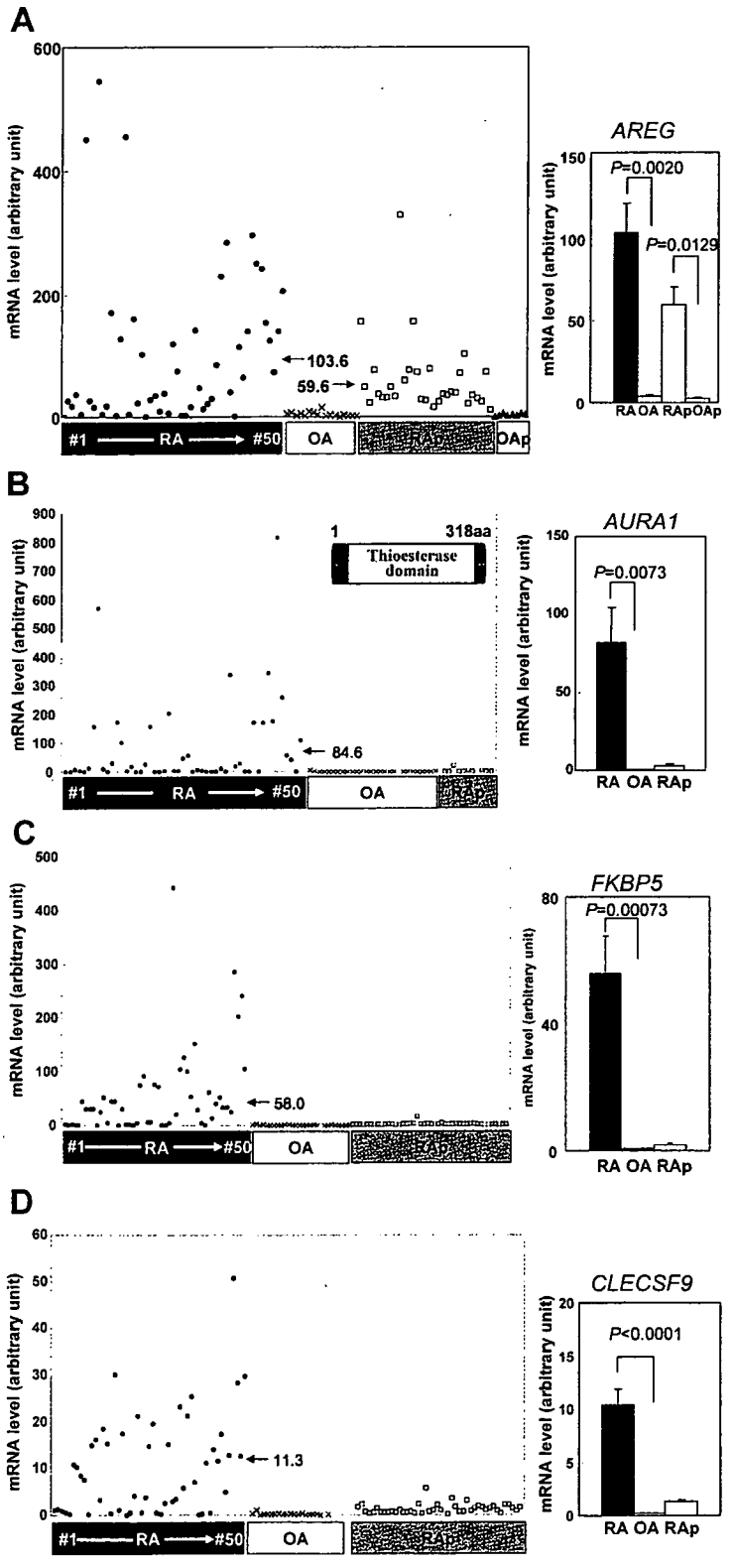
To compare the activation of EGFR signaling between RA and OA patients, we examined the activation of the EGFR signaling pathway in the synoviocytes from the five RA and three OA patients (Fig. 5B). We thus assessed the phosphorylated ERK1/2 expression levels by western blot analysis and expressed the results quantitatively by measuring the intensity of the lower phosphorylated band by densitometry and comparing it with the ERK1/2 band intensity (Fig. 5C). We found that the synoviocytes from the RA and OA patients expressed equivalent levels of EGFR and ERK1/2 proteins, regardless of AREG treatment. In contrast,



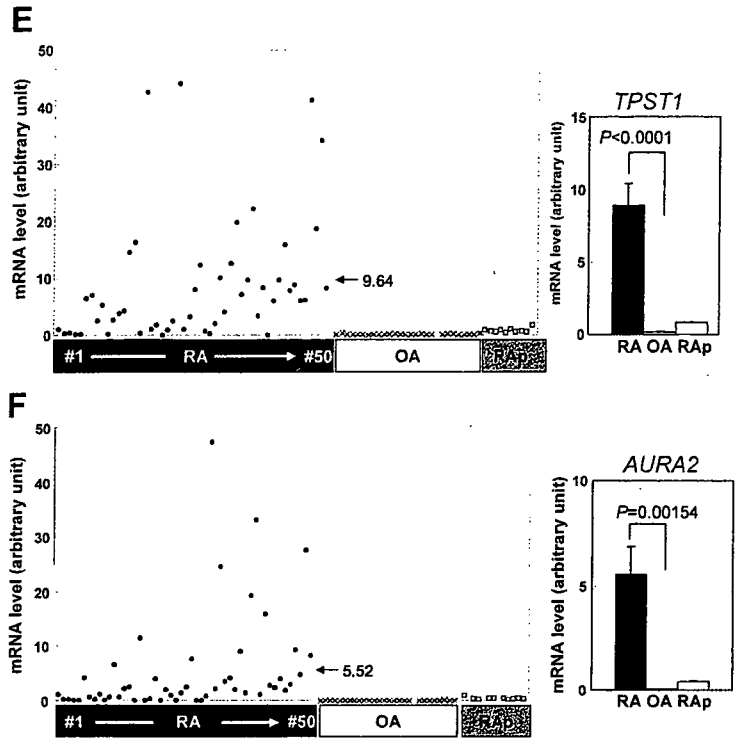


Figure 2. Expression levels of *AURA* genes in individual RA and OA patients. QRT-PCR analyses show that the mRNA levels of (A) *AREG*, (B) *AURA1*, (C) *FKBP5*, (D) *CLECSF9*, (E) *TPST1* and (F) *AURA2* are conspicuously upregulated in RA patient BMMC (and sometimes PBMC), while the BMMC and PBMC of OA patients show negligible upregulation. Expression levels in the BMMC for 50 RA patients (from #1 to 50) are arranged in the denoted order. The inset in (B) shows that the thioesterase domain occupies most of the Aural protein. The mean values of the samples analyzed in triplicate from each individual RA BMMC, RA PBMC, OA BMMC and OA PBMC are indicated by filled circles, open squares, x's, or filled triangles, respectively. The average values for the RA patient group are shown by the horizontal arrows. The bar graphs in the right panels show the average ± SE values of these measurements using the RA or OA BMMC or PBMC. All measurements are statistically significant when RA and OA are compared ($P < 0.01$).

AREG treatment upregulated the phosphorylated ERK1/2 expression levels much more strongly in the synoviocytes from RA2, RA3 and RA4 than in the synoviocytes of any of the OA patients. RA1 is an exception to this pattern as its limited phosphorylated ERK1/2 expression levels were similar to those in OA1–3. The AREG-induced upregulation of ERK1/2 phosphorylation was less apparent in the RA5 synovial cells because ERK1/2 was already activated in the absence of AREG.

Synoviolin plays a role in the synovial hyperplasia of RA by controlling the ERAD system.[10] To determine if the RA synovial cells have an abnormal ERAD system, we measured their levels of the ER stress proteins GRP78/BiP and GRP94, which protect cells from the stress-induced ER dysfunction that could lead to the accumulation of unfolded proteins.[33] We found that while the synovial cells of the RA and OA patients have similar levels of GRP78/BiP (Fig. 5B and D), the RA synoviocytes show enhanced levels of GRP94, irrespective of whether they have been stimulated with AREG. This suggests that at least part of the ER-stress responsive pathway, namely, that mediated by GRP94, is more activated in RA synoviocytes than in OA synoviocytes. Thus, the ERAD pathway does appear to be abnormally upregulated in RA synoviocytes. We confirmed by QRT–PCR that the BMMC and PBMC cells of RA patients RA1–5 show enhanced AREG mRNA levels, unlike the BMMC and PBMC of OA patients OA1–3 (Supplementary Figure S5A). Thus, chronic activation of AREG/EGFR signaling appears to be augmented in RA patients. Since AREG is expressed as transmembrane precursors that are cleaved in the extracellular domain to release soluble growth factor,[34] we speculated that the sera (PB) and bone marrow fluid (BM) of RA1–5 may show enhanced levels of cleaved AREG compared to the equivalent fluids of OA1–3. We tested this by enzyme-linked immunosorbent assay but found only one patient, RA2, showed levels of cleaved AREG that exceeded the detection level of the assay (Supplementary Figure S5B). Thus, it is not clear whether RA patients indeed secrete higher AREG levels than OA patients.

