TABLE I. Clinical Evaluations of LCAP Therapy in 13 Patients With RA

	Responders (n = 6)		Nonresponders (n = 7)	
ACR core set	Baseline[a,b]	After therapy[b,c]	Baseline[a,b]	After therapy[b,c]
Tender joint counts	8.7 ± 4.6	5.3 ± 3.8	10.4 ± 6.6	15.7 ± 10.1
Swollen joint counts	14.3 ± 8.0	4.3 ± 4.3	10.3 ± 10.4	11.1 ± 7.6
Pain scores (mm)	70.8 ± 11.0	43.3 ± 9.4	69.7 ± 24.8	61.4 ± 24.2
Patient's global assessment of disease activity (mm)	68.3 ± 10.7	38.3 ± 13.4	77.1 ± 20.5	68.6 ± 19.6
Physician's global assessment of disease activity (mm)	65.8 ± 9.3	35.0 ± 9.6	66.3 ± 13.3	71.4 ± 12.5
Health assessment of questionnaire	8.7 ± 5.3	4.0 ± 1.9	9.3 ± 5.0	9.3 ± 5.6
CRP (mg/L)	67.3 ± 42.4	30.6 ± 35.6	53.4 ± 29.3	33.4 ± 17.0

[a]Immediately before the first LCAP session.
[b]Values are mean ± SD.
[c]One week after completion of three sessions of LCAP.

TABLE II. Peripheral Blood Cell Counts in 13 Patients With RA

	Pre-1st LCAP[a,b] (/μL)	Post-1st LCAP[b,c] (/μL)	
Leukocytes	8259.2 ± 2321.0	9320.0 ± 3928.7	$P < 0.005$
Lymphocytes	1007.7 ± 381.5	540.0 ± 281.8	$P < 0.001$
Monocytes	386.8 ± 135.9	163.2 ± 109.2	$P < 0.001$
Neutrophils	6776.9 ± 2502.2	8480.4 ± 3999.1	NS
Platelets ($\times 10^{-4}$)	37.3 ± 10.2	18.1 ± 6.1	$P < 0.001$

[a]Immediately before the first LCAP session.
[b]Values are mean ± SD.
[c]Ten min after completion of the first LCAP session.

therapy). Six patients responded to the therapy according to the ACR criteria (20% improvement), and three of these six patients showed an ACR50 improvement. Seven patients did not respond. The results are shown in Table I.

Blood Cell Kinetics and Neutrophil Dynamics in the First LCAP Session

As shown in Table II, the blood cell kinetics during the LCAP sessions was evaluated in all 13 patients. The blood cell counts (mean ± SD, /μL) immediately before the first LCAP session (pre-LCAP) vs. 10 min after the completion of the first LCAP session (post-LCAP) were as follows: neutrophils, 6,777 ± 2,502 vs. 8,480 ± 3,999 (NS); lymphocytes, 1,008 ± 382 vs. 540 ± 282 ($P < 0.001$); monocytes, 387 ± 136 vs. 163 ± 109 ($P < 0.001$); platelets (10^{-4}), 37.3 ± 10.2 vs. 18.1 ± 6.1 ($P < 0.001$). The post-LCAP/pre-LCAP ratios of the neutrophil counts showed no significant difference between the responders and the nonresponders to LCAP therapy (data not shown).

The G-CSF levels did not change significantly during the LCAP session, and the values ranged from 538.6 ± 677.7 pg/mL to 432.5 ± 548.5 pg/mL (mean ± SD in the first LCAP session in all 13 patients); thus, the neutrophil recruitment in the present study could not be attributed to G-CSF induction. Various proportions of metamyelocytes and myelocytes were found in the post-LCAP peripheral blood of the patients (0–1% and 0–4% respectively, of the total leukocyte counts), similar to the results of a previous study on LCAP in healthy volunteers [16].

The flow cytometry analyses of peripheral blood leukocytes obtained from the Ficoll-interface showed that the post-LCAP blood cell fraction from the Ficoll-interface in the responders group contained a significant number of gating cells in addition to lymphocytes and monocytes. Three representative responders are shown in Figure 1A (Cases 1–3). On the other hand, three representative nonresponders are shown in Figure 1B. Gating cells, which were contained in the post-LCAP from the Ficoll interface in the responders group, were not observed in the nonresponders group (Fig. 1B, Cases 4–6).

