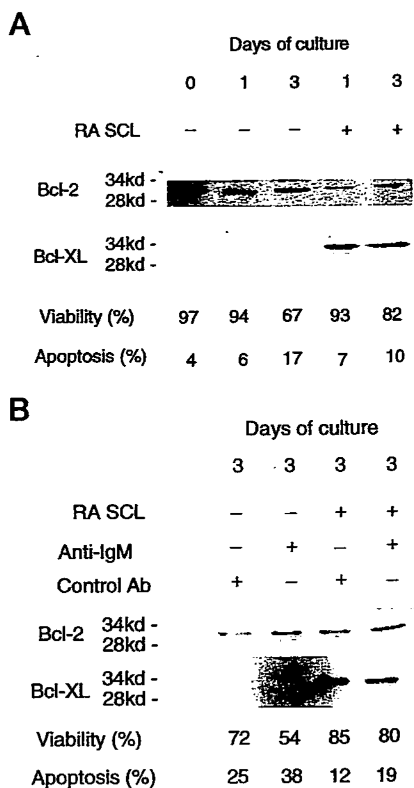

FIGURE 3. The RA SCL induce Bcl-x_L expression by B cells. *A*, After B cells (1×10^6) were cultured with RA SCL (Sy77; 4×10^4) for 1 or 3 days, expression of Bcl-2 and Bcl-x_L proteins (40 μg of each sample) was examined with Western blotting and compared with that by fresh B cells. Percentages of viable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

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

Results

The SCL from RA synovium can rescue B cells from apoptosis

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

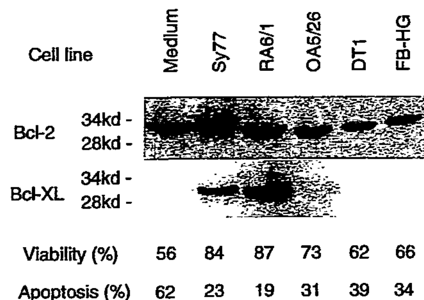

FIGURE 4. The RA SCL rescue B cell from apoptosis more effectively than other cell lines. After B cells (1×10^6) were cultured with various cell lines (4×10^4) for 3 days, expression of Bcl-2 and Bcl-x_L proteins (40 μg of each sample) was examined with Western blotting. Percentages of viable and apoptotic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

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

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

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

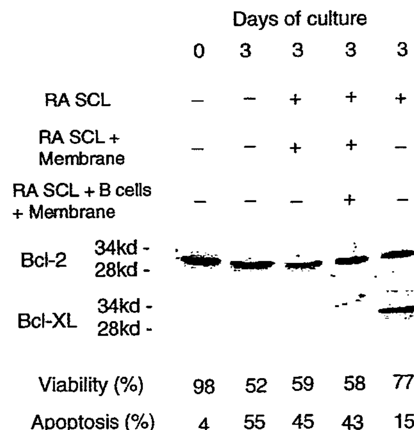

FIGURE 5. Cell contact is required to induce Bcl-x_L and rescue B cells from apoptosis. B cells (1×10^6) and SCL (4×10^4) were cultured together or separated by a 0.4-μm pore size membrane. Where indicated B cells (1×10^6) were added to the bottom chamber with SCL to examine the influence of soluble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

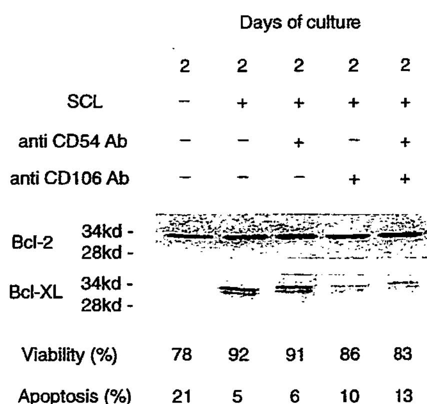

FIGURE 6. Roles of CD54 (ICAM-1) and CD106 (VCAM-1) in SCL-mediated up-regulation of Bcl-x_L and inhibition of B cell apoptosis. B cells (1×10^6) were cultured with or without SCL (4×10^4) for 2 days in the presence or the absence of mAbs to CD54 and/or CD106. Afterward, B cells were analyzed for Bcl-2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

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

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

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

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

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

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

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

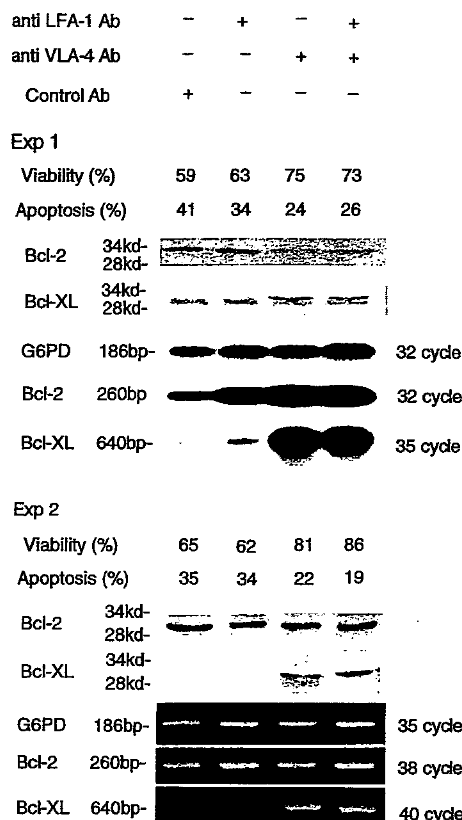

FIGURE 7. Stimulation by VLA-4 can induce Bcl-x_L protein and mRNA in B cells. B cells (1×10^6) were cultured for 3 days with mAbs to CD11a/CD18 and/or CD29/CD49d in 24-well culture plates coated with rabbit anti-mouse IgG. Afterward, the expression of Bcl-2 and Bcl-x_L protein was assessed by Western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

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

Discussion

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

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

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

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

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

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

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

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

Acknowledgments

We thank Dr. Amrie C. Grammer for her careful review of the manuscript.

References

1. Harris, E. D. 1990. Rheumatoid arthritis: pathology and implications for therapy. *N. Engl. J. Med. 322:1277.*
2. McInnes, I. B., J. Al-Mughales, M. Field, B. P. Leung, F. P. Huang, R. Dixon, R. D. Sturrock, P. C. Wilkinson, and F. Y. Liew. 1996. The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. *Nat. Med. 2:175.*
3. Iannone, F., V. M. Corrigall, G. H. Kingsley, and G. S. Panayi. 1994. Evidence for the continuous recruitment and activation of T cells into the joints of patients with rheumatoid arthritis. *Eur. J. Immunol. 24:2706.*
4. Qu, Z., C. H. Garcia, L. M. O'Rourke, S. R. Planck, M. Kohli, and J. T. Rosenbaum. 1994. Local proliferation of fibroblast-like synoviocytes contributes to synovial hyperplasia. *Arthritis Rheum. 37:212.*
5. Firestein, G. S., M. Yeo, and N. J. Zvaifler. 1995. Apoptosis in rheumatoid arthritis synovium. *J. Clin. Invest. 96:1631.*
6. Gomez, J., A. Gonzalez, C. Martinez-A, and A. Rebollo. 1998. IL-2-induced cellular events. *Crit. Rev. Immunol. 18:185.*
7. Lichtenstein, A., Y. Tu, C. Fady, R. Vescio, and J. Berenson. 1995. Interleukin-6 inhibits apoptosis of malignant plasma cells. *Cell. Immunol. 162:248.*
8. Schwarze, M. M., and R. G. Hawley. 1995. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-x_L. *Cancer Res. 55:2262.*
9. Armant, M., G. Delespesse, and M. Sarfati. 1995. IL-2 and IL-7 but not IL-12 protect natural killer cells from death by apoptosis and up-regulate bcl-2 expression. *Immunology 85:331.*
10. Vella, A. T., S. Dow, T. A. Potter, J. Kappler, and P. Marrack. 1998. Cytokine-induced survival of activated T cells in vitro and in vivo. *Proc. Natl. Acad. Sci. USA 95:3810.*

11. Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. 1991 Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature 352:345.*
12. Teague, T. K., P. Marrack, J. W. Kappler, and A. T. Vella. 1997. IL-6 rescues resting mouse T cells from apoptosis. *J. Immunol. 158:5791.*
13. Cohen, S. B., J. B. Crawley, M. C. Kahan, M. Feldmann, and B. M. Foxwell. 1997. Interleukin-10 rescues T cells from apoptotic cell death: association with an upregulation of Bcl-2. *Immunology 92:1.*
14. Poe, J. C., D. H. Wagner, Jr., R. W. Miller, R. D. Stout, and J. Suttles. 1997. IL-4 and IL-10 modulation of CD40-mediated signaling of monocyte IL-1β synthesis and rescue from apoptosis. *J. Immunol. 159:846.*
15. Meazza, R., S. Basso, A. Gaggero, D. Detotero, L. Trentin, R. Pereno, B. Azzarone, and S. Ferrini. 1998. Interleukin (IL)-15 induces survival and proliferation of the growth factor-dependent acute myeloid leukemia M-07e through the IL-2 receptor β/γ. *Int. J. Cancer 78:189.*
16. Dooms, H., M. Desmedt, S. Vancaeneghem, P. Rottiers, V. Goossens, W. Fiers, and J. Grooten. 1998. Quiescence-inducing and antiapoptotic activities of IL-15 enhance secondary $CD4^+$ T cell responsiveness to antigen. *J. Immunol. 161: 2141.*
17. Adachi, S., M. Kubota, Y. W. Lin, A. Okuda, K. Matsubara, Y. Wakazono, H. Hirota, K. Kuwakado, and Y. Akiyama. 1994. In vivo administration of granulocyte colony-stimulating factor promotes neutrophil survival in vitro. *Eur. J. Haematol. 53:129.*
18. Suda, T., H. Hashimoto, M. Tanaka, T. Ochi, and S. Nagata. 1997. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. *J. Exp. Med. 186: 2045.*
19. Hashimoto, H., M. Tanaka M, T. Suda, T. Tomita, K. Hayashida, E. Takeuchi, M. Kaneko, H. Takano, S. Nagata, and T. Ochi. 1998. Soluble Fas ligand in the joints of patients with rheumatoid arthritis and osteoarthritis. *Arthritis Rheum. 41:657.*
20. Koopman, G., R. M. J. Keehnen, E. Lindhout, W. Newman, Y. Shimizu, G. A. van Seventer, C. de Groot, and S. T. Pals. 1994. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. *J. Immunol. 152: 3760.*
21. Pezzano, M., Y. Li, D. Philip, C. Omene, M. Cantey, G. Saunders, and J. C. Guyden. 1995. Thymic nurse cell rescue of early $CD4^+$ $CD8^+$ thymocytes from apoptosis. *Cell. Mol. Biol. 41:1099.*
22. Shimaoka, Y., J. F. Attrep, T. Hirano, K. Ishihara, R. Suzuki, T. Toyosaki, T. Ochi, and P. E. Lipsky. 1998. Nurse-like cells from bone marrow and synovium of patients with rheumatoid arthritis promote survival and enhance function of human B cells. *J. Clin. Invest. 102:606.*
23. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S.R. Kaplan, M.H. Liang, H.S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum. 31:315.*
24. Nicoletti, I., G. Migliorati, M. C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods 139:271.*
25. Adams, J. M., and S. Cory. 1998. Apoptosis: the Bcl-2 protein family: arbiters of cell survival. *Science 281:1322.*
26. Salmon, M., D. Scheel-Toellner, A. P. Huissoon, D. Pilling, N. Shamsadeen, H. Hyde, A. D. D'Angeac, P. A. Bacon, P. Emery, and N. Akbar. 1997. Inhibition of T cell apoptosis in rheumatoid synovium. *J. Clin. Invest. 99:439.*
27. Tuscano, J. M., K. M. Druey, A. Riva, J. Pena, C. B. Thompson, and J. H. Kehrl. 1996. Bcl-x rather than Bcl-2 mediates CD40-dependent centrocyte survival in the germinal center. *Blood 88:1359.*
28. Zhang, X., L. Li, J. Choe, S. Krajewski, J. C. Reed, C. Thompson, and Y. S. Choi. 1996. Up-regulation of Bcl-XL expression protects CD40-activated human B cells from Fas-mediated apoptosis. *Cell. Immunol. 173:149.*
29. Li, L., S. Krajewski, J. C. Reed, and Y. S. Choi. 1997. The apoptosis and proliferation of SAC-activated B cells by IL-10 are associated with changes in Bcl-2, Bcl-x_L, and Mcl-1 expression. *Cell. Immunol. 178:33.*
30. Krajewski, S., M. Krajewska, A. Shabaik, H. G. Wang, S. Irie, L. Fong, and J. C. Reed. 1994. Immunohistochemical analysis of in vivo patterns of Bcl-x expression. *Cancer Res. 54:5501.*
31. Lomo, J., H. K. Blomhoff, S. E. Jacobsen, S. Krajewski, J. C. Reed, and E. B. Smeland. 1997. Interleukin-13 in combination with CD40 ligand potently inhibits apoptosis in human B lymphocytes: upregulation of Bcl-x_L and Mcl-1. *Blood 89:4415.*
32. Choi, M. S. K., L. H. Boise, A. R. Gottschalk, J. Quintans, C. B. Thompson, and G. G. B. Klaus. 1995. The role of bcl-x_L in CD40-mediated rescue from anti-u-induced apoptosis in WEHI-231 B lymphoma cells. *Eur. J. Immunol. 25:1352.*
33. Bras, A., C. Martinez-A, and E. Baixeras. 1997. B cell receptor cross-linking prevents Fas-induces cell death by inactivating the IL-1β-converting enzyme protease and regulation Bcl-2/Bcl-x expression. *J. Immunol. 159:3168.*
34. Clark, E. A. K. H. Grabstein, A. M. Gown, M. Skelly, T. Kaisho, T. Hirano, and G. L. Shu. 1995. Activation of B lymphocyte maturation by a human follicular dendritic cell line, FDC-1. *J. Immunol. 155:545.*
35. Freedman, A. S., J. M. Munro, G. E. Rice, M. P. Bevilacqua, C. Morimoto, B. W. McIntyre, K. Rhynhart, J. S. Pober, and L. M. Nadler. 1990. Adhesion of human B cells to germinal centers in vitro involves VLA-4 and INCAM-110. *Science 249:1031.*
36. Koopman, G., H. K. Parmentier, H. J. Schuurman, W. Newman, C. J. L. M. Meijer, and S. T. Pals. 1991. Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular adhesion molecule 1 pathways. *J. Exp. Med. 173:1297.*
37. Takeuchi, E., T. Tomita, T. Toyosaki-Maeda, M. Kaneko, H. Takano, H. Hashimoto, K. Sugamoto, R. Suzuki, and T. Ochi. 1999. Establishment and characterization of nurse cell-like stromal cell lines from synovial tissues of patients with rheumatoid arthritis. *Arthritis Rheum. 42:221.*
38. Fujio, Y., K. Kunisada, H. Hirota, K. Yamauchi-Takahira, and T. Kishimoto. 1997. Signals through gp130 upregulate *bcl-x* gene expression via STAT1-binding *cis*-element in cardiac myocytes. *J. Clin. Invest. 99:2898.*
39. Quelle, F. W., J. Wang, J. Feng, D. Wang, J. L. Cleveland, J. N. Ihle, and G. P. Zambetti. 1998. Cytokine rescue of p53-dependent apoptosis and cell cycle arrest is mediated by distinct Jak kinase signaling pathways. *Gene Dev. 12:1099.*
40. Sato, T., K. Tachibana, Y. Nojima, N. D'Avirro, and C. Moromoto. 1995. Role of the VLA-4 molecule in T cell costimulation; Identification of the tyrosine phosphorylation pattern induced by the ligation of VLA-4. *J. Immunol. 155:2938.*
41. Maguire, J. E., K. M. Danahey, L. C. Burkly, and G. A. van Sevevter. 1995. T cell receptor- and $\beta1$ integrin-mediated signals synergize to induce tyrosine phosphorylation of focal adhesion kinase (pp125FAK) in human T cells. *J. Exp. Med. 182:2079.*
42. Chen, H. C., and J. L. Guan. 1994. Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA 91: 10148.*
43. Kennedy, S. G., A. J. Wagner, S. D. Conzen, J. Jordan, A. Bellacosa, P. N. Tsichlis, and N. Hay. 1997. The PI3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev. 11:701.*
44. Grillot, D. A. M., M. Gonzale-Gracia, D. Ekhterae, L. Duan, N. Inohara, S. Ohta, M. Seldin, and G. Nunez. 1997. Genomic organization, promoter region analysis, and chromosome localization of the mouse *bcl-X* gene. *J. Immunol. 158:4750.*
45. Dechanet, J., P. Merville, I. Durand, J. Banchereau, and P. Miossec. 1995. The ability of synoviocytes to support terminal differentiation of activated B cells may explain plasma cell accumulation in rheumatoid synovium. *J. Clin. Invest. 95: 456.*
46. Robinson, E., E. C. Keystone, T. J. Schall, N. Gillett, and E. N. Fish. 1995. Chemokine expression in rheumatoid arthritis (RA): evidence of RANTES and macrophage inflammatory protein (MIP)-1β production by synovial T cells. *Clin. Exp. Immunol. 101:398.*
47. Hosaka, S., T. Akahoshi, C. Wada, and H. Kondo. 1994. Expression of the chemokine superfamily in rheumatoid arthritis. *Clin. Exp. Immunol. 97:451.*
48. Rathanaswami, P., M. Hachicha, M. Sadick, T. J. Schall, and S. R. McColl. 1993. Expression of the cytokine RANTES in rheumatoid synovial fibroblasts: differential regulation of RANTES and interleukin-8 genes by inflammatory cytokines. *J. Biol. Chem. 268:5834.*