We also examined whether RA synoviocytes expressed higher synoviolin mRNA levels than OA synoviocytes in the presence or absence of AREG. However, we could not detect any significant differences between the RA and OA patients in this regard (Supplementary

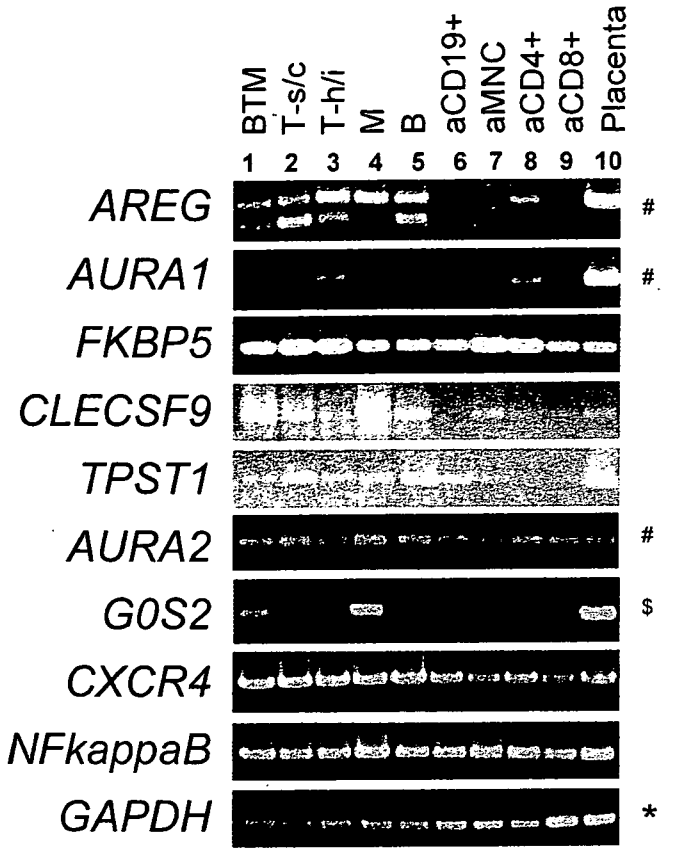


Figure 3. Determination by RT–PCR of the human blood cells that express *AREG*, *AURA1*, *FKBP5*, *CLECSF9*, *TPST1*, *AURA2*, *G0S2*, *CXCR4* and *NFκB*. RT–PCR was performed using the multiple tissue cDNA panel for human blood fractions (MTC, Clontech). *GAPDH* was also amplified as a loading control. PCR amplifications were conducted at 55°C and over 30 cycles except as indicated on the right of the panels: 55°C and 35 cycles (#), 55°C and 27 cycles (*) or 53°C and 25 cycles ($). Lane 1, mononuclear cells (B, T cells and monocytes). Lane 2, resting CD8+ cells (T-suppressor/cytotoxic cells). Lane 3, resting CD4+ cells (T-helper/inducer). Lane 4, resting CD14+ cells (monocytes). Lane 5, resting CD19+ cells (B cells). Lane 6, activated mononuclear cells. Lane 7, activated CD4+ cells. Lane 8, activated CD8+ cells. Lane 9, activated CD19+ cells. Lane 10, human placenta control cDNA served as a DNA size marker.

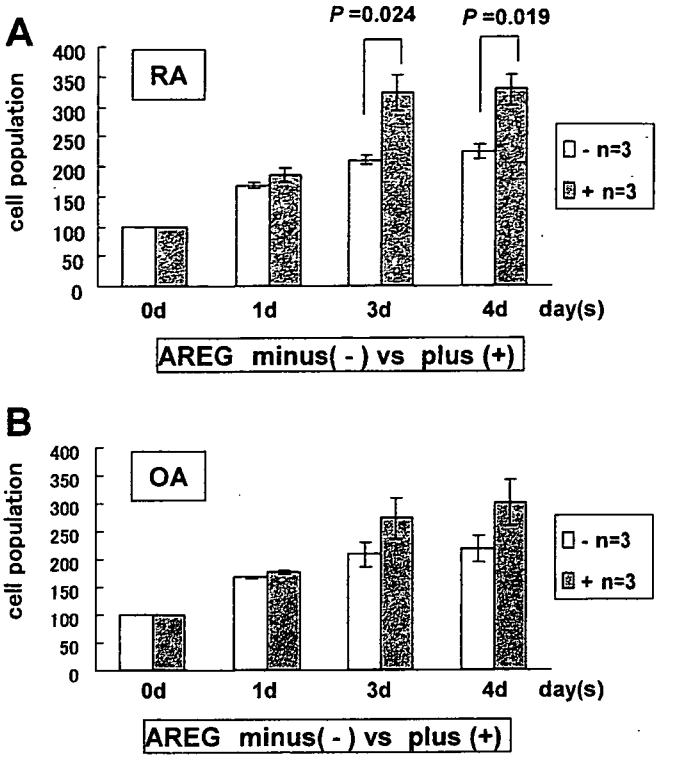


Figure 4. The effect of AREG on the proliferation of synoviocytes from RA and OA patients. The synovial cells from three RA patients (RA1, RA2 and RA3) (A) and three individual OA patients (OA1, OA2 and OA3) were counted on days 0, 1, 3 and 4 after incubation with or without AREG. The cell counts on days 1, 3 and 4 are expressed relative to 0 day. Statistically significant measurements are indicated ($P < 0.05$).

Figure S5C). It is not clear whether the synovial tissues of the patients would, like their cultured derivatives, show a similar lack of synoviolin upregulation.

4. Discussion

In this study, we report our comprehensive isolation of *AURA* genes that show augmented mRNA expression in the BMMC of RA patients as compared to their expression in OA patient BMMC (Fig. 1 and Table 1). Since RA patients suffer from defective central and peripheral B-cell tolerance checkpoints, and often display unusual immunoglobulin light chain repertoires that suggest impaired secondary recombination regulation,[18] we had expected that many immune response genes would be identified as *AURA* genes. Indeed, >10% of the *AURA* genes are directly related to immune responses; moreover, while the other *AURA* genes may seem at first glance to be unrelated to immune responses, many of these can also be linked to immune responses (Table 1). QRT–PCR analysis on individual patient samples revealed that the *AURA* genes discussed below are significantly increased in the BMMC of many of the 50 RA patients we tested (Fig. 2). Thus, the identification of these genes may help us to understand the pathogenesis of RA.

FKBP5, one of the cellular receptors for the immunosuppressant FK506, was expressed at higher mRNA levels in many RA patients than in the OA patients; this was true for the BMMC of the RA patients but not for their PBMC (Fig. 2C). FK506 has been suggested to be an effective drug for reducing the pain associated with RA.[35] This is because it can suppress inflammation by inhibiting the production by synovial cells of prostaglandin E2; it does so by suppressing the IL-1β production by leukocytes.[36] The enhanced FKBP5 expression in RA BMMC is not due to FK506 treatment since at the time of this study, treatment with FK506

