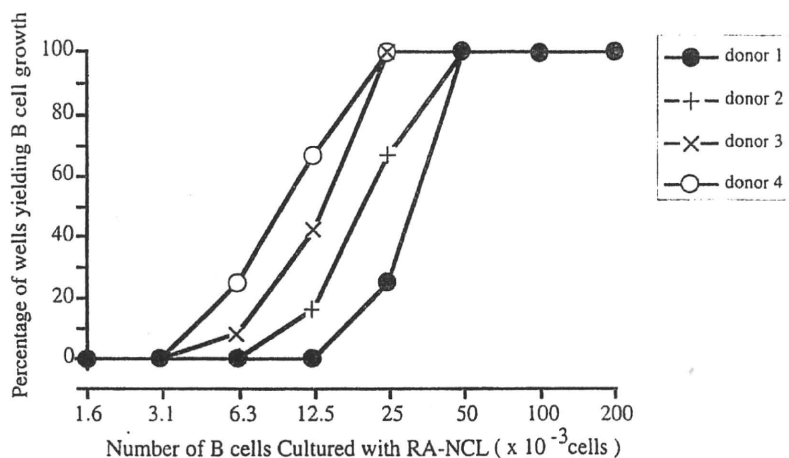


Figure 9. RA-NLC promote outgrowth of a small population of B cells. Several densities of peripheral B cells from four different donors were cultured with RA-NLC91BM for 4 wk in 96-well flat-bottomed plates. The vertical axis shows percentage of wells yielding B cell growth, and horizontal axis shows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

more effective than fibroblasts in maintaining B cell viability by blocking spontaneous apoptosis. Moreover, the mechanism involved the activity of two surface molecules constitutively expressed by RA-NLC, CD106, and CD157. As mentioned above, CD157 expressing bone marrow stromal cells previously have been shown to enhance proliferation of a pre-B cell line (16), but this is the first indication that CD157 may have a functional impact on mature peripheral B cells. Although recombinant soluble CD157 alone had no effect on spontaneous B cell apoptosis, together with RA-NLC, enhanced protection from apoptosis was noted. This finding, along with the data from blocking studies with antibodies to CD157, strongly implied that RA-NLC protected B cells from apoptosis by delivering two signals to B cells, one of which may be provided by CD157 engaging an unknown ligand on the B cell.

The second signal that protected B cells from apoptosis was provided by CD106, a molecule that was also constitutively expressed by RA-NLC and could be further upregulated by IFN- $\gamma$ . An interaction between CD106 expressed by the RA-NLC and its ligand, CD49d/CD29 ( $\alpha_4\beta_1$ , VLA-4) expressed by the B cell, appeared to be essential for maintenance of B cell viability and prevention of apoptosis. Previous evidence has shown that CD106 is essential for other functions of nurse cells such as the induction of pseudoemperipolesis (7). Moreover, CD49d/CD29 engagement has recently been shown to provide a costimulatory signal to human B cells (50). Thus, interactions between RA-NLC-expressed CD106 and B cell-expressed CD49d/CD29 are reasonable candidates to provide viability promoting signals to B cells.

Although RA-NLC failed to support initial B cell proliferation, they supported increased Ig production by otherwise unstimulated peripheral B cells or B cells activated with anti-CD3-stimulated T cells. Of note, RA-NLC were much more effective than fibroblasts at stimulating resting B cells to produce Ig. Although the mechanism of this phenomenon is unclear, it does not appear to be related to the CD40 signaling pathway as the RA-NLC failed to express CD40 ligand (CD154), even after stimulation with IFN- $\gamma$ . It is possible that the molecules involved in the induction of Ig production are the same as those promoting viability, namely either CD157, CD106, or both. The possibility that these molecules provide signals to mature B cells that facilitate Ig production is consistent with the expression of CD157 by reticular cells in spleen and lymph node (44) and the recently described role of signaling via CD49d/CD29 in facilitating Ig production by peripheral B cells (50). This possibility is currently being examined. Regardless of the mechanism, the data are consistent with the conclusion that NLC in the rheumatoid synovium and bone marrow play a role in the local and systemic overreactivity of B cells characteristic of RA.

RA-NLC also supported the outgrowth of EBV-transformed B lymphoblastoid cells from normal B cells. Of importance, the RA-NLC were not infected with EBV, as has been reported for some synovial membrane-derived fibroblasts (51). Moreover, RA-NLC did not facilitate growth of EBV-transformed B lymphoblastoid cell lines, suggesting that they facilitated outgrowth of latently transformed B cells from peripheral blood. The precursor frequency of B cells undergoing long-term growth when cultured on RL-NLC (1:12,500) was comparable to the frequency of B cells latently infected with EBV in adult peripheral blood (52, 53) supporting this conclusion. The mechanism whereby RA-NLC promote the out-

growth of EBV-transformed B lymphoblastoid cell lines from human peripheral B cells is unknown, but is likely to involve more than nonspecific support of viability, as human fibroblasts supported B cell viability somewhat, but failed to facilitate the outgrowth of EBV-transformed B lymphoblastoid cell lines. Whether similar surface molecules are involved in this process as were involved in the maintenance of B cell viability is currently unknown, but is the subject of ongoing investigation.

In rheumatoid synovium, germinal center-like structures containing B cells aggregated around cells resembling follicular dendritic cells (FDC) develop (12). The exact lineage of these FDC-like cells is unclear, but they and FDC in secondary lymphoid organs have certain characteristics in common with RA-NLC. For example, synovial FDC-like cells express CD106 at high levels (12, 54). Constitutive expression of CD106 appears to be a feature of both NLC and FDC. In this regard, some (55) but not all (56) FDC lines derived from human tonsil express high levels of CD106. Importantly, CD106 plays a critical role in the interaction of FDC with B cells both *in vivo* and *in vitro* (55, 57, 58). Moreover, at least one FDC-like line has been shown to express CD157 (55). In addition, FDC-like lines are similar to nurse cell lines *in vitro*, in that both can promote pseudoemperipolesis (59). Finally, both FDC and nurse cells can promote B cell viability. Thus, an FDC-like line promoted the viability of tonsillar B cells (56), whereas freshly prepared FDC promoted the viability of germinal center tonsillar B cells in a CD49d/CD29-dependent manner (60). There are, however, differences between FDC lines and RA-NLC, including the cytokines produced (61) and whether they interact uniquely with B cells (58, 61) or with both T and B cells (59), as is typical of nurse cells. In addition, FDC lines appear to support emperipolesis (56, 59, 60) as well as the pseudoemperipolesis (59) typical of nurse cells (6–9). Despite these discrepancies, the results suggest the possibility that RA-NLC and FDC-like cells at inflammatory sites or even in secondary lymphoid organs have many overlapping functional and phenotypic features. However, RA-NLC fail to express CD21 or CD35, characteristic markers of classic FDC (62–64). The absence of these markers on RA-NLC may be related to the observation that although CD21 and CD35 are expressed by FDC *in situ*, they are rapidly lost when these cells are cultured *in vitro* (55, 56, 59, 61). Of note, these markers can be induced on synoviocytes by stimulation with the proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  (12). In this study, IFN- $\gamma$  upregulated expression of both CD106 and CD157 by RA-NLC. If this cytokine, in conjunction with TNF- $\alpha$  also upregulated expression of CD21 and CD35 by RA-NLC, the cells would then exhibit the phenotype of synovial FDC. In conjunction with the capacity of RA-NLC to facilitate B cell functional activity, cytokine-activated RA-NLC may well account for the aggregates of B cells and CD106 expressing FDC-like cells (54) and the local activation of B cells and immunoglobulin production characteristic of rheumatoid synovitis (36, 37).

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# Bidirectional Regulation of Human B Cell Responses by CD40-CD40 Ligand Interactions<sup>1</sup>

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Positive and negative effects of CD40 ligation on human B cell function were suggested by the observation that mAb to CD40 ligand partially blocked the suppressive influences of anti-CD3-stimulated control CD4<sup>+</sup> T cells, as well as the B cell stimulatory effects of anti-CD3 activated mitomycin C-treated CD4<sup>+</sup> T cells. To examine the negative effects of CD40 ligation in greater detail, B cells were cultured with anti-CD3 activated mitomycin C-treated CD4<sup>+</sup> T cells that expressed optimal levels of CD40 ligand; additional recombinant human CD40 ligand significantly suppressed Ig production, but not proliferation. In contrast, when B cells were stimulated with SAC (formalinized Cowan I strain *Staphylococcus aureus*) and IL-2 in the absence of T cells, small amounts of recombinant CD40 ligand-stimulated Ig production, whereas larger quantities directly suppressed Ig secretion. The suppressive action of CD40 ligation on Ig production was most apparent after initial B cell activation. Moreover, IgD<sup>-</sup> memory B cells were significantly more sensitive to inhibition by CD40 ligation than IgD<sup>+</sup> naive B cells. Engagement of CD40 not only suppressed Ig secretion by IgD<sup>-</sup> memory B cells, but also expression of CD38. Finally, activated B cells acquired the capacity to down-regulate CD40 ligand expression by stimulated CD4<sup>+</sup> T cells more effectively than resting B cells. These results indicate that during T cell-B cell collaboration, engagement of CD40 can influence Ig production both positively and negatively, depending on the density of CD40 ligand as well as the stage of B cell activation and differentiation. *The Journal of Immunology*, 1997, 158: 4620-4633.