A microscopic examination after the gating-in and sorting of this fraction revealed that most of these low-density cells, which were obtained from the Ficoll-interface after centrifugation, were band neutrophils, as confirmed by May-Giemsa staining (Figs. 2A and 2B). Figure 2C shows CD49dim positive and CD3-CD14-CD19-neutrophils. In contrast, the Gate 2 cells are recognized as CD49d+CD3+T cells in Figure 2D.

Band neutrophils were clearly identified in the responders, but not in the nonresponders, and the difference in these cell counts between the responders and the nonresponders was significant: 349.8/μL ± 84.4/μL (n = 6) vs. 48.0/μL ± 37.6/μL (n = 7), $P < 0.005$ (Fig. 3).

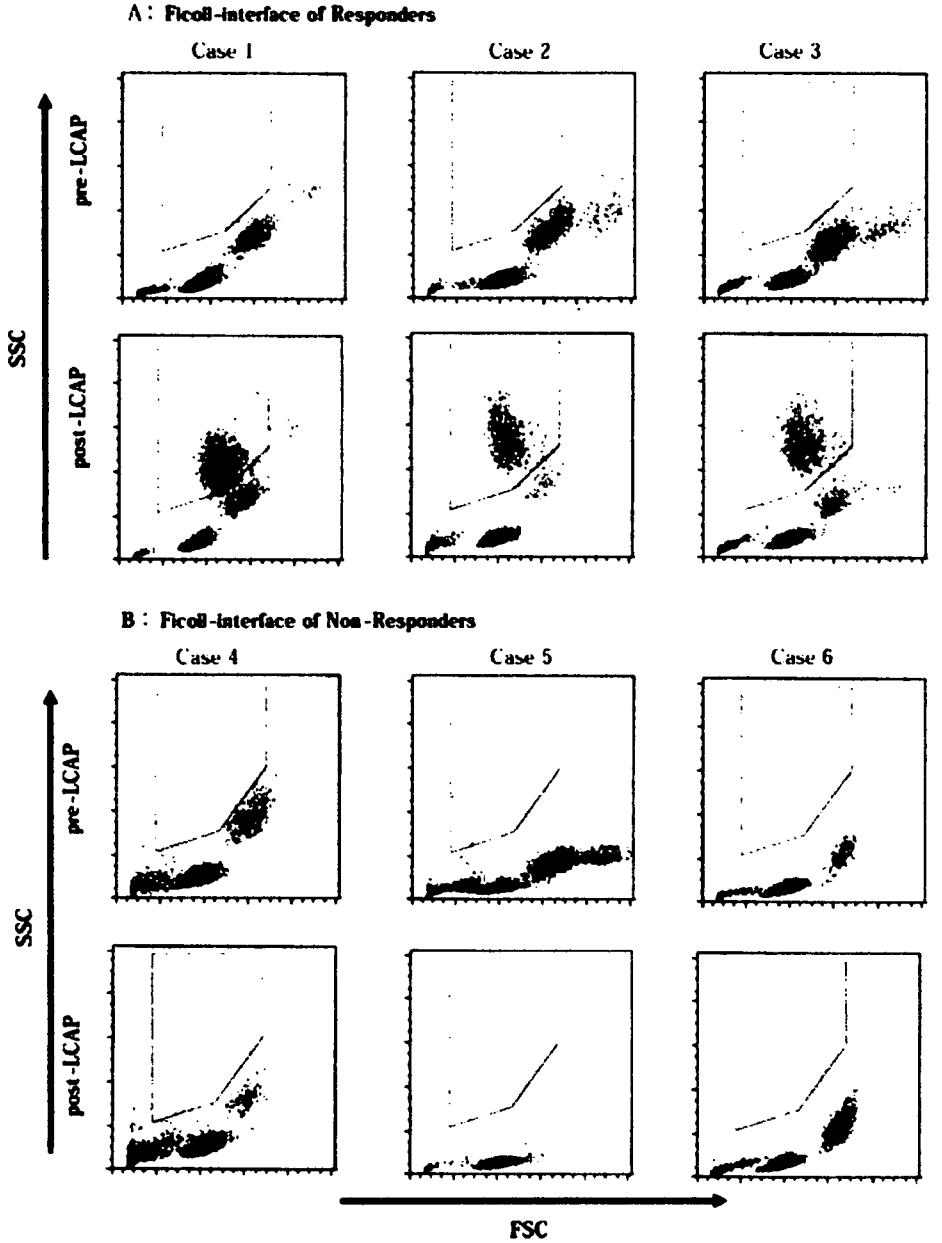

Fig. 1. Low-density band neutrophils found in the post-LCAP peripheral blood of RA patients. Pre-LCAP and post-LCAP mean "immediately before the first LCAP session" and "10 min after the completion of the first LCAP session", respectively. Leukocytes obtained from the Ficoll-interface were analyzed in all 13 patients. Three representative cases of both responders (A; Cases 1–3) and nonresponders (B; Cases 4–6) were shown as flow cytometry patterns. A cell population different from the lymphocytes or monocytes in the post-LCAP blood of the responders (Cases 1–3) was found (A, post-LCAP).