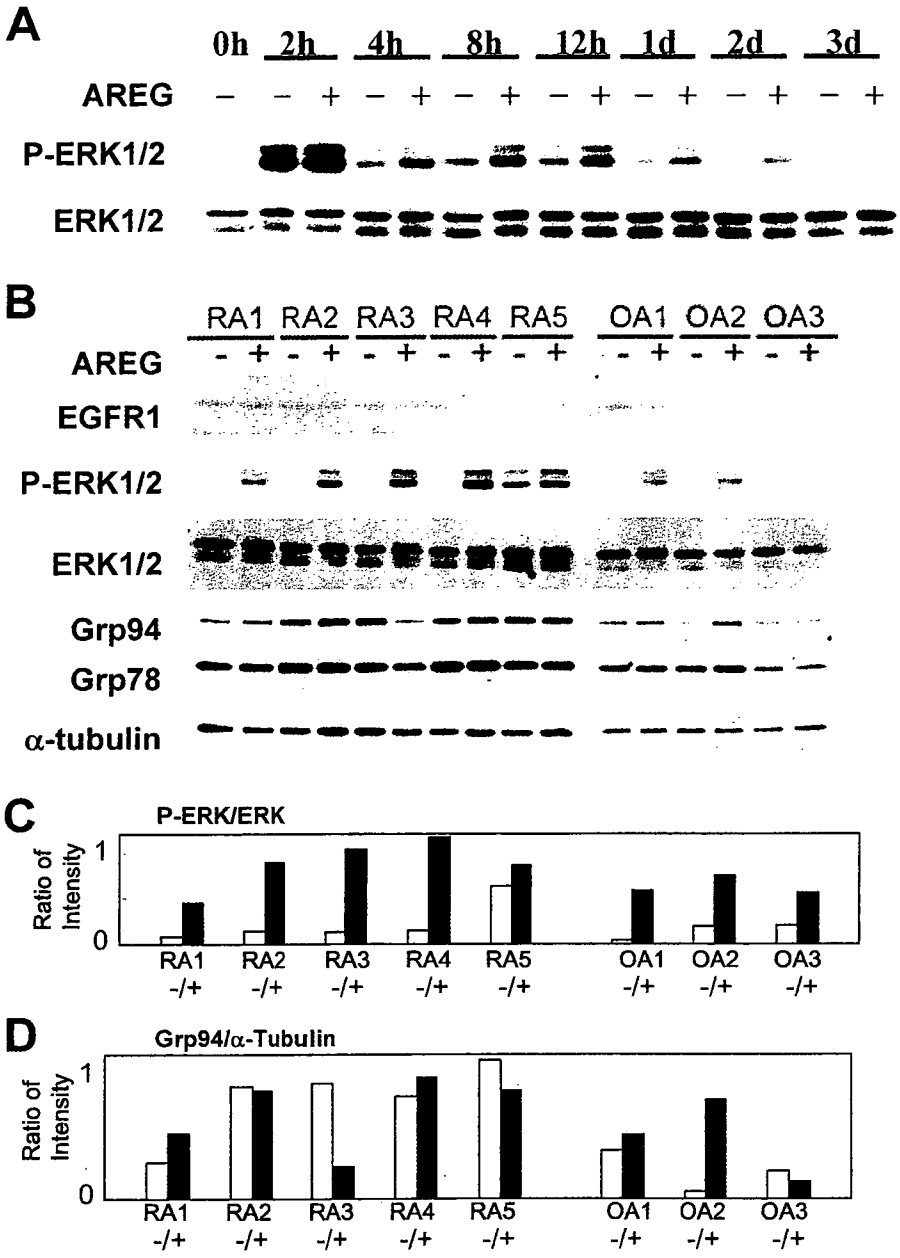


Figure 5. Western blot analysis of RA and OA synovial cells incubated in the presence or absence of AREG. (A) Expression levels of ERK1/2 and its phospho-form (P-ERK1/2) that is phosphorylated at Thr202 and Tyr204. Pooled synovial cells from five RA patients were incubated with (100 ng/ml) or without AREG for varying periods ranging from 0 h to 3 days. (B) Expression levels of EGFR1, ERK1/2, P-ERK1/2, Grp94, Grp78 and synoviolin in synovial cells from individual RA and OA patients that were incubated with or without AREG (100 ng/ml) for 8 h. Alpha-tubulin served as a loading control. (C) Relative optical densities of the western blot bands in (B) to determine P-ERK1/2 expression relative to ERK1/2 expression. (D) Relative optical densities of the western blot bands in (B) to determine Grp94 expression relative to alpha-tubulin expression.

was not permitted in Japan; consequently, none of the patients tested here have ever received FK506. In addition, the enhanced FKBP5 expression by RA BMMC does not correlate with therapeutic treatment using steroids. It remains possible, however, that the increased FKBP5 mRNA levels in the BMMC of RA patients may be due to treatment with other drugs. Alternatively, it may reflect genuine and spontaneous pathological events. Nevertheless, regardless of the cause of its elevated expression, the augmented FKBP expression may strongly inhibit the phosphatase activity of calcineurin, which could increase the dephosphorylation and thus inactivation of various substrates, including the NFAT family proteins and cytokines that are required for the expression of immunoregulatory molecules.

TPST1 mediates tyrosine sulfation within the trans-Golgi system, which affects 1% of all tyrosines in eukaryotic cells. It has been previously suggested that this post-translational modification may play an

important role in the pathogenesis of autoimmune diseases because it regulates mononuclear cell function at various stages of the immune response by enhancing interactions between ligands and receptors.[37] Notably, of the 62 identified target proteins of tyrosine sulfation, nine are cell adhesion molecules and chemokine receptors, which are both central players in leukocyte trafficking. Thus, the augmented expression of *TPST1* in RA patients may elevate the sulfation of crucial tyrosine residues in chemokine receptors that could constitutively increase their binding affinities with their ligands (e.g. the binding of CXCL12–CXCR4).

CLECSF9 belongs to the macrophage-inducible C-type lectin that serves multiple functions by recognizing carbohydrate chains; it plays important roles in macrophage function. Notably, a C-type lectin called DC-specific intercellular adhesion molecule 3-grabbing non-integrin is also highly expressed by macrophages in the synovium of RA patients.[38] However, the HH mRNA expression of macrophage-inducible C-type lectins is strongly induced in response to several inflammatory stimuli. Thus, the augmented expression of *CLECSF9* in the BMMC of RA patients may simply be due to the inflammation in the joint.

Unlike *FKBP5* and *TPST1* genes, the mRNA levels of G0S2, CXCR4 and NF-κB are increased in both the BMMC and PBMC of RA patients (Fig. 2 and Supplementary Figure S3). We previously showed that the PBMC of both systemic lupus erythematosus (SLE) patients and healthy young females express enhanced levels of *G0S2* mRNA.[26] Thus, G0S2 may not actually be involved in the pathogenesis of RA. With regard to the chemokine receptor CXCR4, it was also identified as a inflammation-related gene that is upregulated in synovial cells of patients with pigmented villonodular synovitis (PVNS), which is a joint problem that usually affects the hip or knee and involves the lining of the joint becoming swollen and growing.[8] The enhanced tyrosine sulfation of CXCR4 by augmented TPST1 activity, as described above, may also activate CXCR4, thereby elevating the ability of the CXCR4 ligand to induce the migration of bone marrow cells that could enhance the growth of synovial cells.[39] CXCR4 expression is also upregulated in the spinal cord of animals with experimental autoimmune encephalomyelitis, which is an animal model of autoimmune central nervous system inflammation.[40] With regard to NF-κB, this molecule along with the receptor activator of NF-κB (RANK) and its ligand RANKL have been found to play pivotal roles in the pathophysiological process of RA.[41] Thus, the increased mRNA levels of NF-κB in both the BMMC and PBMC of RA patients may contribute to the bone destruction mediated by activated NF-κB signaling pathway.[42]

AURA1 encodes a novel protein that is similar to thioesterase. Since the thioesterase homologs are widespread, functions of thioesterase vary in the human genome.[43] Thus, the physiological function of *AURA1* remains unknown. A possible role that it could play in RA pathogenesis is suggested by the following observations. First, the stable overexpression of acyl-CoA thioesterase III in human and murine T-cell lines increased both peroxisome numbers and lipid droplet formation, which suggests that it participates in the metabolic regulation of peroxisome proliferation in T cells.[44] Second, altered immune responsiveness is observed in mice deficient in palmitoyl protein thioesterase (PPT1) gene that is mutated in infantile neuronal ceroid lipofuscinosis.[45] Third, $CD4^+$ T cells are the prime mediators of RA in a mouse model SKG strain,[46] and *AURA1* expression is detected predominantly in resting and activated $CD4^+$ T cells (Fig. 3).

AREG is not directly related to immune responses but of all the genes examined, it showed the most conspicuously enhanced expression in both the BMMC and PBMC of many RA patients (Fig. 2A). We also found that the synovial cells of RA patients showed higher sensitivity to AREG, in terms of proliferation, than those of OA patients (Fig. 4). This is not due to augmented expression of EFGR (Fig. 5B, uppermost pane), but due to elevated activation of EGFR signaling pathway because the phosphorylation of ERK1/2 was more enhanced in AREG-treated RA patient synovial cells than that of AREG-treated OA patient synovial cells (Fig. 5). We here present a working hypothesis to explain how augmented AREG expression in BMMC and PBMC of RA patients and subsequent activation of EGFR signaling pathway lead to hyperproliferation of synovial cells in the joints of the RA patients (Fig. 6). Namely, this enhanced phosphorylation of ERK1/2 elevates the expression of many downstream target genes, which may also require the activation of the ERAD system. [12] Given that the Ets-binding site (EBS) of the proximal promoter of the synoviolin gene is responsible for its expression,[47] and that EBS-carrying genes are also activated by signaling events from the ERK pathway,[48] it is possible that the enhanced activation of EGFR signaling induced by AREG may directly activate the expression of synoviolin as well as that of other genes, thereby inducing the hyperproliferation of synovial cells. Thus, it is possible that the ERAD system in RA patients is hyperactivated by synoviolin because of augmented AREG expression in blood cells, possibly in the macrophages that occur in the vicinity of the synovial cells of RA patients, releasing augmented amount of AREG. This hypothesis should be tested more rigourously *in vivo* in the future because the experiments using the isolated synoviocyte cells in tissue culture medium may display distinct response to AREG. Likewise, examination of other EGF family proteins *in vivo* can also be interesting future subjects.