A variety of receptor-ligand pairs have been reported to play important roles in contact-dependent T-B collaboration. Among these, CD40-CD40 ligand interaction plays a central role (1). The importance of this interaction has been confirmed by in vivo studies that have documented that interference with CD40 signaling by targeted gene disruption or by administration of mAb inhibited both primary and secondary immune responses to T cell-dependent Ags (2, 3) and prevented the formation of germinal centers as well as the generation of memory B cells (4-6). Many in vitro studies also have demonstrated that ligation of CD40 induces B cell activation, resulting in proliferation and secretion of Ig, as well as Ig heavy chain switch recombination in the presence of appropriate cytokines (7-10), and also leading to the generation of memory B cells (11).

Most of these studies have focused on the positive influences of CD40 ligation. Recent evidence, however, has also suggested that CD40 engagement may inhibit B cell responses under certain circumstances. In this regard, it has been demonstrated that mAb to CD40 inhibited the growth of certain B cell lymphomas (12-14). Signaling through CD40 has also been shown to induce the death of transformed cells of mesenchymal and epithelial origin (15).

Moreover, intense signaling through CD40 was noted to induce apoptosis of Ig secreting hybridoma by a Fas- and TNF- $\alpha$ -independent mechanism (16). Finally, CD40 ligation inhibited the differentiation of tonsillar B cells into plasma cells or Ig-secreting cells in Ag-specific (17, 18) and Ag-nonspecific responses (11). In these reports, the negative function of CD40 ligation was investigated in cultures of tonsillar B cells, murine B cells, B cell lines, or B cell hybridomas. The possibility that CD40 ligation during T cell-B cell collaboration might limit responses of circulating B cells has not previously been explored.

Here, we show evidence that CD40-CD40 ligand interaction either enhances or suppresses Ig production by normal human peripheral B cells during T cell-B cell collaboration. The functional impact of engagement of CD40 is dependent on the density of CD40 ligand expressed by activated CD4<sup>+</sup> T cells, as well as on the stage of B cell activation and differentiation. The data show that low concentrations of CD40 ligand enhanced Ig production by B cells, whereas high concentrations suppressed Ig production, but not proliferation of B cells. IgD<sup>-</sup> memory B cells were far more sensitive to the suppressive influences of CD40 ligation than IgD<sup>+</sup> naive B cells. IgD<sup>-</sup> memory B cells responded to ligation of CD40 by producing less IgG and IgA and also expressing a lower intensity of CD38. Regulation of the impact of CD40 ligation was noted in that activated B cells strongly suppressed CD40 ligand expression by activated CD4<sup>+</sup> T cells. These results indicate that CD40 ligation mediates complex, often opposing effects on B cells and B cell subpopulations and that B cells have the capacity to escape the suppressive action of CD40 ligation during T cell-B cell collaboration.

## Materials and Methods

### *Monoclonal Abs, cytokines, and reagents*

Various Ab were used, including 64.1, a mouse IgG2a mAb directed at the CD3 molecular complex on mature T cells (19); 4D9-8, a mouse IgG1

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<sup>3</sup> Abbreviations used in this paper: SAC, formalinized Cowan I strain *Staphylococcus aureus*; MFI, mean fluorescence intensity; PE, phycoerythrin.

mAb against the human CD40 ligand molecule that was a kind gift of Dr. R. J. Noelle (Dartmouth Medical School, Hanover, NH); MOPC, a control mouse IgG1 mAb; MR1, a hamster IgG mAb against mouse CD40 ligand; 145-2C11, a hamster IgG mAb against mouse CD3; OKT8 (American Type Culture Collection (ATCC), Bethesda, MD), a mouse IgG2a mAb directed at the CD8 molecule; L243 (ATCC), a mouse IgG2a mAb directed at monomorphic HLA-DR determinants; Coulter clone B4-BIOTIN, a biotinylated mouse IgG1 mAb directed at human CD19 (Coulter Corp., Miami, FL); a PE-conjugated mouse IgG1 mAb directed at human CD19 (Sigma Chemical Co., St. Louis, MO); a sheep anti-mouse IgG1 FITC conjugate (The Binding Site, Birmingham, U.K.); an FITC-conjugated mouse IgG1 mAb directed at human CD38 (Caltag Laboratories, Burlingame, CA); a control FITC-conjugated mouse IgG1 mAb (Caltag Laboratories); and an FITC-conjugated goat F(ab')<sub>2</sub> anti-human IgD Ab (Caltag Laboratories, So. San Francisco, CA).

Purified rIL-2 was obtained from Hoffmann-La Roche (Nutley, NJ) and used at 20 U/ml. Formalinized Cowan I strain *Staphylococcus aureus* (SAC, Pansorbin) was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and was used at a final concentration of 1/60,000 (v/v).

#### Expression of murine or human CD40 ligand by baculovirus-infected Sf9 cells and preparation of Sf9 cell membranes

Sf9 cells were incubated at 26°C with wild-type AcMNPV or human or murine CD40 ligand-encoding recombinant baculovirus (kind gifts of Dr. Marilyn Kehry, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, or Dr. Kathryn Meek, University of Texas Southwestern Medical Center at Dallas, respectively). After a 72-h incubation, cells were harvested, washed twice in PBS, and resuspended in cold homogenization buffer (0.2 M Tris/Cl, pH 7.4) and Pefabloc SC (AEBSF hydrochloride, Boehringer Mannheim GmbH, Mannheim, Germany). Cells were disrupted at 4°C by a Polytron (Brinkman, Westbury, NY) homogenizer at speed 7. Seven to ten 15-s homogenizations were usually found to be sufficient to break >95% of the cells. Afterward, the homogenate was centrifuged at 200 × g for 3 min, and the supernatant was collected and centrifuged again at 7,000 × g for 10 min. The membrane pellet was resuspended in PBS at a final concentration of 20 to 25 × 10<sup>6</sup> cell equivalents per milliliter based on the starting cell number. The membrane suspension was kept at -80°C until used. The final concentration of total protein in the membrane suspension was approximately 0.5 μg/μl. These experiments were conducted with three separate preparations of human CD40 ligand expressing Sf9 membranes and one preparation of mouse CD40 ligand expressing Sf9 membranes. Each lot of membranes was shown to have stimulatory capacity for human B cells using previously described methods (20, 21).

The level of functionally active CD40 ligand expressed by Sf9 cell membranes was determined by a competitive binding assay using a previously described CD40 ligand-CD8 construct as a standard (16). In this assay, Ramos B lymphoma cells were incubated with various concentrations of the CD40 ligand-CD8 construct, and the degree of binding was determined with an FITC-conjugated mAb to CD8 (YTS169.4, ATCC) and analysis by flow cytometry. The capacity of various amounts of membranes prepared from wild-type or recombinant CD40 ligand-expressing baculovirus-infected Sf9 cells to block binding of the CD40 ligand-CD8 construct by Ramos cells was then assessed. Using this approach, it was determined that 2, 4, 6, and 8 μl of CD40 ligand-expressing Sf9 membranes bound 10, 25, 40, and 60%, respectively, of CD40 expressed by 1 × 10<sup>5</sup> Ramos cells.

#### Culture medium

All cultures were conducted in RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with penicillin G (200 U/ml), gentamicin (10 μg/ml), L-glutamine (0.3 mg/ml), and 10% FBS (Life Technologies).

#### Cell preparation

PBMC were isolated from heparinized blood of healthy adult volunteers by centrifugation over sodium diatrizoate/Ficoll gradients (Pharmacia, Inc., Piscataway, NJ). B cells were purified by two different procedures. First, the CEPRATE LC Kit (CellPro, Bothell, WA), a disposable cell separation system for isolating specific cell populations, was used to prepare CD19<sup>+</sup> B cells following the manufacturer's protocol. Briefly, PBMC were reacted with subsaturating concentrations of biotinylated mouse anti-human CD19 mAb (Coulter) at 4°C for 25 min, and after washing, applied to a column containing avidin-coated beads. After unbound cells passed through the column, bound B cells were recovered by mechanical manipulation of the avidin bead column. The resultant population of B cells contained >97% CD19<sup>+</sup> B cells. Peripheral B cells prepared in this manner from normal donors were almost entirely small resting cells as determined by flow cy-

tometric analysis of side and forward scatter. The population was routinely devoid of activated CD69<sup>+</sup> B cells.