The low-density neutrophils found in the responders were CD49d diminished positive ($CD49d^{dim+}$) cells (Fig. 2C). CD49d (VLA-4) molecules are highly expressed in lymphocytes, as shown in Figure 2D, and are also expressed on neutrophils at various stage of neutrophil development in bone marrow, disappearing with maturation [19,20]. Thus, the low-density neutrophils found in our experiments were suggested to be of bone marrow origin. These neutrophil absolute counts may be very small in the blood of the responders, probably because the cells may acquire a higher density within a day and/or migrated out of the peripheral blood on the day after the LCAP session (data not shown). The earlier findings suggested that the recruitment of bone-marrow-derived band neutrophils is associated with the therapeutic efficacy of LCAP and that

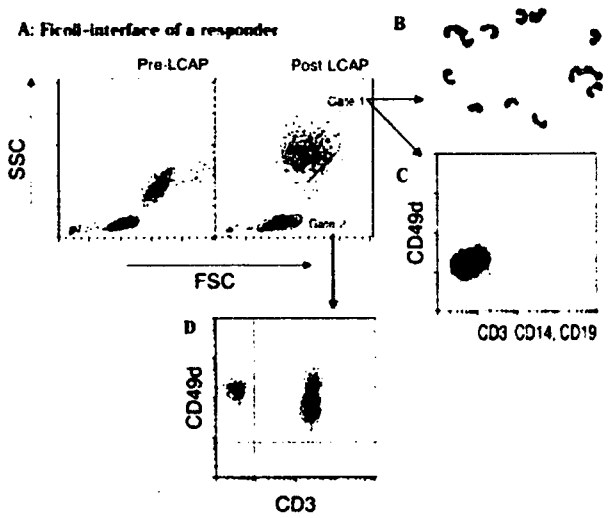

Fig. 2. Characteristics of low-density band neutrophils found in the post-LCAP peripheral blood of a responder. (A) Flow cytometry patterns of cells from the Ficoll-interface are shown for pre- and post-LCAP blood samples from a responder. (B) Microscopic appearance of the gating cells after sorting and May-Giemsa staining. (C) Multiple staining of the Gate-1 cells with FITC- and PE-conjugated mAbs. (D) Lymphocytes (Gate-2) stained with FITC- and PE-conjugated mAbs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