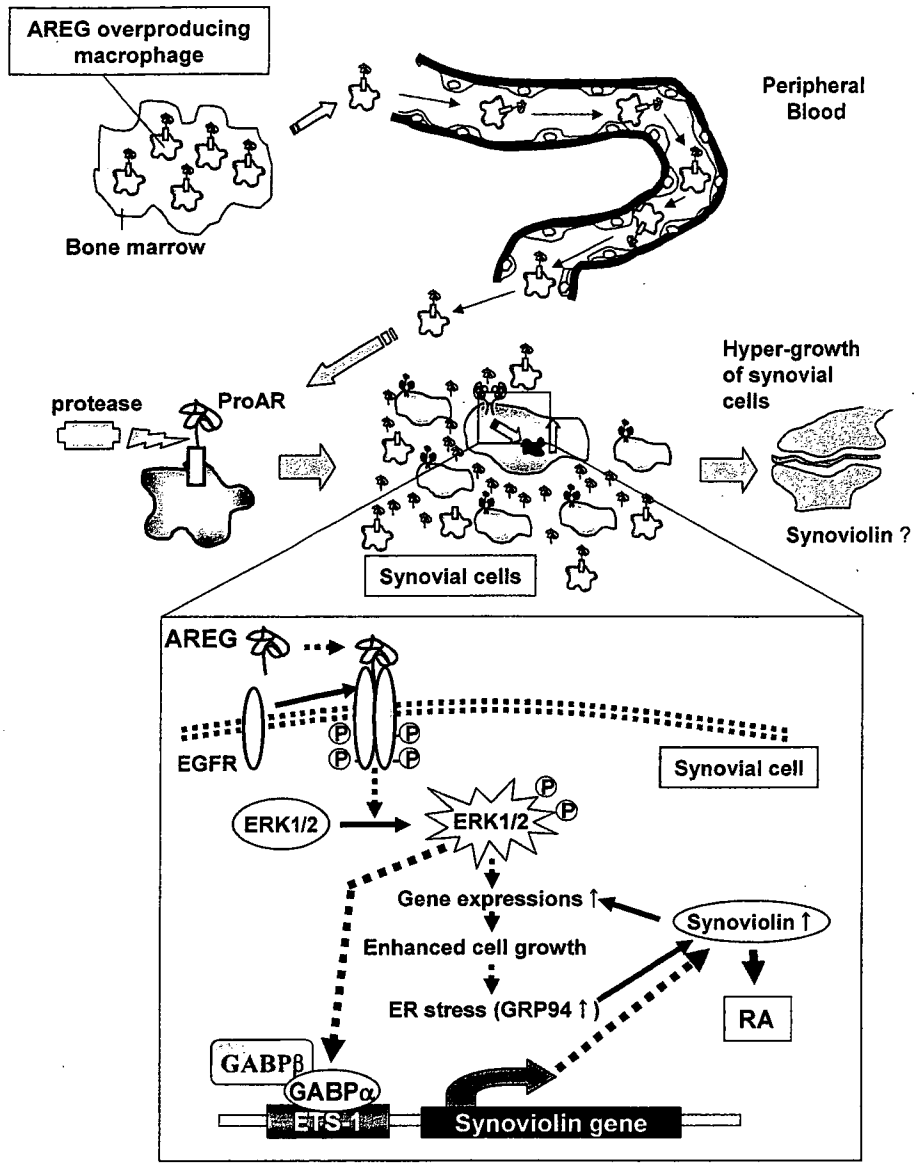


Figure 6. A working hypothesis to explain how augmented level of AREG in BMMC of RA patients may lead to hyperproliferation of synovial cells. Putative macrophages with enhanced expression of AREG precursor (ProAR) may approach to the synovial cells of the joint through blood flow, where they release AREG and activate the EGFR signaling pathway of synovial cells. Since Ets-binding site (ETS-1) of the proximal promoter of the synoviolin gene is one of the downstream targets of ERK pathway, the enhanced activation of EGFR signaling may directly activate the expression of synoviolin gene. The enhanced level of synoviolin activates the ERAD system, which may lead to hyperproliferation of synovial cells.

Overexpression of AREG has been linked to psoriasis in mice and humans.[49,50] Psoriasis is characterized by the hyperproliferation of keratinocytes and the loss of epidermal barrier function that leads to the infiltration of inflammatory cells into the epidermis and dermis.[51] AREG is also upregulated in a synoviocyte cell line derived from an RA patient in which the wild type and a dominant negative form of the orphan nuclear receptor Nurr1 were overexpressed.[52] Interestingly, AREG overexpression in the basal epidermis of transgenic mice induces a phenotype that is associated with synovial membrane inflammation.[49] Moreover, we showed previously that AREG expression is also enhanced in the PBMC of SLE and idiopathic thrombocytopenic purpura patients,[26] which suggests that AREG overexpression may also be associated with other autoimmune diseases. Notably, metalloprotease-mediated AREG shedding and the subsequent activation of EGFR appears to play a critical role in the secretion of IL-8 by the human airway epithelium-like NCI-H292 cells that is induced by tumor necrosis factor-α (TNF-α), a potent multifunctional cytokine that plays a central role in the pathogenesis of many inflammatory diseases like RA.[53] Since TNF-α-induced IL-8 secretion was completely inhibited by the neutralizing antibody against AREG,[53] this antibody could constitute a novel therapeutic tool for RA. Taken

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

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Supplementary Data: Supplementary data are available online at www.dnaresearch.oxfordjournals.org.

References

1. Oertelt, S., Selmi, C., Invernizzi, P., Podda, M., and Gershwin, M. E. 2005, Genes and goals: an approach to microarray analysis in autoimmunity, *Autoimmune Rev.*, **4**, 414–422.
2. Glocker, M. O., Guthke, R., Kekow, J., and Thiesen, H. J. 2006, Rheumatoid arthritis, a complex multifactorial disease: on the way toward individualized medicine, *Med. Res. Rev.*, **26**, 63–87.
3. van der Pouw Kraan, T. C., van Gaalen, F. A., Huizinga, T. W., Pieterman, E., Breedveld, F. C., and Verweij, C. L. 2003, Discovery of distinctive gene expression profiles in rheumatoid synovium using cDNA microarray technology: evidence for the existence of multiple pathways of tissue destruction and repair, *Genes Immun.*, **4**, 187–196.
4. Ruschpler, P., Lorenz, P., Eichler, W., et al. 2003, High CXCR3 expression in synovial mast cells associated with CXCL9 and CXCL10 expression in inflammatory synovial tissues of patients with rheumatoid arthritis, *Arthritis Res. Ther.*, **5**, 241–252.
5. Devauchelle, V., Marion, S., Cagnard, N., et al. 2004, DNA microarray allows molecular profiling of rheumatoid arthritis and identification of pathophysiological targets, *Genes Immun.*, **5**, 597–608.
6. Cagnard, N., Letourneur, F., Essabbani, A., et al. 2005, Interleukin-32, CCL2, PF4F1 and GFD10 are the only cytokine/chemokine genes differentially expressed by in vitro cultured rheumatoid and osteoarthritis fibroblast-like synoviocytes, *Eur. Cytokine Netw.*, **16**, 289–292.
7. Lindberg, J., af Klint, E., Ulfgren, A. K., et al. 2006, Variability in synovial inflammation in rheumatoid arthritis investigated by microarray technology, *Arthritis Res. Ther.*, **8**, R47.
8. Finis, K., Sultmann, H., Ruschhaupt, M., et al. 2006, Analysis of pigmented villonodular synovitis with genome-wide complementary DNA microarray and tissue array technology reveals insight into potential novel therapeutic approaches, *Arthritis Rheum.*, **54**, 1009–1019.
9. Bovin, L. F., Rieneck, K., Workman, C., et al. 2004, Blood cell gene expression profiling in rheumatoid arthritis: discriminative genes and effect of rheumatoid factor, *Immunol. Lett.*, **93**, 217–226.
10. Amano, T., Yamasaki, S., Yagishita, N., et al. 2003, Synoviolin/Hrd1, an E3 ubiquitin ligase, as a novel pathogenic factor for arthropathy, *Genes Dev.*, **17**, 2436–2449.
11. Yagishita, N., Ohneda, K., Amano, T., et al. 2005, Essential role of synoviolin in embryogenesis, *J. Biol. Chem.*, **280**, 7909–7916.
12. Yamasaki, S., Yagishita, N., Tsuchimochi, K., Nishioka, K., and Nakajima, T. 2005, Rheumatoid arthritis as a hyper-endoplasmic reticulum-associated degradation disease, *Athritis Res. Ther.*, **7**, 181–186.
13. Ochi, T., Hakomori, S., Adachi, M., et al. 1988, The presence of a myeloid cell population showing strong reactivity with monoclonal antibody directed to difucosyl type 2 chain in epiphyseal bone marrow adjacent to joints affected with rheumatoid arthritis (RA) and its absence in the corresponding normal and non-RA bone marrow, *J. Rheumatol.*, **15**, 1609–1615.
14. Hayashida, K., Ochi, T., Fujimoto, M., et al. 1992, Bone marrow changes in adjuvant-induced and collagen-induced arthritis, *Arthritis Rheum.*, **35**, 241–245.
15. Jongen-Lavrencic, M., Peeters, H. R., Wognum, A., Vreugdenhil, G., Breedveld, F. C., and Swaak, A. J. 1997, Elevated levels of inflammatory cytokines in bone marrow of patients with rheumatoid arthritis and anemia of chronic disease, *J. Rheumatol.*, **24**, 1504–1509.
16. Hirohata, S., Yanagida, T., Nampei, A., et al. 2004, Enhanced generation of endothelial cells from CD34+ cells of the bone marrow in rheumatoid arthritis: possible role in synovial neovascularization, *Arthritis Rheum.*, **50**, 3888–3896.
17. Nakamura-Kikuoka, S., Takahi, K., Tsuboi, H., et al. 2006, Limited VH gene usage in B-cell clones established with nurse-like cells from patients with rheumatoid arthritis, *Rheumatology*, **45**, 549–557.
18. Samuels, J., Ng, Y. S., Coupillaud, C., Paget, D., and Meffre, E. 2005, Human B cell tolerance and its failure in rheumatoid arthritis, *Ann. N. Y. Acad. Sci.*, **1062**, 116–126.
19. Wardemann, H., Yurasov, S., Schaefer, A., Young, J. W., Meffre, E., and Nussenzweig, M. C. 2003, Predominant autoantibody production by early human B cell precursors, *Science*, **301**, 1374–1377.
20. Bugatti, S., Caporali, R., Manzo, A., Vitolo, B., Pitzalis, C., and Montecucco, C. 2005, Involvement of subchondral bone marrow in rheumatoid arthritis: lymphoid neogenesis and in situ relationship to subchondral bone marrow osteoclast recruitment, *Arthritis Rheum.*, **52**, 3448–3459.
21. Jimenez-Boj, E., Redlich, K., Turk, B., et al. 2005, Interaction between synovial inflammatory tissue and bone marrow in rheumatoid arthritis, *J. Immunol.*, **175**, 2579–2588.
22. Fujii, T., Tamura, K., Masai, K., Tanaka, H., Nishimune, Y., and Nojima, H. 2002, Use of stepwise