A second procedure was also used to purify B cells from PBMC by negative selection as previously described (22). Briefly, PBMC were depleted of monocytes and NK cells by incubation with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co.) in serum-free RPMI 1640 as described (23). The treated cell population was washed and incubated with neuraminidase-treated SRBC (24). The rosetting and nonrosetting populations were then separated by centrifugation on diatrizoate/Ficoll gradients. The nonrosetting cells were obtained from the interface and were again rosetted and centrifuged on diatrizoate/Ficoll gradients to remove residual T cells. The resultant population of B cells contained <2% esterase-positive monocytes and <1% T cells as determined by staining with OKT3 and OKT11, pan-T cell mAb, followed by flow cytometric analysis. The cells were additionally characterized as containing >90% CD19<sup>+</sup> B cells and no Leu 11b<sup>+</sup> NK cells.

In some experiments, IgD<sup>+</sup> or IgD<sup>-</sup> B cells were separated from purified B cells by flow cytometry. Purified B cells were incubated with saturating concentrations of a FITC-conjugated goat F(ab')<sub>2</sub> anti-human IgD Ab for 30 min at 4°C, washed with PBS, and resuspended at a concentration of 5 × 10<sup>6</sup>/ml in PBS containing 2% FBS. Cells were sorted at rates of 2000 to 2500 cells per second using a fluorescence-activated cell sorter (FACStar<sup>Plus</sup>, Becton Dickinson, San Jose, CA). The threshold was set to abort all dead cells and debris as determined by light scattering. After sorting, IgD<sup>+</sup> or IgD<sup>-</sup> B cell populations were washed with PBS and resuspended in RPMI-10% FBS. The viability of each cell population was >98%.

The sedimented rosette-forming cells were treated with isotonic NH<sub>4</sub>Cl to lyse the SRBC and were passed over a nylon wool column. Afterward, purified CD4<sup>+</sup> T cells were prepared from the rosette-forming cells by negative selection, using a panning technique (25) to deplete contaminating HLA-DR<sup>+</sup> cells and CD8<sup>+</sup> T cells. Cells were reacted with saturating concentrations of the anti-HLA-DR mAb, L243, and OKT8. After washing, 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<sup>+</sup> T cell population obtained in this manner contained <0.1% esterase-positive cells, <1% CD8<sup>+</sup> T cells, and >96% CD4<sup>+</sup> T cells.

In some experiments, CD4<sup>+</sup> T cells were treated with mitomycin C before culture. This was accomplished by suspending them in culture medium at approximately 5 × 10<sup>6</sup>/ml and incubating them on a rotator for 50 min at 37°C with mitomycin C at a concentration of 40 μg/ml. Afterward, the cells were washed four times and suspended in culture medium for use.

#### Culture conditions

All cultures were conducted in 96-well microtiter plates with round-bottom wells (Costar, Cambridge, MA). When B cells were activated with CD4<sup>+</sup> T cells, microtiter wells were coated with mAb to CD3 before cells were added, as previously described (26, 27). Briefly, anti-CD3 mAb 64.1 was dissolved in Tris-buffer (50 mM, pH 9.5) at a concentration of 4 μg/ml, unless otherwise specified, and 50 μl was placed in each of the wells of 96-well microtiter plates with round-bottom wells (Costar) and incubated at 37°C for 2 h. The wells were then washed twice with RPMI to remove nonadherent mAb before addition of cells. Approximately 14 to 20% of the added mAb adhered to the wells (27). B cells (2.5 × 10<sup>4</sup>/well) were incubated with various numbers of CD4<sup>+</sup> T cells. Routine cultures were conducted in duplicate in a total volume of 200 μl. The cells were incubated routinely for 10 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

In some experiments, a two-step assay of B cell stimulation was conducted. B cells were activated for various periods of time (usually 48 h) with CD4<sup>+</sup> T cells stimulated by immobilized mAb to CD3 or with SAC plus IL-2 in the presence or absence of membrane-expressed CD40 ligand or control membranes. At the end of the initial culture, activated B cells were separated from CD4<sup>+</sup> T cells by rosetting with neuraminidase-treated SRBC, or B cells stimulated by SAC and IL-2 were harvested. The B cells were washed and recultured with CD4<sup>+</sup> T cells in anti-CD3 mAb-coated microtiter wells or were recultured with IL-2 in the presence or absence of membrane-bound CD40 ligand. The total combined length of the first and second cultures was 10 days.

#### 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

serum and 0.1% sodium azide, cells were reacted with saturating concentrations of various mAb at 4°C for 30 min. For staining with 4D9-8 (anti-CD40 ligand), secondary FITC-conjugated sheep anti-mouse IgG1 (The Binding Site) was used. To detect CD40 ligand expression on CD4<sup>+</sup> T cells cultured with B cells, cells were reacted with 4D9-8, secondary FITC-conjugated Ab and PE-conjugated anti-CD19. Afterward, CD40 ligand expression on the CD19-negative cell population was analyzed. 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 ( $\Delta$ MFI) for staining, with the mAb of interest calculated by subtracting the MFI of staining with the control mAb.

#### Measurement of Ig production

At the end of 10 days of incubation, the supernatants from each culture well were aspirated and assayed for Ig content. Ig in the culture supernatants was quantitated using isotype-specific ELISAs as previously described (28). Briefly, microtiter plates (Cooke, Dynatech Laboratories, Alexandria, VA) were coated with affinity-purified goat Abs to human  $\alpha$ -,  $\mu$ -, or  $\gamma$ -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-20 saline and incubated with serial dilutions of standard Ig preparation (IgA and IgG, Behring Diagnostics, La Jolla, CA; IgM, Tago) or diluted culture supernatants in PBS containing 0.5% Tween-20 (Sigma Chemical Co.) at 37°C for 2 h. Bound Ig was detected with peroxidase-conjugated affinity-purified isotype-specific Abs (Tago) in the presence of *o*-phenylenediamine dihydrochloride (Sigma Chemical Co.) as substrate. Absorbance was determined at a wavelength of 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). Triplicates for each culture condition were performed and differed by less than 10%. The sensitivities of the specific assays were 12 ng/ml for IgA and IgG and 24 ng/ml for IgM.

#### Measurement of B cell DNA synthesis

To examine B cell DNA synthesis, the cells were incubated for 4 days at 37°C with 1  $\mu$ Ci [<sup>3</sup>H]thymidine (6.7 Ci/mM; ICN Pharmaceuticals, Inc., Irvine, CA) present for the last 18 h. The cells were then harvested onto glass fiber filter paper, and [<sup>3</sup>H]thymidine incorporation was determined by liquid scintillation spectroscopy.

#### Assay of B cell growth

To assay cellular growth, a rapid colorimetric method was used (29). Briefly, B cells were cultured with SAC and IL-2 in the presence or absence of recombinant CD40 ligand. Afterward, B cells were harvested, washed, and then re-cultured with IL-2 in the presence or absence of recombinant CD40 ligand for the indicated length of time, after which 180  $\mu$ l of the supernatant was removed without disturbing the pellet. Ten microliters of a yellow tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.), and 100  $\mu$ l of PBS were added to the remaining pellet and incubated at 37°C for 4 h, after which time 100  $\mu$ 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 pipetted gently until mixed. The absorbance was read at 570 and 630 nm on the EL312 Biokinetics Reader (Bio-Tek Instruments).

## Results

### Expression of CD40 ligand by anti-CD3-stimulated CD4<sup>+</sup> T cells

Initial experiments investigated CD40 ligand expression by anti-CD3-stimulated CD4<sup>+</sup> T cells in the absence or presence of B cells. As shown in Figure 1 and comparable with previously published results (30), CD40 ligand expression by activated control CD4<sup>+</sup> T cells in the absence of B cells reached a maximum after 24 h of stimulation and thereafter began to decline. CD40 ligand expression was still detected at 72 h, albeit diminished in intensity. Afterward, the expression continued to decrease to basal levels and was not detected at 144 h or later. The expression of CD40 ligand by activated mitomycin C-treated CD4<sup>+</sup> T cells also reached a maximum at 24 h, but the density was significantly lower than that expressed by activated control T cells. CD40 ligand expression by

activated mitomycin C-treated T cells became undetectable by 72 h. The stability of CD40 ligand expression by each activated T cell population was examined by adding cycloheximide at 24 h after stimulation to block de novo protein synthesis. The  $T_{1/2}$  of CD40 ligand expressed by activated control CD4<sup>+</sup> T cells or mitomycin C-treated CD4<sup>+</sup> T cells, which was calculated from the  $\Delta$ MFI of each sample between 24 h and 48 h, was comparable (4.7 and 6.4 h, respectively).