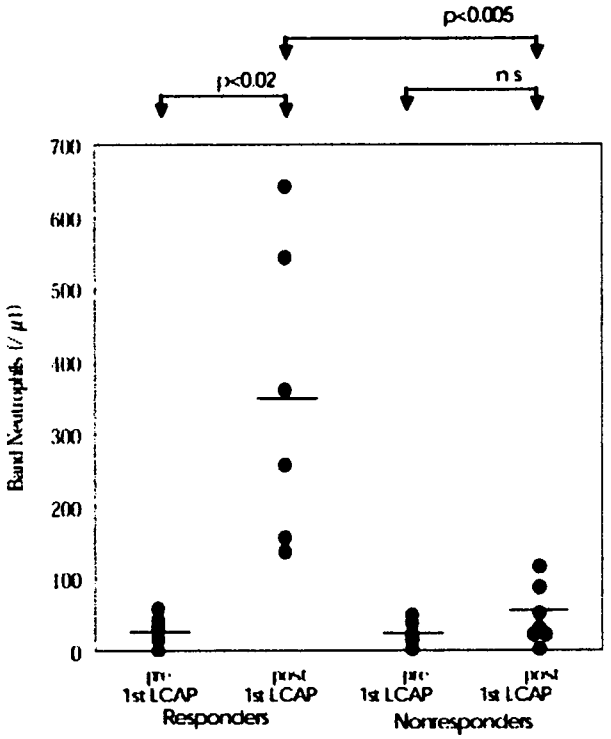

Fig. 3. Low-density band neutrophil counts in pre- and post-LCAP blood samples from RA patients. The definitions of pre-LCAP and post-LCAP are the same as in Figure 1. The cell numbers were calculated from laboratory data for the peripheral blood WBC and neutrophil counts and from the percentage of neutrophil-gated cells on the Ficoll-interface used in the FACS analysis.

the pool of these cells is almost depleted in nonresponders.

Serum IL-10 Levels

An elevation in serum IL-10 levels during the LCAP session was observed in most of the 13 patients. The mean ± SD level immediately before the first LCAP session was 0.9 ± 1.2 pg/mL (range, 0–4.0 pg/mL; n = 13), while that at 10 min after the completion of the first LCAP session (post-LCAP) was 17.1 ± 15.6 pg/mL (range, 2.9–49.9 pg/mL; n = 13; Fig. 4A, $P < 0.001$). No difference in the post-LCAP serum IL-10 levels was observed between the responders and the nonresponders (responders, 18.0 ± 16.4 pg/mL [range, 2.9–49.9 pg/mL; n = 6] vs. nonresponders, 11.6 ± 7.8 [range, 6.0–48.8 pg/mL; n = 7]; Fig. 4B, ns). Thus, the elevated serum IL-10 levels after LCAP appeared to be insufficient to account for the therapeutic effect of LCAP in the RA patients. The increases in after LCAP may play a role in suppressing inflammation, regardless of the lack of a difference between responders and nonresponders, but the increase in IL-10 after LCAP treatment may not be a main factor differentiating responders and nonresponders.

CONCLUSIONS

The primary action of LCAP is the adsorption of peripheral blood leukocytes, followed by prompt neutrophil recruitment. This initial phase of LCAP may be followed by a later phase in which possibly altered interactions among the redistributed immunocompetent cells may lead to antiinflammatory effects on local RA or UC lesions. As reviewed in the introduction [9–11,13], previous investigators have studied the later phase of the therapeutic effects, based on data obtained several days after repeated cytapheresis, in contrast to the present study, which examined the initial phases.

In our preliminary analyses of blood lymphocyte- and monocyte-subsets using monoclonal antibodies against CD3, CD4, CD14, CD19, CD45RA, CD45RO, CD11a, CD11b, CD49d, CD62L, and IL-10 in RA patients, the post-LCAP/pre-LCAP ratios of the CD19+ cell counts were consistently smaller than those of the CD3+ cell counts (data not shown), in accordance with previous observations of LCAP in healthy volunteers [16].

The present study showed that post-LCAP peripheral blood contained a significant number of newly recruited neutrophils, including those with phenotypes not commonly recognized in human peripheral blood. We found that these post-LCAP cells were immature neutrophils that may be pooled and readily recruited