subtraction to comprehensively isolate mouse genes whose transcription is up-regulated during spermiogenesis, *EMBO Rep.*, **3**, 367–372.
23. Arnett, F. C., Edworthy, S. M., Bloch, D. A., et al. 1988, The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis, *Arthritis Rheum.*, **31**, 315–324.
24. Altman, R. D. 1991, Criteria for classification of clinical osteoarthritis, *J. Rheumatol. Suppl.*, **27**, 10–12.
25. Kobori, M., Ikeda, Y., Nara, H., et al. 1998, Large scale isolation of osteoclast-specific genes by an improved method involving the preparation of a subtracted cDNA library, *Genes Cells*, **3**, 459–475.
26. Ishii, T., Onda, H., Tanigawa, A., et al. 2006, Isolation and expression profiling of genes upregulated in the peripheral blood cells of systemic lupus erythematosus patients, *DNA Res.*, **112**, 1–11.
27. Shoyab, M., McDonald, V. L., Bradley, J. G., and Todaro, G. J. 1988, Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7, *Proc. Natl Acad. Sci. USA*, **85**, 6528–6532.
28. Fischer, G. and Aumuller, T. 2003, Regulation of peptide bond cis/trans isomerization by enzyme catalysis and its implication in physiological processes, *Rev. Physiol. Biochem. Pharmacol.*, **148**, 105–150.
29. Russell, L. and Forsdyke, D. R. 1991, A human putative lymphocyte G0/G1 switch gene containing a CpG-rich island encodes a small basic protein with the potential to be phosphorylated, *DNA Cell Biol.*, **10**, 581–591.
30. Santiago, B., Baleux, F., Palao, G., et al. 2006, CXCL12 is displayed by rheumatoid endothelial cells through its basic amino-terminal motif on heparan sulfate proteoglycans, *Arthritis Res. Ther.*, **8**, R43.
31. Ahn, K. S. and Aggarwal, B. B. 2005, Transcription factor NF-kappaB: a sensor for smoke and stress signals, *Ann. N. Y. Acad. Sci.*, **1056**, 218–233.
32. Shin, H. S., Lee, H. J., Nishida, M., et al. 2003, Betacellulin and amphiregulin induce upregulation of cyclin D1 and DNA synthesis activity through differential signaling pathways in vascular smooth muscle cells, *Circ. Res.*, **93**, 302–310.
33. Lee, A. S. 2001, The glucose-regulated proteins: stress induction and clinical applications, *Trends Biochem. Sci.*, **26**, 504–510.
34. Lee, D. C., Sunnarborg, S. W., Hinkle, C. L., et al. 2003, TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase, *Ann. N. Y. Acad. Sci.*, **995**, 22–38.
35. Curran, M. P. and Perry, C. M. 2005, Tacrolimus: in patients with rheumatoid arthritis, *Drugs.*, **65**, 993–1001.
36. Sasakawa, T., Sasakawa, Y., Ohkubo, Y., and Mutohm, S. 2005, FK506 inhibits prostaglandin E2 production from synovial cells by suppressing peripheral blood mononuclear cells, *Int. Immunopharmacol.*, **5**, 1291–1297.
37. Hsu, W., Rosenquist, G.L., Ansari, A. A., and Gershwin, M. E. 2005, Autoimmunity and tyrosine sulfation, *Autoimmune Rev.*, **4**, 429–435.
38. van Lent, P. L., Figdor, C. G., Barrera, P., et al. 2003, Expression of the dendritic cell-associated C-type lectin DC-SIGN by inflammatory matrix metalloproteinase-producing macrophages in rheumatoid arthritis synovium and interaction with intercellular adhesion molecule 3-positive T cells, *Arthritis Rheum.*, **48**, 360–369.
39. Crazzolara, R. and Bernhard, D. 2005, CXCR4 chemokine receptors, histone deacetylase inhibitors and acute lymphoblastic leukemia, *Leuk. Lymphoma.*, **46**, 1545–1551.
40. Glabinski, A. R., O'Bryant, S., Selmaj, K., and Ransohoff, R. M. 2000, CXC chemokine receptors expression during chronic relapsing experimental autoimmune encephalomyelitis, *Ann. N. Y. Acad. Sci.*, **917**, 135–144.
41. Wada, T., Nakashima, T., Hiroshi, N., and Penninger, J. M. 2006, RANKL-RANK signaling in osteoclastogenesis and bone disease, *Trends Mol. Med.*, **12**, 17–25.
42. Jimi, E. and Ghosh, S. 2005, Role of nuclear factor-kappaB in the immune system and bone, *Immunol. Rev.*, **208**, 80–87.
43. Akoh, C. C., Lee, G. C., Liaw, Y. C., Huang, T. H., and Shaw, J. F. 2004, GDSL family of serine esterases/lipases, *Prog. Lipid Res.*, **43**, 534–552.
44. Ishizuka, M., Toyama, Y., Watanabe, H., et al. 2004, Overexpression of human acyl-CoA thioesterase upregulates peroxisome biogenesis, *Exp. Cell Res.*, **297**, 127–141.
45. Jalanko, A., Vesa, J., Manninen, T., et al. 2005, Mice with Ppt1Deltaex4 mutation replicate the INCL phenotype and show an inflammation-associated loss of interneurons, *Neurobiol. Dis.*, **18**, 226–241.
46. Sakaguchi, N., Takahashi, T., Hata, H., et al. 2003, Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice, *Nature*, **426**, 454–460.
47. Tsuchimochi, K., Yagishita, N., Yamasaki, S., et al. 2005, Identification of a crucial site for synoviolin expression, *Mol. Cell. Biol.*, **25**, 7344–7356.
48. Hoffmeyer, A., Avots, A., Flory, E., Weber, C. K., Serfling, E., and Rapp, U. R. 1998, The GABP-responsive element of the interleukin-2 enhancer is regulated by JNK/SAPK-activating pathways in T lymphocytes, *J. Biol. Chem.*, **273**, 10112–10119.
49. Cook, P. W., Brown, J. R., Cornell, K. A., and Pittelkow, M. R. 2004, Suprabasal expression of human amphiregulin in the epidermis of transgenic mice induces a severe, early-onset, psoriasis-like skin pathology: expression of amphiregulin in the basal epidermis is also associated with synovitis, *Exp. Dermatol.*, **13**, 347–356.
50. Bhagavathula, N., Nerusu, K. C., Fisher, G. J., et al. 2005, Amphiregulin and epidermal hyperplasia: amphiregulin is required to maintain the psoriatic phenotype of human skin grafts on severe combined immunodeficient mice, *Am. J. Pathol.*, **166**, 1009–1016.
51. Ritchlin, C. T. 2005, Pathogenesis of psoriatic arthritis, *Curr. Opin. Rheumatol.*, **17**, 406–412.
52. Davies, M. R., Harding, C. J., Raines, S., et al. 2005, Nurr1 dependent regulation of pro-inflammatory mediators in immortalised synovial fibroblasts, *J. Inflamm. (Lond)*, **2**, 15.
53. Chokki, M., Mitsuhashi, H., and Kamimura, T. 2006, Metalloprotease-dependent amphiregulin release mediates tumor necrosis factor-alpha-induced IL-8 secretion in the human airway epithelial cell line NCI-H292, *Life Sci.*, **78**, 3051–3057.

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Research article

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

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Abstract

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

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

Our laboratory has established that nurse-like cells (NLCs) are present in the synovial tissues and bone marrow of patients with rheumatoid arthritis (RA) [1–3]. Such cells, which were first discovered in thymus, play an important role in thymocyte maturation and differentiation [4–6]. *In vitro*, they form unique complexes with thymocytes, which initially adhere to them and then crawl beneath them [7–9]. This phenomenon, which is unique to NLCs at various tissue sites, has been called 'pseudoemperipolesis'. NLCs from RA synovial tissue (RA-NLCs) promote survival of B cells [2,3] and maintain the growth of myeloid cells of patients with RA [1], suggesting that they contribute profoundly to pathogenesis in RA.

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

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

Materials and methods

Isolation of NLCs from RA synovial tissues

RA-NLCs were established from RA synovial tissues as previously described [1]. Briefly, synovial tissues were obtained from knee joints of five patients with RA who fulfilled American College of Rheumatology criteria for RA [13], after informed consent had been obtained. The cells were cultured in DMEM (Dulbecco's modified Eagle's medium [DMEM; Gibco BRL, Gaithersburg, MD, USA] supplemented with 10% fetal calf serum [FCS; Hyclone, Logan, UT, USA], 100 units/ml of penicillin [Gibco BRL], and 100 μg/ml of streptomycin [Gibco BRL] at 37°C in 7.5% CO_2. RA-NLCs were identified by their ability to support pseudoemperipolesis, seen *in vitro* in the migration of a T-cell lymphoma line, MOLT-17, beneath the NLCs, as previously described [3].

Isolation of mononuclear cells from RA synovial fluid

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

Isolation and culture of monocytes from peripheral blood

Peripheral-blood monocytes were collected as plastic-adherent cells, as described previously [14]. Mononuclear cells were isolated from heparinized peripheral blood from five healthy volunteers [15]. Over 97% of the adherent cells were determined to be monocytes by morphology and CD14 expression.