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 IFNγ, whereas the latter secreted G-CSF and more IL-6. Stimulation of both bone marrow and synovial RA-NLC induced expression of CD40 and class II MHC, but not CD154 (CD40L) or CD35. RA-NLC rescued peripheral B cells from spontaneous apoptosis and promoted survival of B cells for > 4 wk. B cell survival was blocked by antibodies to CD106 or CD157. RA-NLC also increased Ig production from B cells. After long-term culture (4–6 wk) with RA-NLC, but not alone or with fibroblasts, outgrowth of B cells was observed. All B cell lines derived from these cultures had been transformed by EBV, although the RA-NLC themselves were not infected with EBV. Precursor frequency analysis indicated that ~ 1 in 12,500 peripheral B cells could give rise to these EBV-transformed B cell lines upon coculture with RA-NLC. These results indicate that RA-NLC from bone marrow and synovium have the capacity to rescue B cells from spontaneous apoptosis, facilitate Ig production, and promote the outgrowth of EBV-transformed B lymphoblastoid cells. These findings suggest that RA-NLC may play a role in the local and systemic hyperreactivity of B cells characteristic of rheumatoid arthritis. (*J. Clin. Invest.* 1998. 102:606–618.) Key words: RA • B cells • apoptosis • stromal cells

Introduction

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

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

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

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Received for publication 19 February 1998 and accepted in revised form 28 May 1998.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/98/08/0595/13 $2.00
Volume 102, Number 3, August 1998, 606–618
http://www.jci.org

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

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

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

Methods

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

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

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

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

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

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

minants (class II MHC); a sheep anti–mouse IgG_1 FITC conjugate (The Binding Site, Birmingham, UK); a control FITC-conjugated mouse IgG_1 mAb (Caltag Laboratories); BEC7, a mouse IgG_1 mAb against human CD157; RF3, a mouse IgG_{2a} 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 IFNγ (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-1α, IL-1β, 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 μg/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 μg/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 (ΔMFI) 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 $MgCl_2$ by using 200 U Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) per 1 μg of total RNA. Each PCR reaction was performed with 50–100 ng of cDNA in a 100-μl vol containing Ultraspec water (Cinna-Biotecx), 50 mM KCl, 10 mM Tris-Cl (pH 9.0), 0.001% gelatin (wt/vol), 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 0.3 μM of each primer. A lower layer of dNTPs, $MgCl_2$, and primers was separated from an upper layer of Taq polymerase, cDNA, and 10× PCR buffer (KCl, Tris-Cl, and gelatin) by an AmpliWax 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× 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 ^{32}P-5′ end-labeled probe, specific for PCR products amplified with a given primer pair. Hybridization was conducted in 5× SSC, 20 mM Na_2HPO_4, 7% SDS, 1× Denhardt's solution with 100 μg/ml denatured salmon sperm DNA at 42°C. Blots were washed in 5× SSC–0.1% SDS at room temperature for 20 min and in 1× SSC–0.1% SDS at 42°C for 15 min and subjected to autoradiography.

Analysis of viable cells. B cells (2×10^5) 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±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, 8×10^5 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 (1×10^4) were analyzed by flow cytometry with a FACScan (Beckon Dickinson) using a 50% logarithmic contour graph with the intensity of propidium iodine 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 μl of the supernatant was removed without disturbing the pellet. 10 μl of a yellow tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.), and 100 μl of PBS were added to the re-

maining pellet and incubated at 37°C for 4 h, after which 100 μl 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 examined 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-

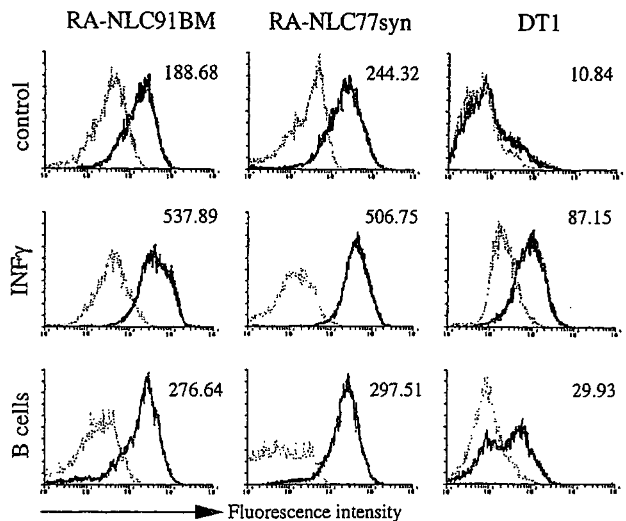

Figure 2. Expression of CD106 by RA-NLC and fibroblasts. Two RA-NLC cell lines and DT1 were cultured in medium alone or with IFNγ (100 U/ml) or with 2×10^6 peripheral B cells for 3 d. Numbers in each histogram indicate the ΔMFI for staining with anti-CD106 mAb calculated by subtracting the MFI of staining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

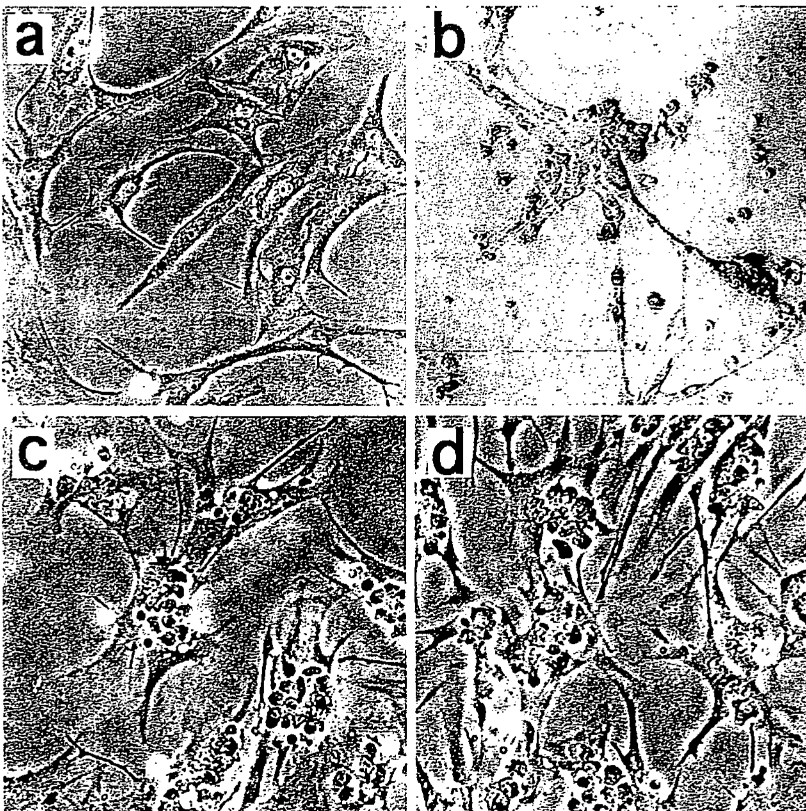

Figure 1. RA-NLC induce pseudoemperipolesis of B and T lymphoma cells and bind peripheral B cells. The morphologic appearance of RA-NLC34syn is shown by phase-contrast microscopy when cultured alone (*a*), with normal peripheral B cells for 4 d (*b*), or with the T cell lymphoma line, MOLT-17 (*c*),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