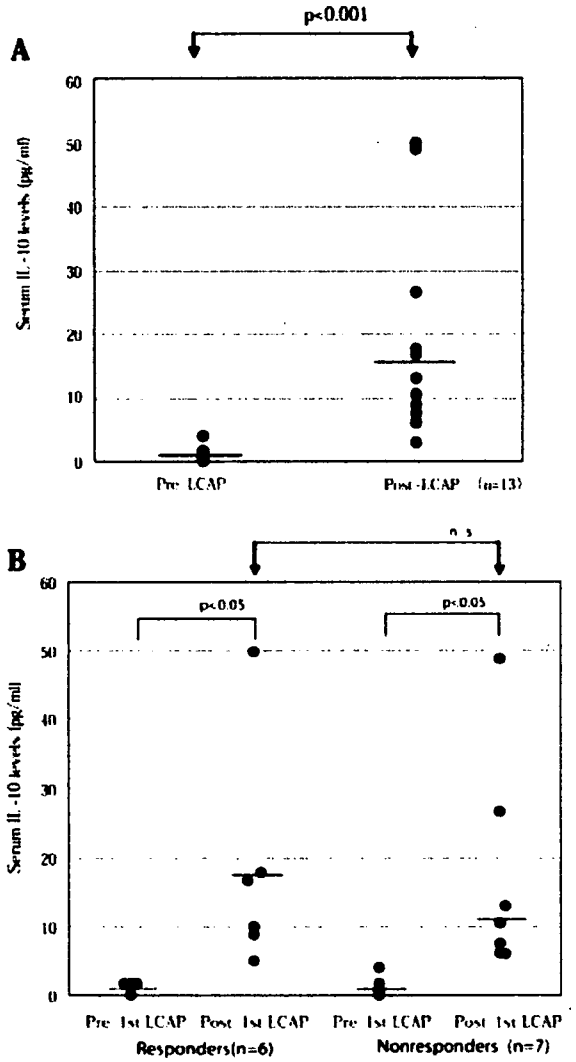

Fig. 4. IL-10 levels in peripheral blood before and after LCAP. Pre- and post-LCAP blood samples were obtained during each session. The IL-10 levels in the post-LCAP samples were significantly elevated in all the patients (A; $n = 13$, $P < 0.001$). The post-LCAP serum IL-10 levels of the responders ($n = 6$) and the nonresponders ($n = 7$) were not significantly different (B, ns).

from bone marrow, especially among the RA patients who responded to LCAP. A previous report indicated that myelocytes and promyelocytes appear in peripheral blood after LCAP in healthy volunteers [16], but the characteristic recruitment of band cells in responders after LCAP and their use as a predictor of LCAP outcome has not been reported to date. In addition, this result has also not been reported for patients with UC.

Mature human neutrophils retain their capacity to synthesize cytokines, including IL-1 [21] and IL-8 [22], when stimulated. A dermatologic study demonstrated a large number of IL-10+ neutrophils, in addition to well-known IL-10+ macrophages that infiltrated ultraviolet-B-irradiated human skin and reported the involvement of these neutrophils in UVB-induced immunosuppression [23].

Another recent study described two novel subsets of mature neutrophils, IL-12+CD11b-CD49d+ neutrophils and IL-10+CD11b+CD49d- neutrophils, in mouse blood [24]. The latter subset was obtained from mice sensitive to infection with methicillin-resistant *Staphylococcus aureus*. These neutrophils induced alternatively activated macrophages, which are known to enhance the expressions of IL-1 receptor antagonists and IL-10 [24]. However, these cells have not been reported in human peripheral blood.

IL-10 production occurs mainly in monocyte/macrophage cells in human peripheral blood, and the increase in IL-10 levels in peripheral blood post-LCAP, which exerts an antiinflammatory function, may participate in the clinical efficacy of LCAP in RA patients. However, monocytes were deleted by LCAP treatment in the same manner in both responders and nonresponders (Fig. 4). Furthermore, the relationship between the IL-10 levels in the peripheral blood and the number of band neutrophils post-LCAP was not significant according to our observations.

In a recent report on pulse-LCAP, large quantities of (5L) LCAP in normal volunteers recruited not only mature neutrophils, but also cells of all stages and differentiations, such as CFU-GEMM, CFU-GM, and juvenile granulocyte precursor cells capable of cell division—from myeloblasts to myelocytes [25]. The data from this previous study showed that the recruitment of various stages of WBCs was important for the effectiveness of LCAP treatment in RA patients. This finding supports our results that the band neutrophils found post-LCAP were only recruited in the LCAP responders.