B cells have been reported to down-regulate CD40 ligand expression by T cells (30). Experiments were conducted, therefore, to determine whether coculture with B cells would alter expression of CD40 ligand by the T cell populations differentially. As shown in Figure 1, CD40 ligand expression by both of the activated CD4<sup>+</sup> T cell populations was markedly diminished in the presence of B cells, although the density of CD40 ligand expressed by control CD4<sup>+</sup> T cells during the initial 48 h remained greater. These results indicate that the major difference between activated control and mitomycin C-treated CD4<sup>+</sup> T cells was the much greater expression of CD40 ligand by the former during the initial 48 to 72 h of culture.

### Monoclonal Ab to CD40 ligand enhances Ig production by B cells cultured with anti-CD3-stimulated control CD4<sup>+</sup> T cells

The next experiments were designed to examine the effects of mAb to CD40 ligand on B cell Ig production induced by anti-CD3-stimulated control or mitomycin C-treated CD4<sup>+</sup> T cells. As seen in Figure 2, anti-CD3-stimulated mitomycin C-treated CD4<sup>+</sup> T cells induced very large amounts of Ig. In contrast, anti-CD3-stimulated control CD4<sup>+</sup> T cells induced the production of minimal amounts of Ig and inhibited the production of Ig supported by anti-CD3-stimulated mitomycin C-treated CD4<sup>+</sup> T cells as previously described (31). When mAb to CD40 ligand was added at the initiation of culture, B cell Ig production induced by anti-CD3-stimulated mitomycin C-treated CD4<sup>+</sup> T cells was inhibited. This effect was noted only when the mAb was added at the initiation of culture. In contrast, mAb to CD40 ligand enhanced Ig production by B cells cultured with anti-CD3-stimulated control CD4<sup>+</sup> T cells. This effect was most apparent when mAb to CD40 ligand was added between 24 and 48 h of culture. Suppression of Ig production in cultures of B cells stimulated with mitomycin C-treated and control T cells was also partially relieved when mAb to CD40 ligand was added between 24 and 48 h of culture. mAb to CD40 ligand had no effect on Ig production when added 72 to 96 h after stimulation or later.

### Supplemental rCD40 ligand suppresses B cell Ig production

The next experiments were conducted to determine whether excess CD40 ligand would alter Ig production in cultures of B cells and activated mitomycin C-treated CD4<sup>+</sup> T cells. As a control, the effect of the same amount of exogenous CD40 ligand on Ig production by purified B cells stimulated with SAC and IL-2, but no T cells, was examined. Preliminary experiments determined the number of activated mitomycin C-treated T cells that induced the maximal amount of Ig production. As shown in Figure 3A, when B cells were cultured with activated mitomycin C-treated CD4<sup>+</sup> T cells, Ig production was dramatically suppressed by the addition of Sf9 membranes expressing recombinant human CD40 ligand at the initiation of culture. The apparent suppression of IgM production was not related to Ig class switching because production of IgG and IgA was also suppressed. When B cells were stimulated with SAC and IL-2 in the absence of T cells, small concentrations of recombinant human CD40 ligand dramatically enhanced Ig production, whereas larger concentrations suppressed Ig secretion



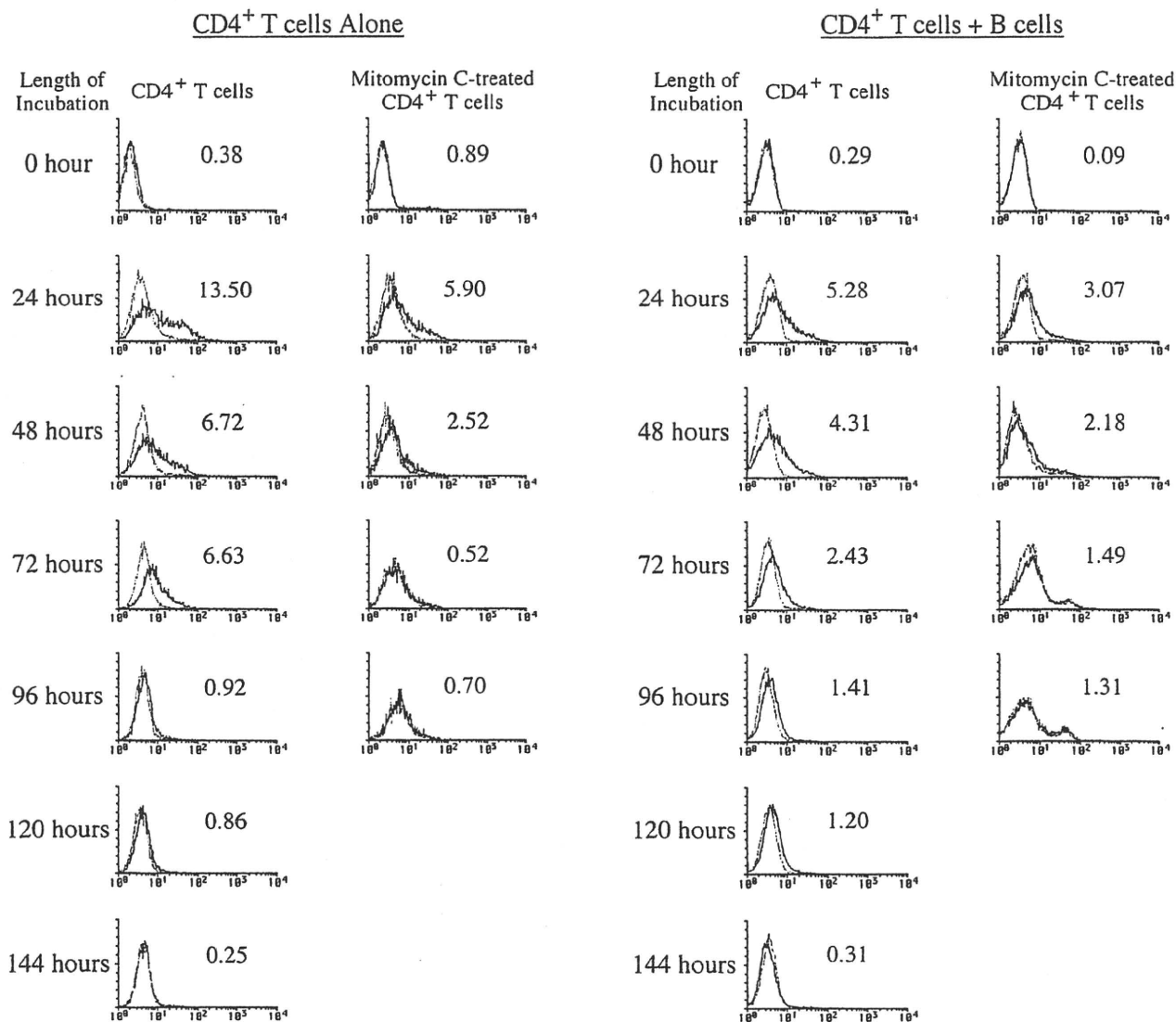


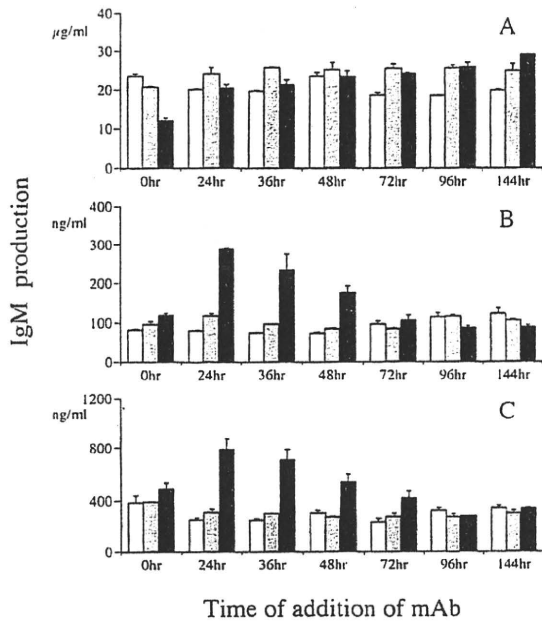
FIGURE 1. Kinetics of CD40 ligand expression by anti-CD3-stimulated CD4<sup>+</sup> T cells cultured with or without B cells. Either control CD4<sup>+</sup> T cells or those treated with mitomycin C before stimulation ( $1 \times 10^5$ /well) were incubated with or without B cells ( $2.5 \times 10^4$ /well) in anti-CD3 (200 ng/well)-coated wells for the length of time indicated. Then, cells were stained with CD19-PE mAb and either 4D9-8 or a control mAb followed by anti-mouse IgG1-FITC. CD40 ligand expression by CD19-negative cells was analyzed by flow cytometry. Numbers in each histogram indicate the  $\Delta$ MFI for staining with 4D9-8 calculated by subtracting the MFI of staining with the control mAb.