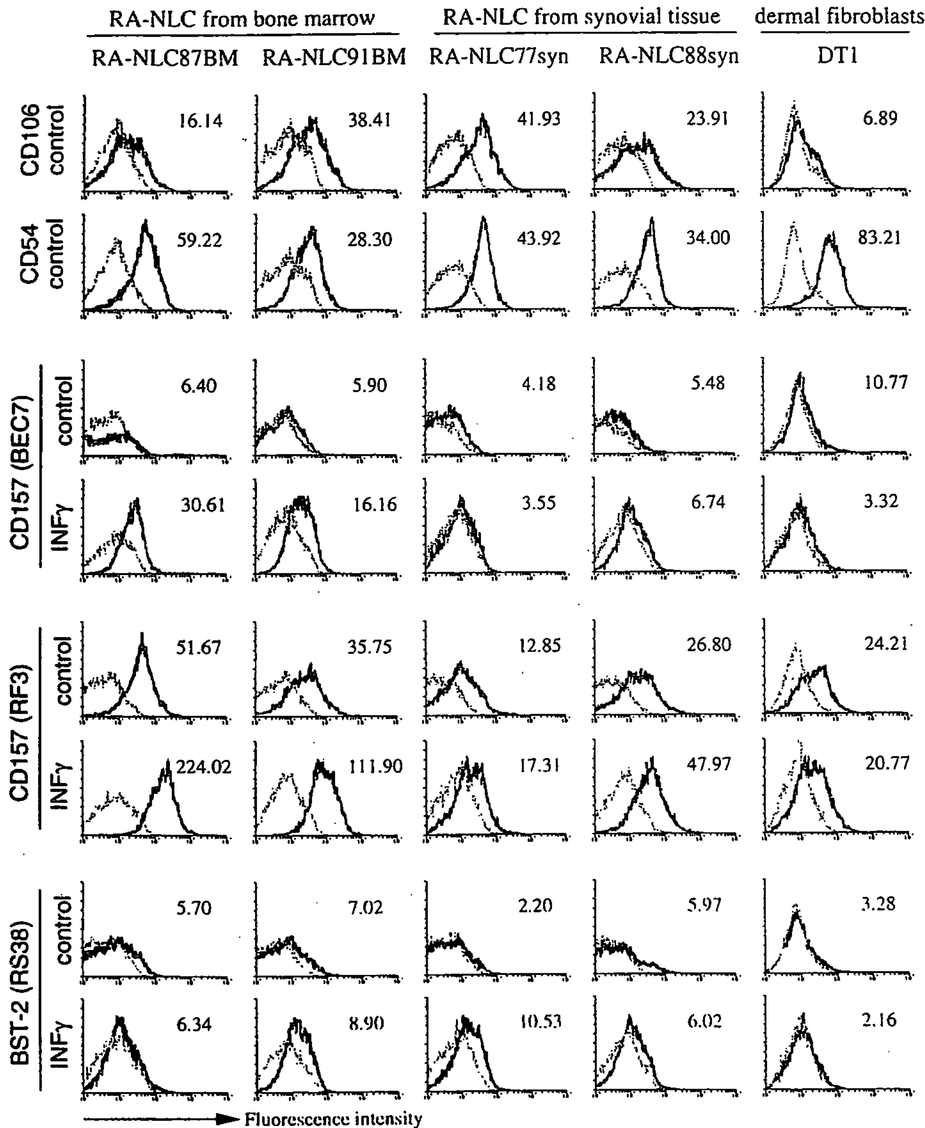

Figure 3. CD106, CD54, CD157, and BST-2 expression by RA-NLC cells and fibroblasts. Four RA-NLC cell lines or human dermal fibroblasts (DT1) were cultured in medium alone (control) or with IFNγ (100 U/ml) for 3 d, and were detached with 0.05% trypsin-EDTA in PBS at room temperature for 5 min. They were 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 ΔMFI 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. IFNγ (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 IFNγ. In contrast, expression of CD106 by human dermal fibroblasts was much less marked after stimulation with IFNγ 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: Bone marrow 87BM	91BM	Synovial tissue 77syn	88syn	45syn	Human skin fibroblasts (DT1)
CD29	Resting	+	+	+	+	+	+
CD35	Resting	–	–	–	–	–	–
	IFNγ	–	–	–	–	–	–
CD40	Resting	–	–	–	–	–	–
	IFNγ	+	+	+	+	+	+
CD40L	Resting	–	–	–	–	–	–
	IFNγ	–	–	–	–	–	–
CD49c	Resting	+	+	+	+	+	+
CD54	Resting	+	+	+	+	+	+
CD106	Resting	+	+	+	+	+	–/+
	IFNγ	+	+	+	+	+	+
HLA-A,B,C	Resting	+	+	+	+	+	+
HLA-DR	Resting	–	–	–	–	–	–
	IFNγ	+	+	+	+	+	+

RA-NLC or human skin fibroblasts (DT1) were cultured with medium alone (resting) or with IFNγ (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 IFNγ. This was much more marked with bone marrow–derived compared with synovium-derived RA-NCL. Using mAb BEC7, only RA-NLC87BM and RA-NLC91BM were found to express CD157 and then only after stimulation with IFNγ. Even after IFNγ stimulation expression of BEC7-identified CD157 was modest. BST-2 was not expressed by any of the cells constitutively, and IFNγ 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-1α	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-7	IL-8	G-CSF	GM-CSF	TNFα	TNFβ
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.