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

Formation of multinucleated giant cells by TRAP-positive mononuclear cells

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

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

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

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

Examination of bone resorption

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

Results

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

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

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

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

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

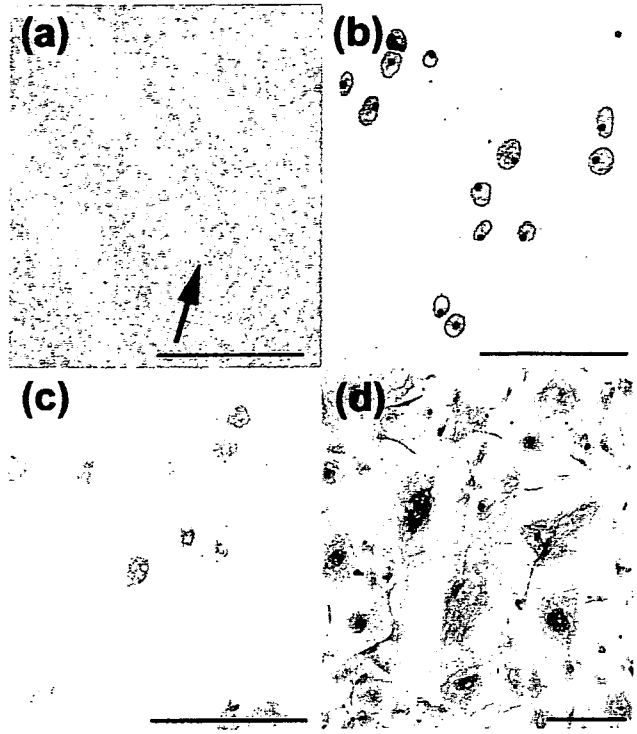
Differentiation of TRAP-positive mononuclear cells into multinucleated giant bone-resorbing cells in the absence of RA-NLCs

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

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

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

Figure 1



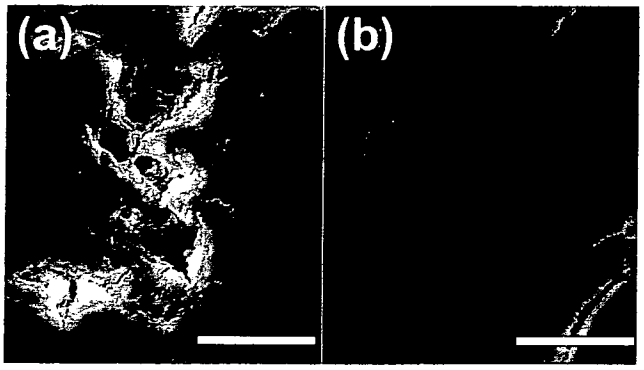
Morphology of TRAP-positive mononuclear cells induced from peripheral-blood monocytes with RA-NLCs. **(a)** Phase-contrast micrograph of monocytes co-cultured with RA-NLCs. Mononuclear cells (arrow) are growing on the RA-NLCs. **(b)** Mononuclear cells collected from the culture shown in (a). May–Grunwald–Giemsa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

Discussion

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

Fujikawa *et al* reported that synovial macrophages differentiated into osteoclasts after incubation in the presence of a rat osteoblast-like cell line [20]. The fibroblasts isolated from RA synovia induced differentiation of monocytes into multinucleated cells in the presence of 1,25-dihydroxyvitamin D_3 and macrophage-colony-

Figure 2



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

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

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

Table 1

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

Stimulator	Concentration	Fusion index (%) [a]
None		1.8
Conditioned medium[b]	(10% v/v)	86.1
IL-1α	(1 ng/ml)	1.1
IL-1β	(1 ng/ml)	1.3
IL-2	(250 U/ml)	7.7
IL-3	(5 ng/ml)	64.8
IL-4	(100 U/ml)	1.3
IL-5	(1 ng/ml)	66.1
IL-6	(20 ng/ml)	5.6
IL-6 + sIL-6R	(sIL-6R: 200 ng/ml)	5.3
IL-7	(20 ng/ml)	72.4
IL-8	(20 ng/ml)	2.1
IFN-γ	(100 U/ml)	7.1
GM-CSF	(1 ng/ml)	73.8
M-CSF	(25 ng/ml)	5.8
TNF-α	(1 ng/ml)	6.9
VD_3	(10^{-7} mol/l)	3.4
Dexamethasone	(10^{-8} mol/l)	0.8
M-CSF + IL-4	(M-CSF, 25 ng/ml; IL-4,100 U/ml)	0.1
IL-3 + IL-7		75.4
IL-7 + GM-CSF		77.3
IL-3 + IL-7 + GM-CSF		78.7
Phytohemagglutinin	(1% v/v)	2.0
RANKL[c]	(100 ng/ml)	2.6
RANKL + M-CSF [c]	(RANKL/ODF, 100 ng/ml; M-CSF, 25 ng/ml)	38.1

[a]TRAP-positive mononuclear cells were stimulated with various cytokines for 96–120 h. Fusion indices were calculated using the formula given in the text and previously [16]. Data are representative of three independent experiments using TRAP-positive cells induced from monocytes. [b]See [15], and Supplementary material. [c]The culture was maintained for 14 days. GM-CSF = granulocyte macrophage-colony-stimulating factor; IFN = interferon; IL = interleukin; M-CSF = macrophage-colony-stimulating factor; ODF = osteoclast differentiation factor; RANKL = receptor activator of nuclear-factor-κB ligand; sIL-6R = soluble interleukin-6 receptor; TNF = tumor necrosis factor; VD_3 = 1,25-dihydroxyvitamin D_3.

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

Conclusion

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

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References

1. Tomita T, Takeuchi E, Toyosaki-Maeda T, Oku H, Kaneko M, Takano, Sugamoto H, Ohzono K, Suzuki R, Ochi T: **Establishment of nurse-like stromal cells from bone marrow patients with rheumatoid arthritis: indication of characteristic bone marrow microenvironment in patients with rheumatoid arthritis.** *Rheumatology (Oxford)* 1999, **38**:854-863.
2. Takeuchi E, Tomita T, Toyosaki-Maeda T, Hashimoto H, Kaneko M, Takano H, Sugano K, Suzuki R, Ochi T: **Establishment and characterization of nurse-cell like stromal cell lines from synovial tissues of patients with rheumatoid arthritis.** *Arthritis Rheum* 1999, **42**:221-228.
3. Shimaoka Y, Attrep JF, Hirano T, Ishihara K, Suzuki R, Toyosaki T, Ochi T, Lipsky PE: **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* 1998, **102**:606-618.
4. Pezzano M, Li Y, Phlip D, Omene C, Cantey M, Saunders G, Guyden JC: **Thymic nurse cell rescue of early CD4+ CD8+ thymocytes from apoptosis.** *Cell Mol Biol* 1995, **41**:1099-1111.
5. Pezzano M, Phlip D, Stephenson S, Li Y, Reid V, Maitta R, Guyden JC: **Positive selection by thymic nurse cells requires IL-1 beta and is associated with an increased Bcl-2 expression.** *Cell Immunol* 1996, **169**:174-184.
6. Iwabuchi K, Nakayama K, McCoy RL, Wang F, Nishimura T, Habu S, Murphy KM, Loh, DY: **Cellular and peptide requirements for *in vitro* clonal deletion of immature thymocytes.** *Proc Natl Acad Sci U S A* 1992, **89**:9000-9004.
7. Itoh T, Doi H, Chin S, Nishimura T, Kasahara S: **Establishment of mouse thymic nurse cell clones from a spontaneous BALB/c thymic tumor.** *Eur J Immunol* 1988, **18**:821-824.
8. Miyake K, Hasunuma Y, Yagita H, Kimoto, M: **Requirement for VLA-4 and VLA-5 integrins in lymphoma cells binding to and migration beneath stromal cells in culture.** *J Cell Biol* 1992, **119**:653-662.
9. Hiai H, Nishi Y, Miyazawa T, Matsudaira Y, Nishizuka Y: **Mouse lymophoid leukemias: symbiotic complexes of neoplastic lymphocytes and their microenvironments.** *J Natl Cancer Inst* 1981, **66**:713-722.
10. Gravallese EM, Harada Y, Wang JT, Gom AH, Thomhil TS, Goldring, SR: **Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis.** *Am J Pathol* 1998, **152**:943-951.
11. Chang JS, Quinn JM, Demaziere A, Bulstrode CJ, Francis MJ, Duthie RB, Athanasou NA: **Bone resorption by cells isolated from rheumatoid synovium.** *Ann Rheum Dis* 1992, **51**:1223-1229.
12. Fujikawa Y, Shingu M, Torisu T, Itonaga I, Masumi S: **Bone resorption by tartrate-resistant acid phosphatase-positive multinuclear cells isolated from rheumatoid synovium.** *Br J Rheumatol* 1996, **35**:213-217.
13. Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HA, Medsger TA, Jr, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JG, Wilder RL, Hunder GG: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.** *Arthritis Rheum* 1988, **31**:315-324.
14. Akagawa KS, Takatsuka N, Nozaki Y, Komuro I, Azuma M, Ueda M, Naito M, Takahashi K: **Generation of CD1+RelB+ dendritic cells and tartrate-resistant acid phosphatase-positive osteoclast-like multinucleated giant cells from human monocytes.** *Blood* 1996, **88**:4029-4039.
15. Toyosaki T, Tsuruta Y, Yoshioka T, Takemoto H, Suzuki R, Tomita T, Ochi T: **Recognition of rheumatoid arthritis synovial antigen by CD4+, CD8− T cell clones established from rheumatoid arthritis joints.** *Arthritis Rheum* 1998, **41**:92-100.
16. Higuchi S, Tabata N, Tajima M, Ito M, Tsurudome M, Sudo A, Uchida A, Ito Y: **Induction of human osteoclast-like cells by treatment of blood monocytes with anti-fusion regulatory protein-1/CD98 monoclonal antibodies.** *J Bone Miner Res* 1998, **13**:44-49.
17. Zhou, LJ, Tedder TF: **CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells.** *Proc Natl Acad Sci U S A* 1996, **93**:2588-2592.
18. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashino K, Udagawa N, Takahashi N, Suda T: **Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL.** *Proc Natl Acad Sci U S A* 1998, **95**:3597-3602.
19. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. **Ostreoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.** *Cell* 1998, **93**:165-178.
20. Fujikawa Y, Sabokbar A, Neale S, Athanasou NA: **Human osteoclast formation and bone resorption by monocytes and synovial macrophages in rheumatoid arthritis.** *Ann Rheum Dis* 1996, **55**:816-822.
21. Takayanagi H, Oda H, Yamamoto S, Kawaguchi H, Tanaka S, Nishikawa T, Koshihara Y: **A new mechanism of bone destruction in rheumatoid arthritis: synovial fibroblasts induce osteoclastogenesis.** *Biochem Biophys Res Commun* 1997, **240**: 279-286.
22. Burger JA, Zvaifler NJ, tsukada N, Firestein GS, Kipps TJ: **Fibroblast-like synoviocytes support pseudoemperipolesis via a stromal cell-derived B-cell factor-1-and CD106 (VCAM-1)-dependent mechanism.** *J Clin Invest* 2001, **107**:305-315.
23. Shigeyama Y, Pap T, Kunzler P, Simmen BR, Gay RE, Gay S: **Expression of osteoclast differentiation factor in rheumatoid arthritis.** *Arthritis Rheum* 2000, **43**:2523-2530.