(Fig. 3B). To insure the specificity of the inhibitory effect, similar experiments were conducted with recombinant murine CD40 ligand, which is known to stimulate human B cells (20, 21). Murine CD40 ligand also caused marked suppression of T cell-dependent Ig production that was completely reversed by a mAb specific for the murine CD40 ligand (Fig. 4). Finally, in contrast to the effect on Ig production, recombinant CD40 ligand did not suppress T cell-dependent B cell [<sup>3</sup>H]thymidine incorporation and modestly, but significantly, stimulated [<sup>3</sup>H]thymidine incorporation by purified B cells (Fig. 5). Larger concentrations of CD40 ligand induced less [<sup>3</sup>H]thymidine incorporation by highly purified B cells.

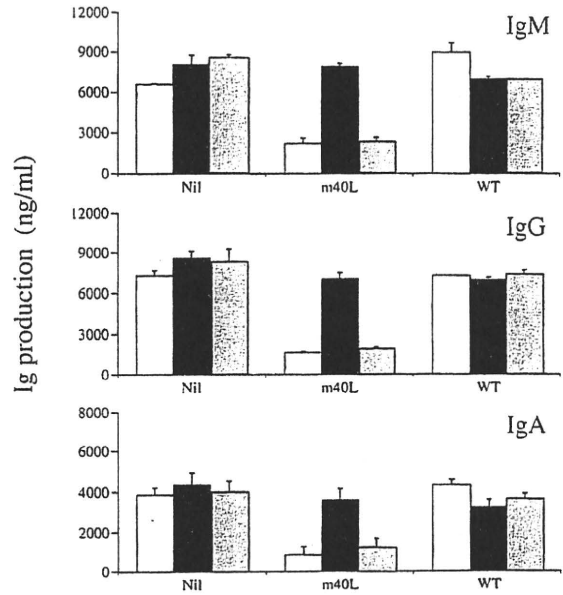
*The effect of supplemental rCD40 ligand is dependent on the quantity of physiologic CD40 ligand expressed by activated CD4<sup>+</sup> T cells*

The relationship between CD40 ligand expression by CD4<sup>+</sup> T cells and the intensity of stimulation by anti-CD3 was examined by

activating mitomycin C-treated CD4<sup>+</sup> T cells with various concentrations of immobilized anti-CD3 in the presence of B cells. Low concentrations of anti-CD3 (3 ng/well) induced minimal, if any, CD40 ligand expression, whereas maximal CD40 ligand expression was noted with 9 ng/ml or more of anti-CD3 (data not shown). To relate this to the functional outcome of T cell-B cell collaboration, recombinant CD40 ligand was added to cultures stimulated with various concentrations of anti-CD3, and the impact on Ig production was examined (Fig. 6). When a low concentration of anti-CD3 (3 ng/well) was used as a stimulus, the addition of CD40 ligand enhanced Ig production by six- to eightfold. When higher concentrations of anti-CD3 were employed to stimulate the CD4<sup>+</sup> T cells, the addition of the same concentration of recombinant CD40 ligand suppressed Ig production. These results are consistent with the conclusion that the impact of supplemental CD40 ligand is dependent on the level of endogenous CD40 ligand expression.

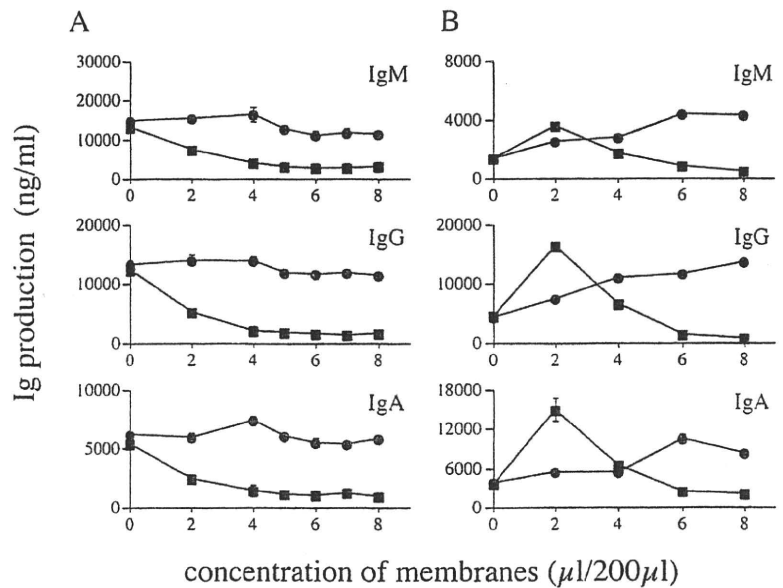


**FIGURE 2.** The role of CD40-CD40 ligand interaction in T cell-dependent Ig secretion by B cells. B cells ( $2.5 \times 10^4$ /well) were cultured with (A) anti-CD3 (200 ng/well)-stimulated mitomycin C-treated CD4<sup>+</sup> T cells, (B) anti-CD3 (200 ng/well)-stimulated control CD4<sup>+</sup> T cells, or (C) a 1/1 mixture of both populations ( $1 \times 10^5$ /well). Either 4D9-8 (black), a control mAb (gray), or no mAb (white) was added at a final concentration of 10 µg/ml at various times after stimulation. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data from three experiments are shown, expressed as mean  $\pm$  SEM. Data shown are for analysis of IgM secretion. Similar results were noted when IgG or IgA secretion was analyzed.



**FIGURE 4.** mAb to murine CD40 ligand (MR1) completely blocks the suppressive effect of recombinant murine CD40 ligand on Ig secretion by B cells. B cells ( $2.5 \times 10^4$ /well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated CD4<sup>+</sup> T cells ( $1 \times 10^5$ /well) in the presence of 6 µl of membranes from Sf9 cells infected with either recombinant murine CD40 ligand or wild-type baculovirus. Either an anti-mouse CD40 ligand mAb (MR1, black), a control mAb (145-2C11, gray), or no mAb (white) was added at a concentration of 1 µg/ml where indicated. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.

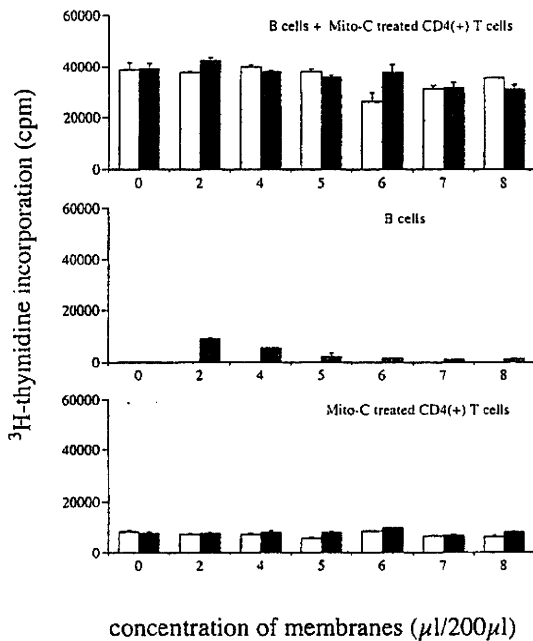
**FIGURE 3.** Supplemental CD40 ligand suppresses Ig secretion by B cells in a dose-dependent manner. B cells ( $2.5 \times 10^4$ /well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated CD4<sup>+</sup> T cells ( $2 \times 10^5$ /well) (A) or with SAC + IL-2 (B) in the presence of various concentrations of membranes from Sf9 cells infected with recombinant human CD40 ligand (squares) or wild-type (circles) baculovirus. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data from three experiments are shown, expressed as mean  $\pm$  SEM.