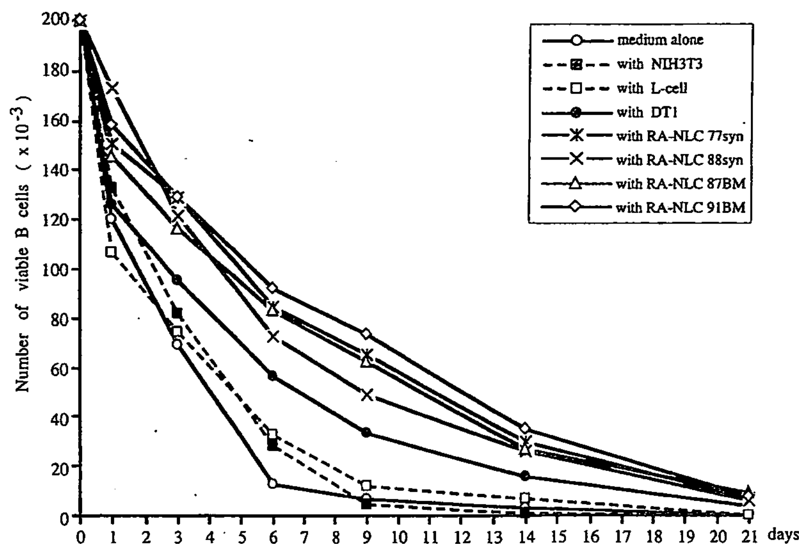

Figure 4. RA-NLC promote survival of peripheral B cells more effectively than fibroblasts. Peripheral B cells (2×10^5) were cultured in medium alone or with RA-NLC or mouse fibroblasts (NIH3T3, L cell) or human dermal fibroblasts (DT1) for 21 d, and the numbers of viable cells were counted at days 1, 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±2.0% (mean±SEM); DT1, 28.4%; medium alone, 6.2%, and at day 9, RA-NLC, 31.4±2.5%; DT1, 16.8%; medium alone 3.4%, and at day 14, RA-NLC, 14.9±1.0%; DT1, 7.8%; medium alone 1.4%, and at day 21, RA-NLC, 3.7±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 ~ 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±SEM [× 10³])		*P
B cells with medium alone	–	35.2±5.7	(17.6%)	< 0.0001 (vs. RA-NLC)
+ Soluble CD157	–	33.9±4.9	(16.9%)	< 0.0001 (vs. RA-NLC)
+ Control soluble CD38	–	31.6±4.2	(15.8%)	< 0.0001 (vs. RA-NLC)
B cells with RA-NLC cells	+	90.5±5.6*	(45.2%)	< 0.0001 (vs. + Soluble CD157)
+ Anti-CD106 mAb (1.G11B1)	+	36.4±5.5*	(18.2%)	< 0.0001
+ Control IgG1 mAb (MOPC)	+	78.8±6.0	(39.4%)	
+ Anti-CD157 mAb (RF3)	+	82.4±5.8	(41.2%)	
+ Control IgG2a mAb (P1.17)	+	81.8±4.9	(40.9%)	
+ Anti-CD157 polyclonal Ab	+	54.6±6.3*	(27.3%)	< 0.01
+ Control preimmune rabbit IgG	+	77.8±4.5	(38.9%)	
+ Soluble CD157	+	147.6±8.3*	(73.8%)	< 0.0001
+ Control soluble CD38	+	79.0±5.1	(39.5%)	

Peripheral B cells were cultured in 96-well flat-bottom culture plates (2×10^5/well, 200 μl) 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_1) 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 μg/ml, and antibodies were used at 4 μg/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±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.

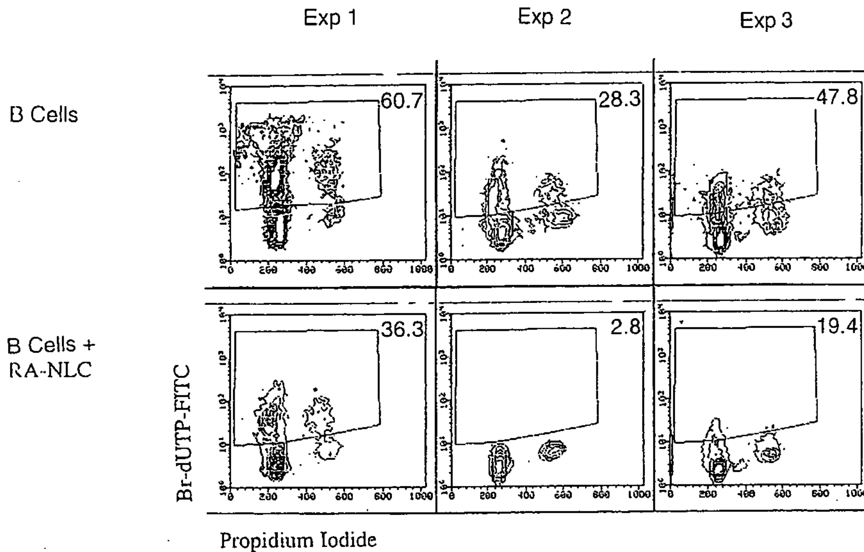

Figure 5. RA-NLC protect B cells from spontaneous apoptosis. Peripheral B cells were cultured in medium alone or with RA-NLC77syn for 3 d and assessed for the percentage of apoptotic cells by the TUNEL technique. The numbers in each box indicate the percentage of apoptotic cells.

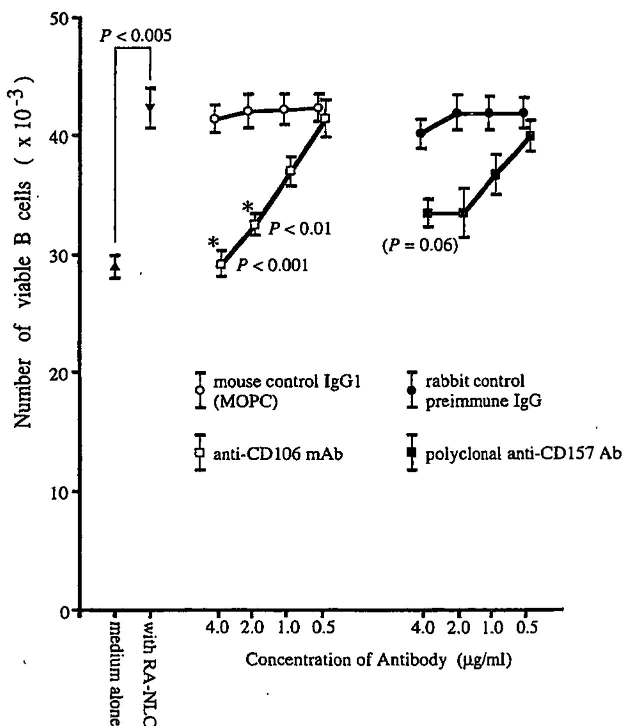

Figure 6. CD106 and CD157 are involved in the maintenance of B cell viability by RA-NLC. Peripheral B cells (2×10^5) were cultured in medium alone or with RA-NLC77syn and several concentrations of anti-CD106 mAb or anti-CD157 Ab or control Ab, and the number of viable B cells was counted at day 7 after 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±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

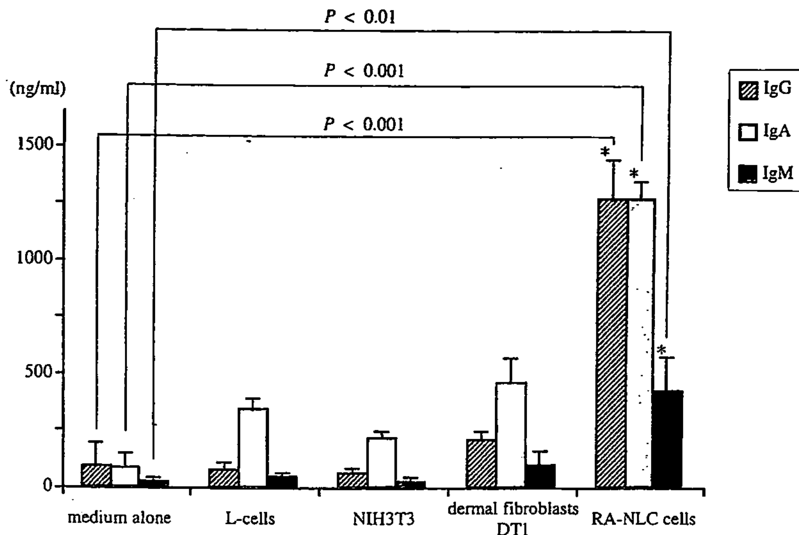

Figure 7. RA-NLC induce secretion of immunoglobulin from peripheral B cells. Peripheral B cells (5×10^4) were cultured in medium alone or with mouse fibroblasts (L-cells, NIH3T3 cells), human dermal fibroblasts (DT1), or with RA-NLC33BM with no other stimulation. The supernatants were harvested after 12 d of culture and Ig content was analyzed by isotype-specific ELISA. Data from three experiments are shown and expressed as mean±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. Sugamoto, 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. Toyasaki-Maeda, H. Oku, M. Kaneko, H. Takano, K. Sugamoto, K.