Although the recruitment of immature neutrophils in peripheral blood following the first session of LCAP for RA may reflect the clinical effect of LCAP, a long-term curative effect does not seem to persist after LCAP treatment. Immature neutrophils found in post-LCAP samples after the first session were not found the following week during the second pre-LCAP session. Immature neutrophils, which were recruited post-LCAP, may mature or move to marginal pools within a short time period. Although the absolute number of band cells among the WBCs in post-LCAP samplings might be relatively low, the numbers of band cells in responders and nonresponders were significantly different.

On the other hand, long-term observations have suggested that patients who undergo LCAP treatment may exhibit an improvement in their clinical symptoms for as long as 4 months after LCAP treatment [26].

In our results, low-density and stab-formed neutrophils in the peripheral blood of responder patients after the first LCAP session were also found after the second or third LCAP sessions in these patients (data not shown). On the other hand, the immature neutrophils

were not found after each LCAP session in nonresponder patients. Therefore, the results of the first LCAP session may indicate whether a curative effect can be attained and whether further LCAP sessions will be beneficial.

Although the direct association of the recruitment of stab neutrophils following the first LCAP session and the improvement in arthralgia could not be confirmed, the appearance of these immature neutrophils found in peripheral blood following the first session of LCAP for RA may predict the clinical efficacy of LCAP.

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Rheumatoid Arthritis Fibroblast-like Synoviocytes Express BCMA and Are Stimulated by APRIL

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Objective. **Fibroblast-like synoviocytes (FLS) are among the principal effector cells in the pathogenesis of rheumatoid arthritis (RA). This study was undertaken to examine the variety of stimulating effects of APRIL and its specific effect on FLS in the affected RA synovium.**

Methods. **Synovium and serum samples were obtained from patients with RA, patients with osteoarthritis (OA), and healthy subjects. Soluble APRIL proteins were assayed by enzyme-linked immunosorbent assay. The relative gene expression of APRIL, BCMA, interleukin-6 (IL-6), tumor necrosis factor α (TNFα), IL-1β, and RANKL was assessed in RA and OA FLS by polymerase chain reaction. Effects of APRIL on the production of proinflammatory cytokines and RANKL in RA FLS were investigated by flow cytometry and with the use of a BCMA-Fc fusion protein.**

Results. **A significantly higher level of soluble APRIL was detected in RA serum compared with normal serum. Among the 3 receptors of APRIL tested, RA FLS expressed only BCMA, whereas OA FLS expressed none of the receptors. APRIL stimulated RA FLS, but not OA FLS, to produce IL-6, TNFα, IL-1β, and APRIL itself. In addition, APRIL increased RA FLS expression of RANKL and also enhanced progression of the cell cycle of RA FLS. Neutralization of APRIL by the BCMA-Fc fusion protein attenuated all of these stimulating effects of APRIL on RA FLS.**

Conclusion. **RA FLS are stimulated by APRIL and express the APRIL receptor BCMA. These results provide evidence that APRIL is one of the main regulators in the pathogenesis of RA.**

Rheumatoid arthritis (RA) is characterized by joint destruction resulting from chronic inflammation in the synovial tissue. The chronicity of the disease is postulated to be maintained by interactions between infiltrating mononuclear cells and synovial cells (1,2), in addition to the autocrine stimulatory effects of proinflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 (1). Fibroblast-like synoviocytes (FLS) act as one of the main effector cells in the joint destruction of RA, through their ability to invade and degrade soft tissue and cartilage (1,3). FLS can also stimulate the differentiation and activation of osteoclasts, resulting in bone erosion (4–6).

Recent research has provided important information about the signaling mechanisms that can target FLS in the affected RA synovium, such as mediators of inflammation, cytokines, and cell–cell and cell–extracellular matrix interactions (7). These signaling mechanisms underlie the ability of RA FLS to drive migration, proliferation, and matrix degradation. Moreover, RA FLS have been shown to proliferate in an anchorage-independent manner, to lack contact inhibition, and to constitutively express cytokines, oncogenes, and cell cycle proteins, in a transformation-related manner (8,9).