*The suppressive effect of CD40 ligand on Ig production by B cells requires its presence between 48 to 96 h of culture with B cells*

The next experiments were designed to determine the stage at which CD40 ligand exerted its suppressive effects on Ig production

by B cells cultured with anti-CD3-stimulated mitomycin C-treated CD4<sup>+</sup> T cells. The results shown in Figure 3A determined the concentration of recombinant CD40 ligand that induced maximal suppression of Ig production, and this amount of CD40 ligand was added at different times after initiation of cultures of B cells with

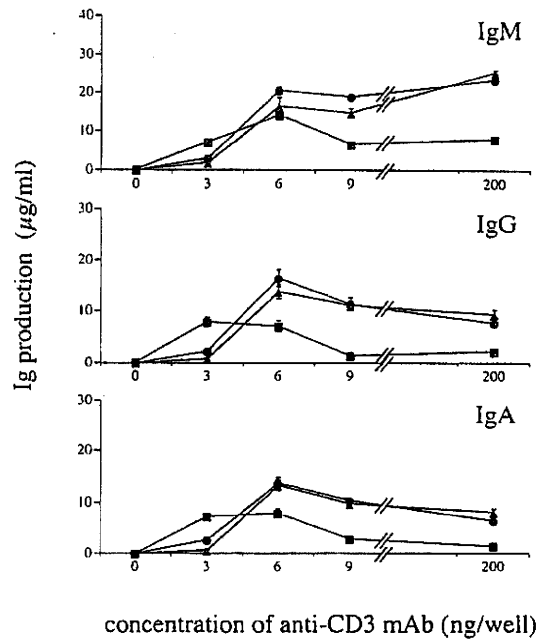


**FIGURE 5.** The effect of supplemental CD40 ligand on DNA synthesis of B cells cultured with anti-CD3 activated CD4<sup>+</sup>T cells. B cells ( $2.5 \times 10^6$ /well) were cultured alone or with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated CD4<sup>+</sup> T cells ( $1 \times 10^5$ /well). Various concentrations of membranes from Sf9 cells infected with recombinant human CD40 ligand (solid) or wild-type (open) baculovirus were added. [<sup>3</sup>H]thymidine incorporation was assessed after a 96-h incubation. Data are expressed as mean  $\pm$  SEM.

anti-CD3-stimulated mitomycin C-treated CD4<sup>+</sup> T cells. As seen in Figure 7, Ig production was strongly suppressed when recombinant CD40 ligand was added at day 0, 2, and 4. In contrast, when CD40 ligand was added at day 6 or later, no effect on Ig production was noted.

To confirm that CD40 ligand suppressed B cell responses after initial activation, B cells were separated from anti-CD3-stimulated mitomycin C-treated CD4<sup>+</sup> T cells after either 48 h or 96 h of stimulation, and recultured with IL-2 in the presence or absence of recombinant CD40 ligand. As shown in Figure 8, recombinant CD40 ligand clearly suppressed Ig production by B cells that had been activated by coculture with T cells for 48 h. Of note, when B cells had been activated for 96 h, they became resistant to the suppressive effects of CD40 ligand and again became responsive to the positive effects of CD40 ligation with enhancement of Ig production observed. These results are consistent with the conclusion that the effect of CD40 ligation varies profoundly based on the length of stimulation of the B cell.

To elucidate the suppressive role of CD40 ligation, purified B cells were activated with SAC and IL-2 in the absence of T cells. Activated B cells were harvested after 48 h and recultured with IL-2. CD40 ligand was added to either the initial cultures or the subsequent cultures of activated B cells or both. As shown in Table I, the addition of CD40 ligand to the first culture enhanced production of IgM, IgG, and IgA. In contrast, when CD40 ligand was added to the subsequent culture of activated B cells, only production of IgM was enhanced. Finally, when CD40 ligand was present in both the initial and subsequent cultures, suppression of the production of all Ig isotypes was noted compared with that found in cultures containing CD40 ligand in the initial culture only. In all circumstances, the degree of suppression was greater when larger

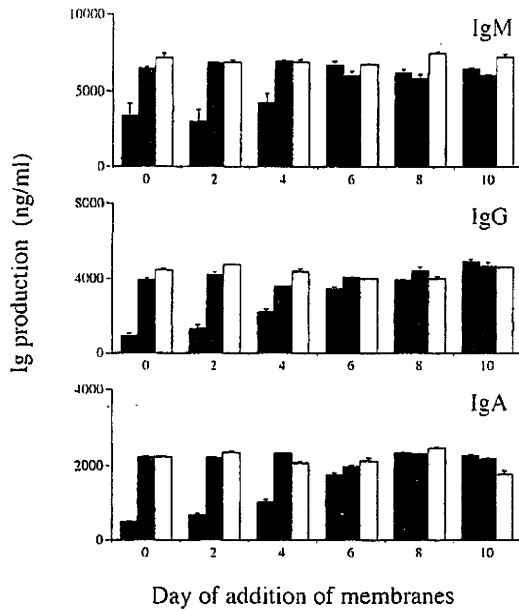


**FIGURE 6.** The impact of recombinant CD40 ligand on Ig secretion by B cells depends on the density of CD40 ligand expressed by activated T cells. B cells ( $2.5 \times 10^6$ /well) were cultured with mitomycin C-treated CD4<sup>+</sup> T cells ( $7.5 \times 10^4$ /well) stimulated with various concentrations of anti-CD3. Membranes (2  $\mu$ l) of Sf9 cells infected with either recombinant human CD40 ligand (squares), wild-type (circles) baculovirus, or no membranes (triangles) were added at the initiation of the cultures. The supernatants were harvested after 10 days of culture, and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.

amounts of CD40 ligand were added to the subsequent culture (data not shown). Finally, addition of CD40 ligand at the initiation of culture or 48 h later substantially increased the number of metabolically active cells found after a 10-day incubation (Table I). These results are consistent with the conclusion that CD40 ligand suppressed Ig production, but not growth of activated B cells, and that inhibition of Ig production by later exposure to CD40 ligand was enhanced when the B cells had been initially activated in the presence of CD40 ligand. Moreover, production of IgM appeared to be regulated differently from production of IgG and IgA.

*CD40 ligand exerts a greater suppressive effect on Ig production by IgD<sup>-</sup> memory B cells*

The next experiments were conducted to investigate the differential effects of CD40 ligation on IgM vs IgG and IgA production. In cultures of human B cells with various polyclonal B cell activators, IgM production largely derives from IgM<sup>+</sup>IgD<sup>+</sup> naive B cells, whereas IgG and IgA is produced by IgD<sup>-</sup> postswitch memory B cells (32). Therefore, the possibility that CD40 ligand exerted a differential regulatory effect on naive vs memory B cells was examined. Unfractionated B cells, IgD<sup>+</sup> naive B cells or IgD<sup>-</sup> memory B cells were stimulated with SAC and IL-2 in the presence or absence of recombinant CD40 ligand. As seen in Figure 9, recombinant CD40 ligand enhanced IgM production by SAC + IL-2-stimulated IgD<sup>+</sup> naive B cells and induced small amounts of IgG production in some experiments (0 ng/ml vs 50 ng/ml in cultures containing wild-type and CD40 ligand membranes, respectively).



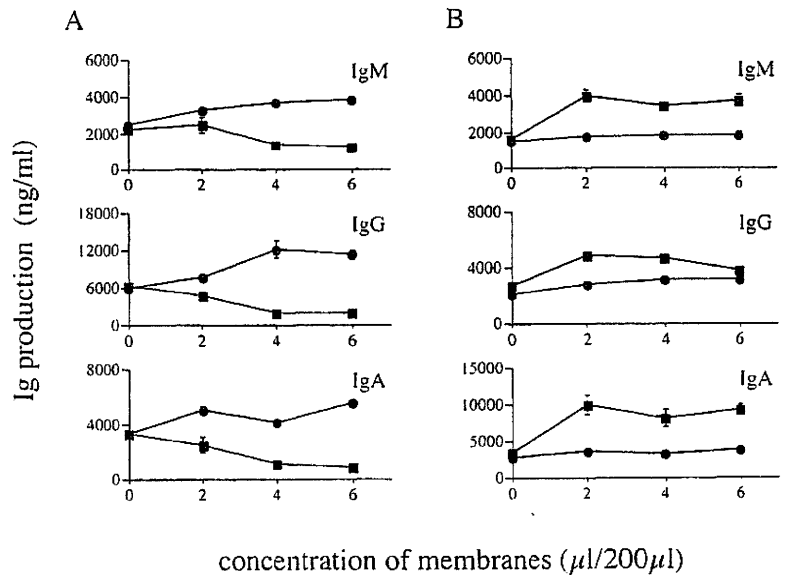
**FIGURE 7.** Kinetics of the suppression of B cell responses by CD40 ligand. B cells ( $2.5 \times 10^4$ /well) were cultured with anti-CD3 (200 ng/well)-stimulated mitomycin C-treated CD4<sup>+</sup> T cells ( $1 \times 10^5$ /well). Membranes (6  $\mu$ l) of Sf9 cells infected with either recombinant human CD40 ligand (black) or wild-type baculovirus (gray) or no membranes (white) were added at the time of stimulation or afterward. The supernatants were harvested after 10 days of culture and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.