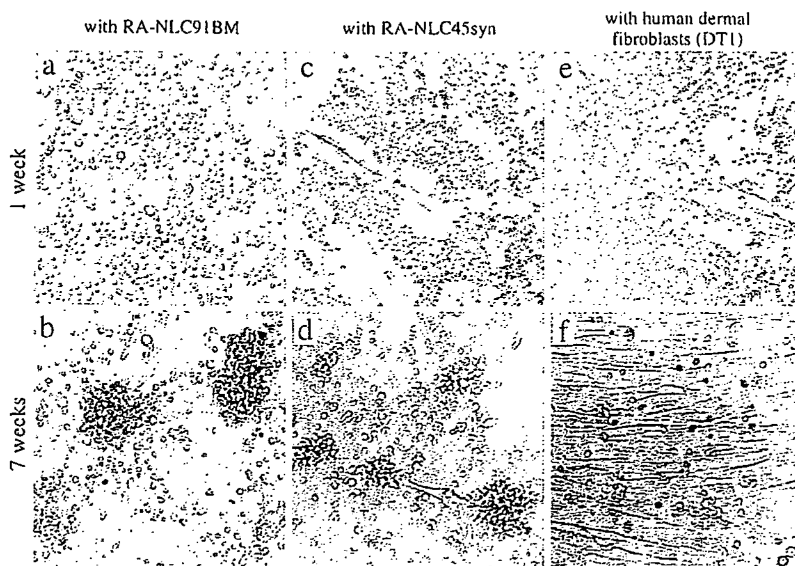

Figure 8. Outgrowth of B cells with RA-NLC. Long-term culture of normal peripheral B cells with RA-NLC (*a–d*) or human dermal fibroblasts (*e* and *f*) was performed. B cells were observed by phase-contrast microscopy after a culture of 1 (*a*, *c*, and *e*) and 7 wk (*b*, *d*, and *f*), respectively.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

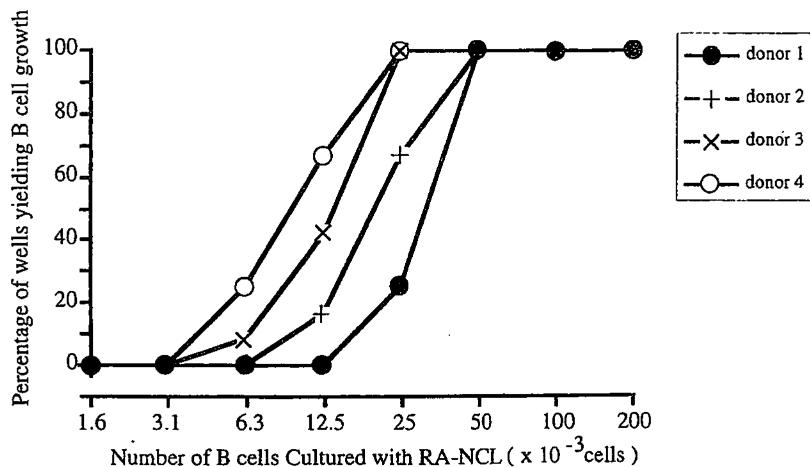

Figure 9. RA-NLC promote outgrowth of a small population of B cells. Several densities of peripheral B cells from four different donors were cultured with RA-NLC91BM for 4 wk in 96-well flat-bottomed plates. The vertical axis shows percentage of wells yielding B cell growth, and horizontal axis shows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

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-γ. 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 IFNγ. 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 outgrowth 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 emperiopolesis (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-α and IFNγ (12). In this study, IFNγ upregulated expression of both CD106 and CD157 by RA-NLC. If this cytokine, in conjunction with TNF-α 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).

Acknowledgments

The authors are grateful to Dr. Amrie C. Grammer for her careful review of the manuscript.

This research was supported by National Institutes of Health Specialized Center of Research grant AR-39169 and by the program for Promotion of Fundamental Studies in Health Science of the Organi-

zation for Drug ADR Relief, Research and Development Promotion and Product Review of Japan.

References

1. Wekerle, H., and U.P. Ketelsen. 1980. Thymic nurse cells-Ia-bearing epithelium involved in T-lymphocyte differentiation? *Nature*. 283:402–404.

2. Wekerle, H., U.P. Ketelsen, and M. Ernst. 1980. Thymic nurse cells. Lymphoepithelial cell complexes in murine thymuses: morphological and serological characterization. *J. Exp. Med.* 151:925–944.

3. Pezzano, M., Y. Li, D. Philp, C. Omene, M. Cantey, G. Saunders, and J.C. Guyden. 1995. Thymic nurse cell rescue of early CD4+CD8+ thymocytes from apoptosis. *Cell. Mol. Biol.* 41:1099–1111.

4. Pezzano, M., D. Philp, S. Stephenson, Y. Li, V. Reid, R. Maitta, and J.C. Guyden. 1996. Positive selection by thymic nurse cells requires IL-1 beta and is associated with an increased Bcl-2 expression. *Cell. Immunol.* 169:174–184.

5. Iwabuchi, K., K. Nakayama, R.L. McCoy, F. Wang, T. Nishimura, S. Habu, K.M. Murphy, and D.Y. Loh. 1992. Cellular and peptide requirements for in vitro clonal deletion of immature thymocytes. *Proc. Natl. Acad. Sci. USA*. 89:9000–9004.

6. Itoh, T., H. Doi, S. Chin, T. Nishimura, and S. Kasahara. 1988. Establishment of mouse thymic nurse cell clones from a spontaneous BALB/c thymic tumor. *Eur. J. Immunol.* 18:821–824.

7. Miyake, K., Y. Hasunuma, H. Yagita, and M. Kimoto. 1992. Requirement for VLA-4 and VLA-5 integrins in lymphoma cells binding to and migration beneath stromal cells in culture. *J. Cell Biol.* 119:653–662.

8. Hiai, H., Y. Nishi, T. Miyazawa, Y. Matsudaira, and Y. Nishizuka. 1981. Mouse lymphoid leukemias: symbiotic complexes of neoplastic lymphocytes and their microenvironments. *J. Natl. Cancer Inst.* 66:713–722.

9. Iwagami, S., S. Furue, T. Toyosaki, T. Horikawa, H. Doi, S. Satomi, T. Itoh, T. Sakata, and R. Suzuki. 1994. Establishment and characterization of nurse cell-like clones from human skin. Nurse cell-like clones can stimulate autologous mixed lymphocyte reaction. *J. Immunol.* 153:2927–2938.

10. Tomita, T., T. Toyosaki, S. Kawamura, H. Oku, R. Suzuki, H. Hashimoto, K. Hayashida, J. Hashimoto, and T. Ochi. 1996. Stromal cells established from bone marrow of RA patients could induce and maintain unusual myeloid cells ($CD14^{+}CD15^{+}$ cells) specific to severe RA patients. *Arthritis Rheum. Suppl.* 39:194.

11. Toyosaki, T., Y. Tsuruta, T. Yoshioka, T. Tomita, R. Suzuki, and T. Ochi. 1996. Recognition of synovial cell antigen by $CD4^{+}CD8^{-}$ T cell clones established from rheumatoid arthritis patients. *Arthritis Rheum. Suppl.* 39:266.

12. Edwards, J.C.W., R.D. Leigh, and G. Cambridge. 1997. Expression of molecules involved in B lymphocyte survival and differentiation by synovial fibroblasts. *Clin. Exp. Immunol.* 108:407–414.

13. Dechanet, J., P. Merville, I. Durand, J. Banchereau, and P. Miossec. 1995. The ability of synoviocytes to support terminal differentiation of activated B cells may explain plasma cell accumulation in rheumatoid synovium. *J. Clin. Invest.* 95:456–463.

14. Arnett, F.C., S.M. Edworthy, D.A. Bloch, D.J. McShane, J.F. Fries, N.S. Cooper, L.A. Healey, S.R. Kaplan, M.H. Liang, H.S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31:315–324.

15. Wysocki, L.J., and V.L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA*. 75:2844–2848.

16. Kaisho, T., J. Ishikawa, K. Oritani, J. Inazawa, H. Tomizawa, O. Muraoka, T. Ochi, and T. Hirano. 1994. BST-1, a surface molecule of bone marrow stromal cell lines that facilitates pre-B-cell growth. *Proc. Natl. Acad. Sci. USA*. 91:5325–5329.

17. Okuyama, Y., K. Ishihara, N. Kimura, Y. Hirata, K. Sato, M. Itoh, and L.B. Ok. 1996. Human BST-1 expressed on myeloid cells functions as a receptor molecule. *Biochem. Biophys. Res. Commun.* 228:838–845.