Supplementary material

Supplementary materials and methods

Cell lines

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

Examination of pseudoemperipolesis

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

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

Long-term maintenance of monocytes by RA-NLCs

Monocytes (2.5×10^5) were co-cultured with RA-NLCs or CCD-19Lu with or without culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) in supplemented DMEM in 48-well culture plates at 37°C in 7.5% CO_2 for up to 70 days. Half of the medium was changed once a week. The TRAP-positive mononuclear cells were collected from the culture, stained with trypan blue, and counted for viability under a microscope.

Antibodies and staining of cells

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

Cytokines and reagents

Conditioned media were prepared as previously reported [15]. Briefly, a mixture of peripheral-blood mononuclear cells from 10 healthy donors was stimulated with phytohemagglutinin at 37°C for 72 h. Culture supernatant fluids were collected, filtered, and used as conditioned media. IL-1α was purchased from Immugenex (Los Angeles, CA). IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, soluble IL-6 receptor (sIL-6R), interferon gamma (IFN-γ), granulocyte/macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor, and tumor necrosis factor (TNF)-α were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). IL-7 and IL-8 were purchased from Genzyme. 1,25-dihydroxyvitamin D_3 and dexamethasone were purchased from Wako Pure Chemical Co. (Osaka, Japan). Receptor activator of nuclear-factor-κB ligand (RANKL) was obtained from Peprotech (London, UK).

Assessment of phagocytic activity

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

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

Neutralizing antibodies specific for IL-3, IL-5, IL-7, and GM-CSF were used for inhibition of the generation of multinucleated giant bone-resorbing cells. Irrelevant polyclonal mouse IgG from Jackson ImmunoResearch (West Grove, PA, USA) was used as a control. The TRAP-positive mononuclear cells (5×10^4) were pre-incubated with each antibody in DMEM containing 10% FCS in microtubes at 37°C for 1 h. The cells were cultured in 4-well chamber slides, and stimulated with a cytokine for 96–120 h at 37°C in 7.5% CO_2. At the end of the culture period, the cells were stained for TRAP and the fusion index was calculated as described in the main paper.

Detection of calcitonin receptors

Calcitonin receptors on the multinucleated giant bone-resorbing cells were detected *in situ* using ^{125}I-human calcitonin were performed as described elsewhere [23]. TRAP-positive mononuclear cells (5×10^4) were stimulated with IL-3, IL-5, IL-7, or GM-CSF at the optimal concentrations in 4-well chamber slides (Nalge Nunc International, Rochester, NY, USA) for 96–120 h at 37°C in 7.5% CO_2. After formation of multinucleated giant bone-resorbing cells had been confirmed microscopically, the cells were incubated in 0.4 ml αMEM (Gibco BRL) with 0.1% bovine serum albumin and 0.2 mCi/ml of ^{125}I-labeled human calcitonin (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 h at 22°C. Nonspecific binding was assessed on each slide in the presence of an excess amount of unlabeled human calcitonin. Then the cells were washed three times with phosphate-buffered saline solution and fixed with 2.5% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The slides were washed and dried as previously described [S1]. Air-dried slides were dipped in photographic emulsion (Kodak NTB3; Eastman Kodak, Rochester, NY, USA), drained, and dried for 2 h and were kept in a light-proof container with desiccant at 4°C for 10 days. The slides were developed in accordance with the manufacturer's instructions.

Supplementary Table 1

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

Antigen		% Positive[a]		
		Monocytes from peripheral blood	TRAP-positive cells induced from monocytes	TRAP-positive cells from RA-SF
CD1a		1.24	0.11	0.24
CD1b		0.22	0.13	0.12
CD2		2.20	0.18	1.01
CD3		0.82	0.85	0.82
CD4		0.28	0.61	0.35
CD5		0.23	0.44	0.29
CD11a	LFA-1	94.72	1.16	1.18
CD11b	CR3 α chain	95.46	99.42	99.56
CD11c	CR4 α chain	99.47	99.22	99.01
CD13		99.44	96.15	93.87
CD14		95.70	99.28	99.63
CD15	Le^x	22.81	0.49	15.97
CD16	FcgR III	0.72	0.24	0.30
CD19		0.33	0.13	0.13
CD20		0.36	0.11	0.10
CD34		0.54	0.18	0.18
CD35	CR1	93.49	0.50	0.60
CD45		98.73	94.28	92.90
CD45RA		0.37	0.15	0.46
RA45RO		0.65	4.18	3.30
CD51/61	VNR[b]	0.22	0.10	0.17
CD54	ICAM-1	0.21	0.30	0.33
CD68		94.59	1.06	1.10
CD80	B7/BB1	8.19	7.41	6.52
CD83		0.37	0.10	1.42
CD86	B70/FUN-1	90.01	0.55	1.21
HLA-A, -B, -C		97.44	99.68	99.53
HLA-DR		96.65	98.45	98.16

[a]Cells were stained with monoclonal antibody specific for various antigens, and analyzed by FACScan. Data are representative of four independent analyses. [b]Vitronectin receptor. RA-SF = synovial fluid from patients with rheumatoid arthritis.

Supplementary results

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

The TRAP-positive mononuclear cells, whether induced from monocytes or collected from the synovial fluid of patients with RA, strongly expressed CD11b, CD11c, CD14, CD45, HLA-A, HLA-B, HLA-C, and HLA-DR but did not express CD1a, CD1b, CD2, CD4, CD5, CD16, CD19, CD20, or CD83. These observations suggest that these cells belong to the monocyte/macrophage lineage (Supplementary Table 1). However, the cells did not express CD11a, CD35, or CD68, which are expressed on freshly isolated monocytes from peripheral blood (Supplementary Table 2). The TRAP-positive mononuclear cells had strong phagocytic activity against heat-killed yeast (data not presented). In addition, the cells were positive for carbonic anhydrase II, actin, and vitronectin receptor (Supplementary Fig. 1), and calcitonin receptors were also