**Table 1.** Differential regulation of Ig secretion by early and later addition of CD40 ligand

Addition to First Culture	Addition to Second Culture		
	Nil	40L	WT
		IgM (ng/ml)	
Nil	1280 $\pm$ 34	5960 $\pm$ 116	1800 $\pm$ 70
40L	4010 $\pm$ 47	2040 $\pm$ 46	6290 $\pm$ 910
WT	2210 $\pm$ 15	4790 $\pm$ 371	2210 $\pm$ 140
		IgG (ng/ml)	
Nil	1240 $\pm$ 22	1740 $\pm$ 163	2010 $\pm$ 78
40L	5830 $\pm$ 430	1210 $\pm$ 55	9880 $\pm$ 744
WT	1640 $\pm$ 106	1130 $\pm$ 169	1530 $\pm$ 134
		IgA (ng/ml)	
Nil	453 $\pm$ 16	942 $\pm$ 34	742 $\pm$ 26
40L	3710 $\pm$ 185	1030 $\pm$ 85	6580 $\pm$ 725
WT	509 $\pm$ 29	800 $\pm$ 53	488 $\pm$ 7.0
		MTT (OD $\times 10^{-3}$ )	
Nil	122 $\pm$ 10.7	240 $\pm$ 6.0	99.7 $\pm$ 2.6
40L	160 $\pm$ 4.5	194 $\pm$ 3.3	158 $\pm$ 6.2
WT	93.5 $\pm$ 0.5	207 $\pm$ 0.5	101 $\pm$ 4.6

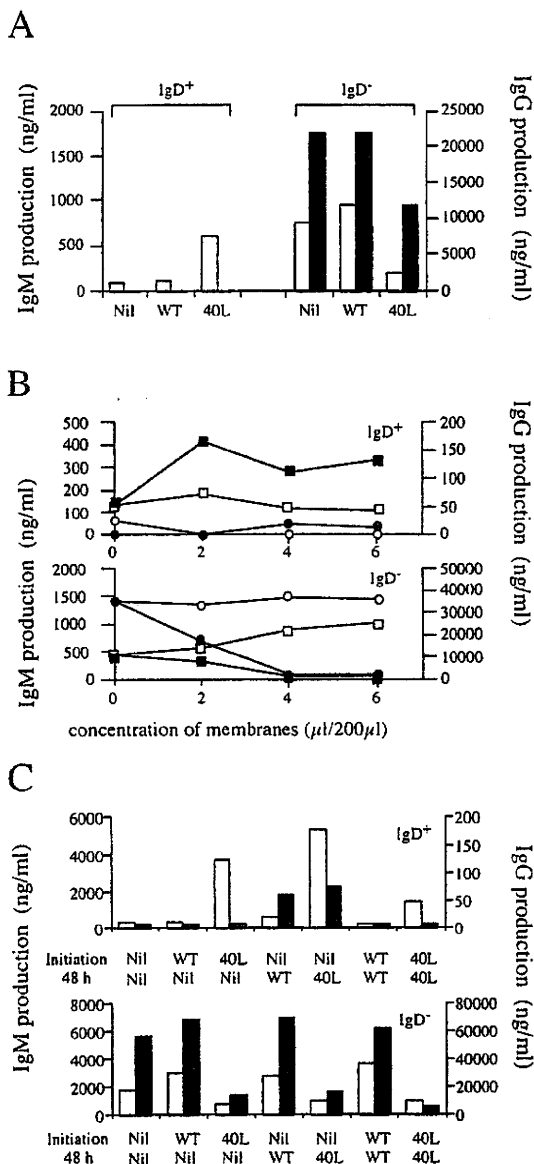
<sup>a</sup> B cells ( $2 \times 10^5$ /well) were cultured for 48 h with SAC (1:60,000) and IL-2 (20 U/ml) in the presence of membranes (6  $\mu$ l) of Sf9 cells infected with either recombinant human CD40 ligand (40L) or wild type (WT) baculovirus. Afterward, cells were harvested from the wells and cultured ( $5 \times 10^4$ /well) with IL-2 (20 U/ml) in the presence of either membrane (4  $\mu$ l). The supernatants were harvested after a total culture of 10 days and Ig content was analyzed by isotype-specific ELISA. The number of metabolically active cells was assessed by MTT assay after a total length of culture of 10 days. OD at the initiation of the second culture was 102, 110, and 91  $\times 10^3$ , for Nil, 40L, and WT in the first culture, respectively. Data presented are representative of at least three independent experiments with similar results.

**FIGURE 8.** Sensitivity of B cells to suppression by CD40 ligand is dependent on the stage of B cell activation. B cells ( $5 \times 10^4$ /well) were cultured with anti-CD3 (200 ng/ml)-stimulated mitomycin C-treated CD4<sup>+</sup> T cells ( $1 \times 10^5$ /well) for either 48 h (A) or 96 h (B). Afterward, cells were harvested from the wells, and B cells were separated from T cells by rosetting with neuraminidase-treated SRBC. The activated B cells ( $5 \times 10^4$ /well) were cultured in the presence of IL-2 (20 U/ml). Various concentrations of membranes of Sf9 cells infected with either recombinant human CD40 ligand (squares) or wild-type baculovirus (circles) were added where indicated. The supernatants were harvested after a total culture of 10 days, and Ig content was analyzed by isotype-specific ELISA. Data presented are representative of at least three independent experiments with similar results.



By contrast, the same concentration of CD40 ligand suppressed both IgM and IgG production by IgD<sup>-</sup> memory B cells. With all of the concentrations of CD40 ligand tested, Ig production by IgD<sup>-</sup> memory B cells was suppressed (Fig. 9B). Only when extremely low concentrations of CD40 ligand were employed, below the concentration capable of stimulating IgD<sup>+</sup> naive B cells, was a positive response from IgD<sup>-</sup> memory B cells observed (data not shown). Finally, as shown in Figure 9C, increased production of IgM was noted when IgD<sup>+</sup> naive B cells were stimulated with CD40 li-

gand at the initiation of culture or 48 h later. Only when IgD<sup>+</sup> naive B cells were stimulated with CD40 ligand at both the initiation and at 48 h was inhibition noted. In contrast, CD40 ligand suppressed responses of IgD<sup>-</sup> memory B cells when added either at the initiation of culture, at 48 h, or at both times. These results are consistent with the conclusion that IgD<sup>+</sup> naive and IgD<sup>-</sup> memory B cells are markedly different in their responses to CD40 ligation, with the former poised toward stimulation and the latter toward suppression by this interaction.



**FIGURE 9.** Difference of the effect of CD40 ligand on Ig secretion by IgD<sup>+</sup> or IgD<sup>-</sup> B cell subpopulations. *A*, IgD<sup>+</sup> (left bars) or IgD<sup>-</sup> (right bars) B cells ( $5 \times 10^4$ /well) were cultured with SAC (1:60,000) and IL-2 (20 U/ml) in the absence or presence of membranes (2 μl) of Sf9 cells infected with either recombinant human CD40 ligand or wild-type baculovirus added at the initiation of culture. After a 10-day culture, IgM production (open bars) and IgG production (closed bars) were assessed. *B*, IgD<sup>+</sup> (upper panel) or IgD<sup>-</sup> (lower panel) B cells ( $5 \times 10^4$ /well) were cultured with SAC and IL-2 in the absence or presence of various concentrations of membranes of Sf9 cells infected with either recombinant human CD40 ligand (closed symbols) or wild-type baculovirus (open symbols) added at the initiation of culture. After a 10-day incubation, IgM (squares) and IgG (circles) were assessed. *C*, IgD<sup>+</sup> (upper panel) or IgD<sup>-</sup> (lower panel) B cells were cultured with SAC and IL-2 alone or in the presence of 2 μl of membranes of Sf9 cells infected with either recombinant human CD40 ligand or wild-type baculovirus added at the initiation of culture or 48 h later, as indicated. After a 10-day incubation, IgM (open bars) and IgG (closed bars) secretion was assessed.

It has previously been suggested that CD40 ligation favors the development of CD38<sup>-</sup> memory cells from germinal center B cells and restricts the development of CD38<sup>+</sup> plasma cells (11). Since

peripheral blood IgD<sup>+</sup> naive and IgD<sup>-</sup> memory B cells represent pre- and post-germinal center cells, respectively, it was of interest to determine the impact of CD40 ligation on CD38 expression by these cells. As shown in Figure 10, SAC + IL-2 increased expression of CD38 on B cells. This effect was blocked by CD40 ligand. Even when CD38 expression was up-regulated by stimulation with SAC + IL-2 in the absence of CD40 ligand, late addition of CD40 ligand caused rapid down-modulation. When B cells were separated into IgD<sup>+</sup> or IgD<sup>-</sup> subpopulations, CD38<sup>+</sup> cells were found in both the IgD<sup>+</sup> and IgD<sup>-</sup> B cell populations. Although SAC + IL-2 induced CD38 expression by both B cell populations, CD38<sup>bright</sup> cells were only found in the activated IgD<sup>-</sup> memory B cell population (Fig. 11). Whereas CD40 ligand down-regulated CD38 expression by activated IgD<sup>-</sup> memory B cells, it enhanced expression by activated IgD<sup>+</sup> naive B cells.