18. Ishikawa, J., T. Kaisho, H. Tomizawa, B.O. Lee, Y. Kobune, J. Inazawa, M. Itoh, T. Ochi, K. Ishihara, et al. 1995. Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. *Genomics*. 26:527–534.

19. Lee, B.O., K. Ishihara, K. Denno, Y. Kobune, M. Itoh, O. Muraoka, T. Kaisho, T. Ochi, and T. Hirano. 1996. Elevated levels of the soluble form of bone marrow stromal cell antigen 1 in the sera of patients with severe rheumatoid arthritis. *Arthritis Rheum.* 39:629–637.

20. Dong, C., D. Willerford, F.W. Alt, and M.D. Cooper. 1996. Genomic organization and chromosomal localization of the mouse Bp3 gene, a member of the CD38-ADP-ribosyl cyclase family. *Immunogen.* 45:35–43.

21. Muraoka, O., H. Tanaka, M. Itoh, K. Ishihara, and T. Hirano. 1996. Genomic structure of human BST-1. *Immunol. Lett.* 54:1–4.

22. Ferrero, E., and F. Malavasi. 1997. Human CD38, a leukocyte receptor and ectoenzyme, is a member of a novel eukaryotic gene family of nicotinamide adenine $dinucleotide^{+}$-converting enzymes: extensive structural homology with the genes for murine bone marrow stromal cells antigen 1 and aplysian ADP-ribosyl cyclase. *J. Immunol.* 159:3858–3865.

23. Hirata, Y., N. Kimura, K. Sato, Y. Ohsugi, S. Takasawa, H. Okamoto, T. Kaisho, K. Ishihara, and T. Hirano. 1994. ADP ribosyl cyclase activity of a novel bone marrow stromal cell surface molecule, BST-1. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 356:244–248.

24. de Galdiero, M.E., and A. Marcatili. 1997. Cytokine and adhesion molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins. *Infect. Immun.* 65:699–707.

25. Nakajima-Iijima, S., H. Hamada, P. Reddy, and T. Kakunaga. 1985. Molecular structure of the human cytoplasmic beta-actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA*. 82:6133–6137.

26. Cocks, B.G., R. de Waal Malefyt, J.P. Galizzi, J.E. de Vries, and G. Aversa. 1993. IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *Int. Immunol.* 5:657–663.

27. Newkirk, M.M., K.N. Watanabe Duffy, J. Leclerc, N. Lambert, and J.B. Shiroky. 1994. Detection of cytomegalovirus, Epstein-Barr virus and herpes virus-6 in patients with rheumatoid arthritis with or without Sjögren's syndrome. *Br. J. Rheumatol.* 33:317–322.

28. Knecht, H., R. Sahli, P. Shaw, C. Meyer, E. Bachmann, B.F. Odermatt, and F. Bachmann. 1990. Detection of Epstein-Barr virus DNA by polymerase chain reaction in lymph node biopsies from patients with angioimmunoblastic lymphadenopathy. *Br. J. Haematol.* 75:610–614.

29. Jumper, M.D., J.B. Splawski, P.E. Lipsky, and K. Meek. 1994. Ligation of CD40 induces sterile transcripts of multiple Ig H chain isotypes in human B cells. *J. Immunol.* 152:438–445.

30. Gorczyca, W., J. Gong, and Z. Darzynkiewicz. 1993. Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 53:1945–1951.

31. Tohma, S., and P.E. Lipsky. 1991. Analysis of the mechanisms of T cell-dependent polyclonal activation of human B cells. Induction of human B cell responses by fixed activated T cells. *J. Immunol.* 146:2544–2552.

32. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. 65:55–63.

33. Itoh, M., K. Ishihara, H. Tomizawa, H. Tanaka, Y. Kobune, J. Ishikawa, and T. Hirano. 1994. Molecular cloning of murine BST-1 having homology with CD38 and Aplysia ADP-ribosyl cyclase. *Biochem. Biophys. Res. Commun.* 203: 1309–1317.

34. Kajimoti, Y., J. Miyagawa, K. Ishihara, Y. Okuyama, Y. Fujitani, M. Itoh, T. Kaisho, T. Matsuoka, H. Watada, T. Hanafusa, Y. Yamasaki, Y. Matsuzawa, and T. Hirano. 1996. Pancreatic islet cells express BST-1, a CD38-like surface molecule having ADP-ribosyl cyclase activity. *Biochem. Biophys. Res. Commun.* 219:941–946.

35. Prasad, G.S., D.E. McRee, E.A. Stura, D.G. Levitt, H.C. Lee, and C.D. Stout. 1996. Crystal structure of Aplysia ADP ribosyl cyclase, a homologue of the bifunctional ectozyme CD38. *Nature Struct. Biol.* 3:957–964.

36. Smiley, J.D., C. Sachs, and M. Ziff. 1968. In vitro synthesis of immunoglobulin by rheumatoid synovial membrane. *J. Clin. Invest.* 47:624–632.

37. Wernick, R., P.E. Lipsky, E. Marban-Arcos, J.J. Maliakkal, D. Edelbaum, and M. Ziff. 1985. IgG and IgM rheumatoid factor synthesis in rheumatoid synovial membrane cell cultures. *Arthritis Rheum.* 28:742–752.

38. Kriegsmann, J., G.M. Keyszer, T. Geiler, R. Brauer, R.E. Gay, and S. Gay. 1995. Expression of vascular cell adhesion molecule-1 mRNA and protein in rheumatoid synovium demonstrated by in situ hybridization and immunohistochemistry. *Lab. Invest.* 72:209–214.

39. Veale, D.J., and C. Maple. 1996. Cell adhesion molecules in rheumatoid arthritis. *Drugs Aging*. 9:87–92.

40. Marlor, C.W., D.L. Webb, M.P. Bombora, J.M. Greve, and M.L. Blue. 1992. Expression of vascular cell adhesion molecule I in fibroblast-like synoviocytes after stimulation with tumor necrosis factor. *Am. J. Pathol.* 140:1055–1060.

41. Morales Ducret, J., E. Wayner, M.J. Elices, G. Firestein, and N. Zvaifler. 1992. $\alpha 4/\beta 1$ Integrin (VLA-4) ligands in arthritis: vascular cell adhesion molecule expression in synovium and on fibroblast-like synoviocytes. *Arthritis Rheum.* 49:1424–1431.

42. Ishihara, K., Y. Kobune, Y. Okuyama, M. Itoh, B.O. Lee, O. Muraoka, and T. Hirano. 1996. Stage-specific expression of mouse BST-1/BP-3 on the early B and T cell progenitors prior to gene rearrangement of antigen receptor. *Int. Immunol.* 8:1395–1404.

43. McNagny, K.M., P.A. Cazenave, and M.D. Cooper. 1988. BP-3 alloantigen. A cell surface glycoprotein that marks early B lineage cells and mature myeloid lineage cells in mice. *J. Immunol.* 141:2551–2556.

44. McNagny, K.M., R.P. Bucy, and M.D. Cooper. 1991. Reticular cells in peripheral lymphoid tissues express the phosphatidylinositol-lined BP3 antigen. *Eur. J. Immunol.* 21:509–515.

45. Vicari, A.P., A.G. Bean, and A. Zlotnik. 1996. A role for BP-3/BST-1 antigen in early T cell development. *Int. Immunol.* 8:183–191.

46. Dong, C., J. Wang, P. Neame, and M.D. Cooper. 1994. The murine BP-3 gene encodes a relative of the CD38/NAD glycohydrolase family. *Int. Immunol.* 6:1353–1360.

47. Okuyama, Y., K. Ishihara, N. Kimura, Y. Hirata, K. Sato, M. Itoh, L.B. Ok, and T. Hirano. 1996. Human BST-1 expressed on myeloid cells functions as a receptor molecule. *Biochem. Biophys. Res. Commun.* 228:838–845.

48. Namen, A.E., S. Lupton, K. Hjerrild, J. Wignall, D.Y. Mochizuki, A.

Schmierer, B. Mosley, C.J. March, D. Urdal, and S. Gillis. 1988. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature.* 333:571–573.