BAFF (also termed B lymphocyte stimulator, or BlyS [trademark of Human Genome Sciences, Rockville, MD]), a member of the TNF family, is essential for B cell generation, maintenance, and autoreactivity (10–12). High levels of BAFF are detectable in the sera of patients with autoimmune rheumatic diseases, particularly systemic lupus erythematosus (SLE) and Sjögren's syndrome (13–15). BAFF is also present at high levels in

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rheumatoid synovial fluid (14–16) and has been shown to be expressed in the synovial tissue of patients with RA. However, the expression profile and role of BAFF in the pathogenesis of RA remain unclear (2,17).

A proliferation-inducing ligand, or APRIL, is a close homolog to BAFF and is produced as a secreted ligand (18). APRIL is expressed by dendritic cells, macrophages, T cells, and B cells, and enhances the proliferation and survival of T and B cells (19). Moreover, the raised levels of APRIL in the serum of patients with SLE suggest that APRIL may be involved in autoimmunity (20). In addition to these immunologic functions, APRIL was originally identified as a ligand involved in the proliferation of tumors (21). APRIL was shown to have a remarkable capacity to stimulate both solid and lymphoid tumor growth (21,22). Currently, 2 TNF receptor family members, TACI and BCMA, have been shown to bind to APRIL with high affinity (23).

In the present series of experiments on BAFF and its related molecules in the affected joints of patients with RA, we found that RA FLS expressed BCMA, but not TACI or the BAFF receptor (BAFF-R), whereas FLS from patients with osteoarthritis (OA FLS) expressed none of these receptors. We thus assessed the effects of APRIL through its receptor, BCMA, on RA FLS in comparison with OA FLS.

PATIENTS AND METHODS

Patients and synovial specimens. Synovial tissue specimens were obtained at the time of total joint replacement surgery from 16 patients who fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the diagnosis of RA (24). Synovial tissue specimens from 12 patients with OA were evaluated as disease controls. For analysis of soluble APRIL proteins, synovial fluid samples were collected from another series of 38 patients with RA who required a therapeutic arthrocentesis of the affected joints. In addition, sera were obtained from 31 of these RA patients and from 51 healthy subjects as normal controls.

All patients who participated in this study provided their informed consent. All of the experiments carried out were approved by the ethics committee of the International Medical Center of Japan.

Enzyme-linked immunosorbent assay (ELISA). Soluble APRIL proteins in the serum and synovial fluid were assayed with a sandwich ELISA using a human APRIL ELISA kit (Bender MedSystems, Burlingame, CA) following the manufacturer's instructions. Optical density was measured with an ImmunoMini NJ-2300 plate reader (InterMed, Tokyo, Japan). Synovial fluid samples were diluted to 1:20 to avoid the possibility of assay error due to high viscosity of the samples. The diluted synovial fluid was then assessed by ELISA for soluble APRIL. As a test of the validity of the APRIL ELISA system in synovial fluid, 3 synovial fluid samples were intentionally spiked with known amounts of standard recombinant APRIL protein. The measured concentration of APRIL was as predicted in all tested assays. The results showed that there was no evidence of assay inhibitors and no difference in the recovery rate between the tested synovial fluid samples.

Cell preparation. Synovial tissue specimens from patients with RA and patients with OA were digested with deoxyribonuclease I (Worthington, Lakewood, NJ), type IV collagenase (Worthington), and hyaluronidase (Sigma, St. Louis, MO), to obtain single-cell suspensions. After overnight culture in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (ICN Biomedicals, Aurora, OH), penicillin–streptomycin (Invitrogen), and gentamycin (Sigma) in a humidified atmosphere of 5% CO_2, adherent cells were cultivated as FLS. At confluence, cells were trypsinized and recultured in medium. FLS from passages 4–6 were used in each experiment. Normal human dermal fibroblasts were used as the normal control cells.