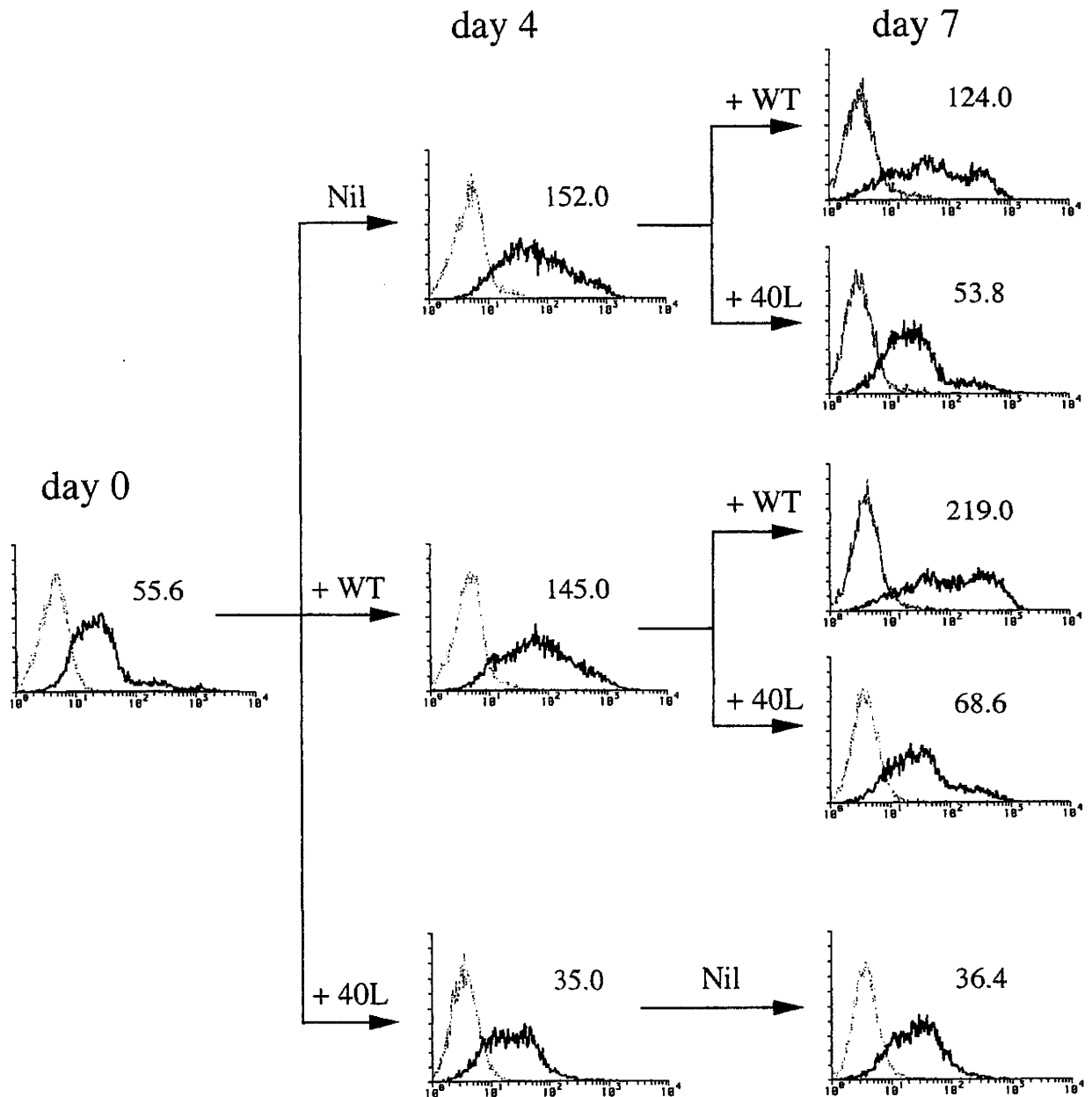
*Activated B cells down-regulate CD40 ligand expression by CD4<sup>+</sup> T cells more effectively*

The final experiments examined the capacity of activated B cells to influence CD40 ligand expression by anti-CD3-stimulated CD4<sup>+</sup> T cells. CD40 ligand expression by CD4<sup>+</sup> T cells was assessed in cultures supplemented with either resting B cells or B cells that previously had been activated with anti-CD3-stimulated CD4<sup>+</sup> T cells. As seen in Figure 12, CD40 ligand expression by activated CD4<sup>+</sup> T cells was down-regulated by coculture with B cells and much more markedly down-regulated by coculture with activated B cells. However, when the activated B cells were removed from culture, the CD4<sup>+</sup> T cells rapidly re-expressed CD40 ligand. On the other hand, when CD4<sup>+</sup> T cells were first activated to express CD40 ligand, coculture with activated but not resting B cells rapidly down-modulated CD40 ligand expression.

**Discussion**

Engagement of B cell CD40 by CD40 ligand expressed by activated T cells plays an essential role in the activation, proliferation, and differentiation of B cells during T cell-B cell collaboration (1-11). Initial studies focused on the stimulatory effects of this interaction on B cell function. However, more recent results have indicated a more complex role of CD40 engagement on B cell function, with the possibility that signals provided through CD40 might inhibit B cell growth and Ab formation (12-14, 16) or induce apoptosis of B cells (16) at specific stages of maturation and/or differentiation. The current studies, therefore, examined the positive and negative influences of ligation of CD40 on the function of human peripheral B cells. As peripheral blood B cells contain both IgM<sup>+</sup>IgD<sup>+</sup> naive B cells that have not yet been expanded in germinal centers and IgD<sup>-</sup> memory B cells that have previously been stimulated by Ag (32), the use of this population permitted an analysis of the differential sensitivity of these subsets to signals conveyed by CD40 ligation. To ensure that CD40 engagement was provided in a physiologically relevant manner, recombinant membrane-bound human CD40 ligand expressed by membranes of baculovirus-infected Sf9 cells was employed. It was anticipated that this form of CD40 ligand would mimic engagement of CD40 accomplished by activated T cells most closely (20, 21), as the avidity and three-dimensional organization of the interaction should be comparable and effects mediated by Fc receptor binding of mAb and hypercross-linking by multimeric constructs would be avoided. The physiologic relevance of this approach was emphasized by the observation that both the positive and negative effects of CD40 ligation were detected in cultures of intact anti-CD3 activated CD4<sup>+</sup> T cells and B cells.





**FIGURE 10.** CD38 expression is decreased by engagement of CD40. B cells ( $5 \times 10^4$ /well) were immediately stained for CD38 expression or were stimulated with SAC (1:60,000) and IL-2 (20 U/ml) in the presence or absence of membranes ( $2 \mu\text{l}$ ) of Sfi9 cells infected with either recombinant human CD40 ligand or wild-type baculovirus. In some experiments, membranes ( $2 \mu\text{l}$ ) were again added to the culture wells on day 4. Cells were harvested after culture for various lengths of time as indicated and stained with FITC-conjugated anti-CD38 mAb or FITC-conjugated control mAb, and CD38 expression was analyzed by flow cytometry. Numbers in each histogram indicate the  $\Delta\text{MFI}$  for staining with anti-CD38 mAb calculated by subtracting the MFI of staining with the control mAb.

The temporally limited expression of CD40 ligand by activated T cells suggests that prolonged engagement of B cell CD40 might negatively influence B cell responses. Previous studies had indicated that engagement of CD40 with mAb or a soluble trimeric CD40 ligand construct could inhibit specific Ab responses by tonsillar memory B cells (11, 17, 18). Inhibition appeared to require simultaneous engagement of surface Ig and limited specific Ab production, but not proliferation or expansion of tonsillar B cells with a memory cell phenotype ( $\text{CD}38^- \text{IgD}^-$ ). As specific Ab pro-

duction originated from memory B cells only (17, 18), effects of CD40 ligation on naive B cells could not be estimated. Moreover, since specific Ag or engagement of surface Ig was required to elicit Ab production (17, 18), the effect of CD40 ligation alone could not be estimated. Finally, differential effects of CD40 ligation dependent on the stage of B cell activation were not assessed. This is of particular relevance because it has been shown that both Ag and CD40 ligation are necessary for secondary Ab responses of peripheral blood B cells from normal volunteers immunized with the