49. Salman, M., D. Scheel-Toellner, A.P. Huissoon, D. Pilling, N. Shansadeen, H. Hyde, A.D. D'Angeac, P.A. Bacon, P. Emery, and A.N. Akbar. 1997. Inhibition of T cell apoptosis in the rheumatoid synovium. *J. Clin. Invest.* 99:439–446.

50. Silvy, A., P. Altevogt, P. Mondiere, C. Bella, and T. Defrance. 1997. A role for the VLA-4 integrin in the activation of human memory B cells. *Eur. J. Immunol.* 27:2757–2764.

51. Koide, J., K. Takada, M. Sugiura, H. Sekine, T. Ito, K. Saito, S. Mori, T. Takeuchi, S. Uchida, and T. Abe. 1997. Spontaneous establishment of an Epstein-Barr virus-infected fibroblast line from the synovial tissue of a rheumatoid arthritis patient. *J. Virol.* 71:2478–2481.

52. Klein, G. 1994. Epstein-Barr virus strategy in normal and neoplastic B cells. *Cell.* 71:791–793.

53. Kieff, E. 1996. Epstein-Barr virus and its replication. *In* Virology. B.N. Fields, D.N. Knipe, P.M. Howley, R.M. Chanock, T.P. Monath, J.L. Melnick, B. Roizman, and S.E. Straus, editors. Lippicott-Raven, Philadelphia. 2343–2396.

54. Edwards, J.C., L.S. Wilkinson, P. Speight, and D.A. Isenberg. 1993. Vascular cell adhesion molecule 1 and alpha 4 and beta 1 integrins in lymphocyte aggregates in Sjögren's syndrome and rheumatoid arthritis. *Ann. Rheum. Dis.* 52:806–811.

55. Clark, E.A., K.H. Grabstein, A.M. Gown, M. Skelly, T. Kaisho, T. Hirano, and G.L. Shu. 1995. Activation of B lymphocyte maturation by a human follicular dendritic cell line, FDC-1. *J. Immunol.* 155:545–555.

56. Lindhout, E., A. Lakeman, M.L.C.M. Mervissen, and C. de Groot. 1994. Functionally active Epstein Barr Virus transformed follicular dendritic cell-like lines. *J. Exp. Med.* 179:1173–1184.

57. Freedman, A.S., J.M. Munro, G.E. Rice, M.P. Bevilacqua, C. Morimoto, B.W. McIntyre, K. Rhynhart, J.S. Pober, and L.M. Nadler. 1990. Adhesion of human B cells to germinal centers in vitro involves VLA-4 and INCAM-110. *Science.* 249:1031–1033.

58. Koopman, G., H.K. Parmentier, H.J. Schuurman, W. Newman, C.J.L.M. Meijer, and S.T. Pals. 1991. Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte function associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular adhesion molecule 1 pathways. *J. Exp. Med.* 173:1297–1304.

59. Tsunoda, R., M. Nakayama, E. Heinen, K. Miyake, K. Suzuki, N. Sugai, and M. Kojima. 1992. Emperipolesis of lymphoid cells by human follicular dendritic cells in vitro. *Virchows Arch. B Cell Pathol.* 62:69–78.

60. Lindhout E., M.L.C.M. Mervissen, J. Kwekkeboom, J.M. Tager, and C. deGroot. 1993. Direct evidence that human follicular dendritic cells rescue germinal center B cells from death by apoptosis. *Clin. Exp. Immunol.* 91:330–336.

61. Clark, E.A., K.H. Grabstein, and G.L. Shu. 1992. Cultured human follicular dendritic cells. Growth characteristics and interactions with B lymphocytes. *J. Immunol.* 148:3327–3335.

62. Bosseloir, A., E. Heinen, T. Defrance, F. Bouzhazha, N. Antoine, and L.J. Simar. 1994. Moabls MAS516 and 5B5, two fibroblast markers, recognize human follicular dendritic cells. *Immunol. Lett.* 42:49–54.

63. Pallesen, G., and O. Myhre-Jensen. 1987. Immunophenotypic analysis of neoplastic cells in follicular dendritic cell sarcoma. *Leukemia.* 7:549–557.

64. Humphrey, J.H., D. Grennan, and V. Sundaram. 1984. The origin of follicular dendritic cells in the mouse and the mechanism of trapping of immune complexes on them. *Eur. J. Immunol.* 14:859–864.

Bidirectional Regulation of Human B Cell Responses by CD40-CD40 Ligand Interactions[1]

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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$^+$ T cells, as well as the B cell stimulatory effects of anti-CD3 activated mitomycin C-treated CD4$^+$ 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$^+$ 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$^-$ memory B cells were significantly more sensitive to inhibition by CD40 ligation than IgD$^+$ naive B cells. Engagement of CD40 not only suppressed Ig secretion by IgD$^-$ memory B cells, but also expression of CD38. Finally, activated B cells acquired the capacity to down-regulate CD40 ligand expression by stimulated CD4$^+$ 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-α-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$^+$ 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$^-$ memory B cells were far more sensitive to the suppressive influences of CD40 ligation than IgD$^+$ naive B cells. IgD$^-$ 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$^+$ 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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Received for publication October 29, 1996. Accepted for publication February 20, 1997.

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

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0022-1767/97/$02.00

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 $F(ab')_2$ 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. Katheryn 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 $200 \times g$ for 3 min, and the supernatant was collected and centrifuged again at $7,000 \times g$ for 10 min. The membrane pellet was resuspended in PBS at a final concentration of 20 to 25×10^6 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 1×10^5 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 $CD19^+$ 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% $CD19^+$ B cells. Peripheral B cells prepared in this manner from normal donors were almost entirely small resting cells as determined by flow cytometric analysis of side and forward scatter. The population was routinely devoid of activated $CD69^+$ 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% $CD19^+$ B cells and no Leu $11b^+$ NK cells.

In some experiments, IgD^+ or IgD^- B cells were separated from purified B cells by flow cytometry. Purified B cells were incubated with saturating concentrations of a FITC-conjugated goat $F(ab')_2$ anti-human IgD Ab for 30 min at 4°C, washed with PBS, and resuspended at a concentration of 5×10^6/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^{Plus}, Becton Dickinson, San Jose, CA). The threshold was set to abort all dead cells and debris as determined by light scattering. After sorting, IgD^+ or IgD^- 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_4Cl to lyse the SRBC and were passed over a nylon wool column. Afterward, purified $CD4^+$ T cells were prepared from the rosette-forming cells by negative selection, using a panning technique (25) to deplete contaminating $HLA\text{-}DR^+$ cells and $CD8^+$ 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 $CD4^+$ T cell population obtained in this manner contained <0.1% esterase-positive cells, <1% $CD8^+$ T cells, and >96% $CD4^+$ T cells.

In some experiments, $CD4^+$ T cells were treated with mitomycin C before culture. This was accomplished by suspending them in culture medium at approximately 5×10^6/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 $CD4^+$ 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 (2.5×10^4/well) were incubated with various numbers of $CD4^+$ 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_2 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 (ΔMFI) 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 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-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 μCi [^{3}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 [^{3}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 recultured with IL-2 in the presence or absence of recombinant CD40 ligand for the indicated length of time, after which 180 μl 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 μl of PBS were added to the remaining pellet and incubated at 37°C for 4 h, after which time 100 μl 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 $CD4^+$ T cells

Initial experiments investigated CD40 ligand expression by anti-CD3-stimulated $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ T cells or mitomycin C-treated $CD4^+$ T cells, which was calculated from the ΔMFI 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 $CD4^+$ T cell populations was markedly diminished in the presence of B cells, although the density of CD40 ligand expressed by control $CD4^+$ T cells during the initial 48 h remained greater. These results indicate that the major difference between activated control and mitomycin C-treated $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ T cells. As seen in Figure 2, anti-CD3-stimulated mitomycin C-treated $CD4^+$ T cells induced very large amounts of Ig. In contrast, anti-CD3-stimulated control $CD4^+$ T cells induced the production of minimal amounts of Ig and inhibited the production of Ig supported by anti-CD3-stimulated mitomycin C-treated $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 $CD4^+$ 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 3*A*, when B cells were cultured with activated mitomycin C-treated $CD4^+$ 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