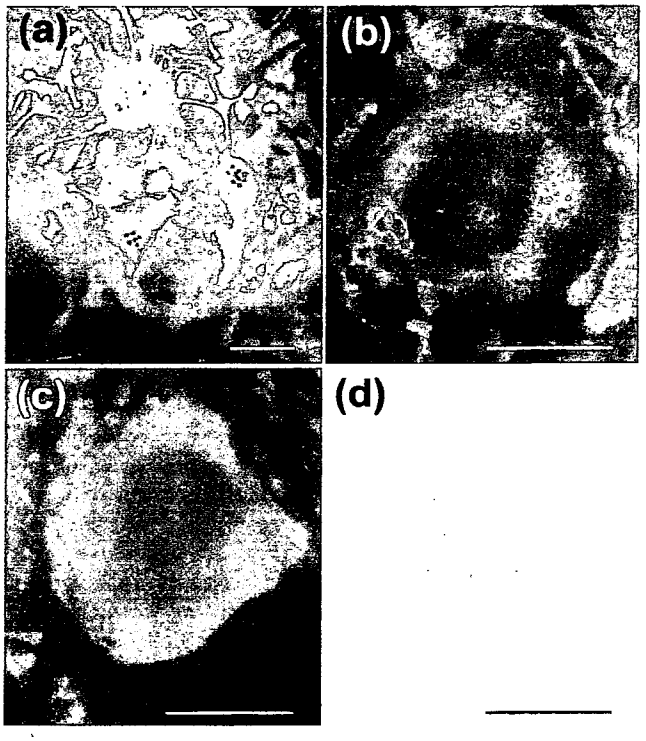
Supplementary Table 2

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

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

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

Supplementary Figure 1



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

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

Long-term maintenance of monocytes by RA-NLCs

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

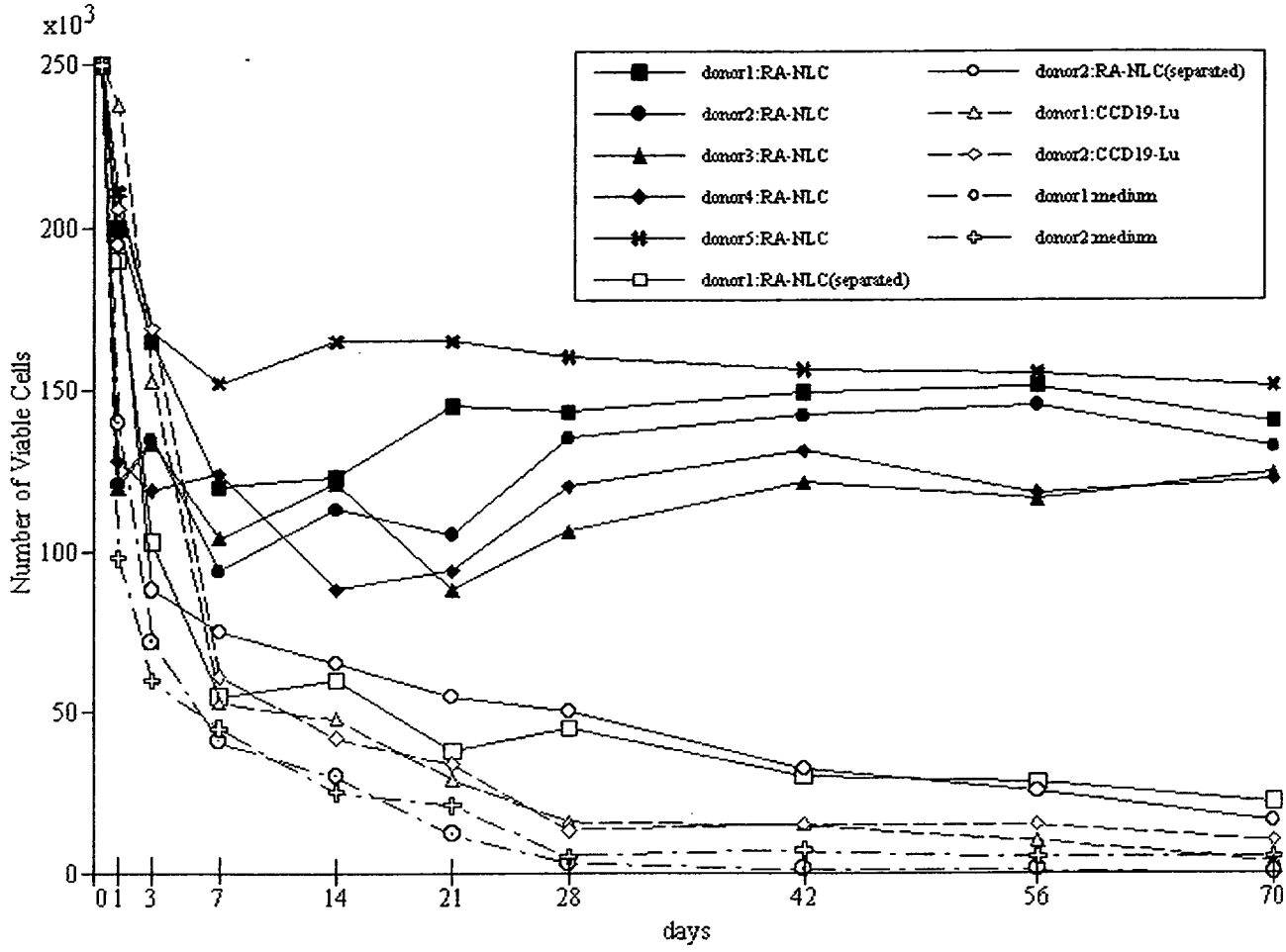
Induction of osteoclasts by IL-3, IL-5, IL-7, or GM-CSF

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

Supplementary reference

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

Supplementary Figure 2



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

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

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

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

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

Materials and Methods

Antibodies and reagents

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

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

Stromal cell lines and fibroblast lines

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

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

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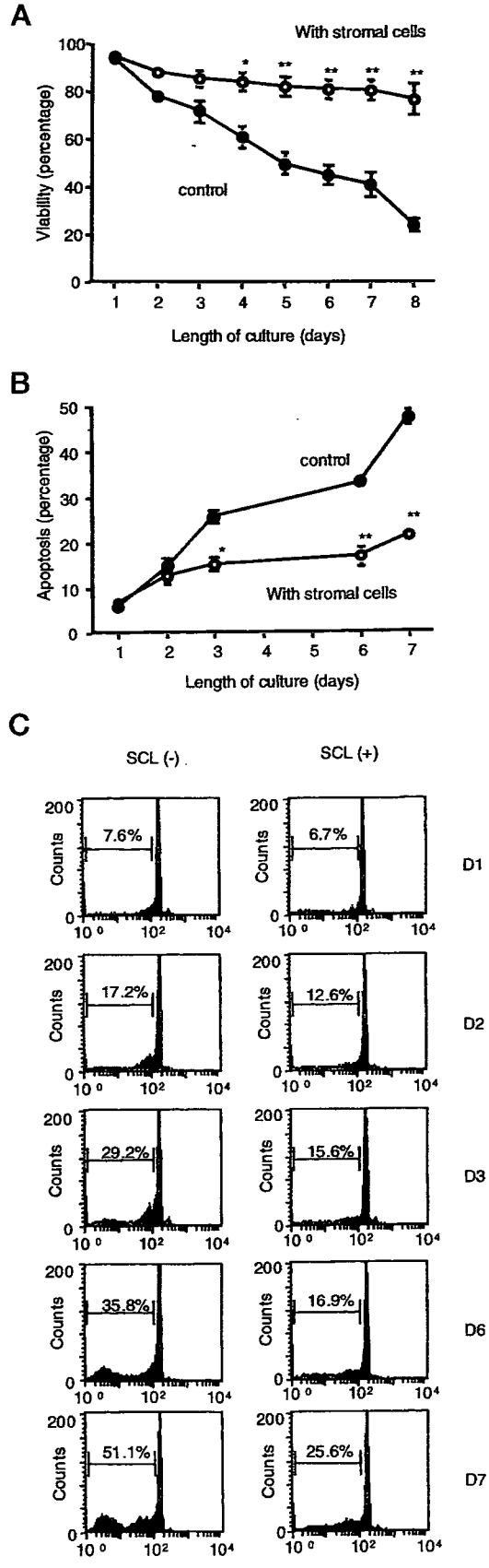

FIGURE 1. The SCL derived from RA synovial tissue rescue peripheral B cells from apoptosis. Peripheral B cells (1×10^5) were cultured with or without RA SCL (Sy77; 4×10^3) in 96-well microtiter plates. After various lengths of culture, B cells were harvested with gentle pipetting, and the percentages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

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

B cell separation

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

B cell culture

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

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

Analysis of viable cells

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

Detection of apoptosis

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

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

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

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

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

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

Western blotting

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

RNA isolation and RT-PCR

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

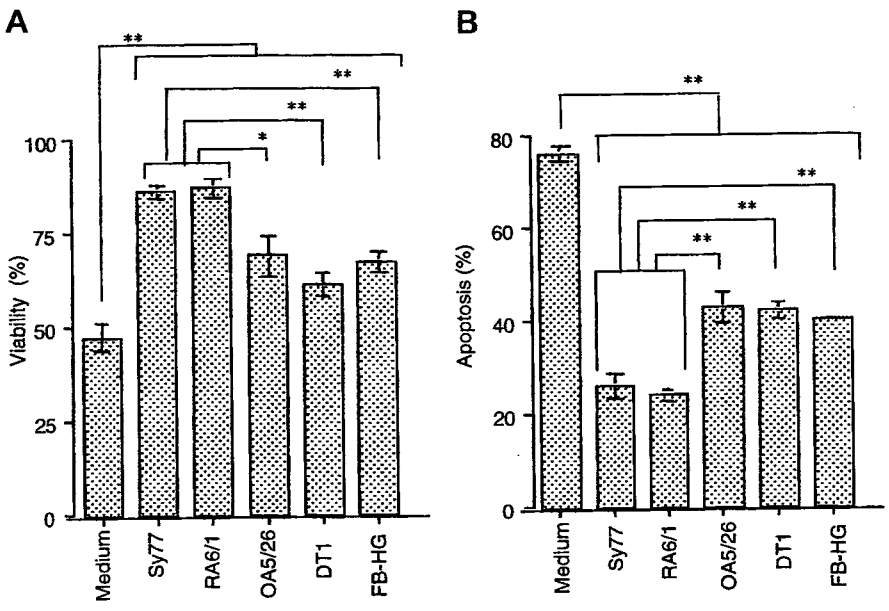

FIGURE 2. The RA SCL rescue B cells from apoptosis more effectively than other cell lines. Peripheral B cells (1×10^5) were cultured with various SCLs or fibroblasts (4×10^3) in 96-well microtiter plates. After 4 days of culture, B cells were harvested with gentle pipetting, and the percentages of viable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