Reverse transcription–polymerase chain reaction (RT-PCR) and real-time PCR analyses. Total RNA was extracted from RA or OA FLS using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 μg) was quantified with spectrophotometry and reverse-transcribed using an oligo$(dT)_{15}$ primer (Promega, Madison, WI) and Superscript II RNase H–reverse transcriptase (Life Technologies, Gaithersburg, MD) at 42°C for 2 hours. The RT-PCR process was carried out in a GeneAmp PCR 9700 system (Applied Biosystems, Foster City, CA). To ensure that each sample contained the same amount of complementary DNA (cDNA), we determined the concentration of GAPDH cDNA in each sample, using GAPDH-specific primers. All samples were amplified for the appropriate number of cycles, so that the amount of the PCR product obtained was within the linear portion of the amplification curve.

The PCR products were electrophoresed on a 2% agarose gel and were visualized by ethidium bromide staining. To check the relative levels of gene expression for APRIL, BCMA, IL-6, TNFα, IL-1β, and RANKL in the RA and OA FLS, real-time PCR was performed with a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) in an ABI PRISM 7700 Sequence Detection system (Applied Biosystems) according to the manufacturer's instructions. Data analysis was performed using ABI PRISM Sequence Detection software, version 1.7 (Applied Biosystems). The specific primer sets used for RT-PCR and real-time PCR were as follows: for GAPDH, 5′-GAAATCCCATCACCATCTTCCAG-3′ (forward) and 5′-ATGAGTCCTTCCACGATACCAAAG-3′ (reverse); for APRIL, 5′-CCAGCCTCATCTCCTTTCTTGC-3′ (forward) and 5′-GGTTGCCACATCACCTCTGTCAC-3′ (reverse); for BAFF-R, 5′-GGTCCTGGTGGGTCTGGTGAG-3′ (forward) and 5′-GGCTGAATGCTGTGGTCTGTAGTG-3′ (reverse); for TACI, 5′-TATGAGATCCTGCCCCGAAGAG-3′ (forward) and 5′-TCTGAGCCTCTGTGCTCCAATC-3′ (reverse); for BCMA, 5′-TCTCTGGACCTGTTTGGGACTGAG-3′ (forward) and 5′-CGTGGTGACAAGAATGGTTGC-3′ (reverse); for IL-6, 5′-CACCTCTTCAGAACGAATTG-3′ (forward) and 5′-GGATCAGGACTTTTGTACTC-3′ (reverse); for TNFα, 5′-CCACGCTCTTCTGCCTGCTG-3′ (forward) and 5′-CTGGAGCTGCCCCTCAGCTT-3′ (reverse); for IL-1β, 5′-AAAGCTTGGTGATGTCTGGT-3′ (forward) and 5′-TCTACACTCTCCAGCTGTAG-3′ (reverse); and for

RANKL, 5′-AGACACAACTCTGGAGAGTCAAG-3′ (forward) and 5′-TACGCGTGTTCTCTACAAGGTC-3′ (reverse).

Flow cytometry. RA FLS (from passages 4–6) from 16 patients with RA and OA FLS (from passages 4–6) from 12 patients with OA were trypsinized and harvested. The RA or OA FLS were stained with a monoclonal antibody (mAb) to biotinylated goat anti-human APRIL (R&D Systems, Minneapolis, MN) with streptavidin–phycoerythrin (PE) (BD Biosciences, San Diego, CA), rat anti-human BCMA–fluorescein isothiocyanate (Alexis, Nottingham, UK), mouse anti-human RANKL-PE (eBioscience, San Diego, CA), and an appropriate isotype control antibody. Cells were incubated on ice for 30 minutes in phosphate buffered saline (PBS) containing 2% FCS. Before the staining of cytoplasmic APRIL and RANKL, cells were incubated in cold 4% paraformaldehyde fixative in PBS at room temperature for 10 minutes, and then washed with 0.05% saponin (ICN Biochemicals, Irvine, CA) in Hanks' balanced salt solution (Sigma) for permeabilization. Analysis of the cells was performed using a BD FACSCalibur system (BD Immunocytometry Systems; BD Biosciences) and FlowJo software (version 6.1.1; Tree Star, Ashland, OR).

Immunohistochemistry. The antibodies used for immunohistochemical visualization were mouse anti-human APRIL (Aprily-2) mAb (1:400; Alexis) and goat anti-human BCMA (N-16) polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Frozen sections from 8 RA synovial tissue samples and 2 OA synovial tissue samples were fixed in cold acetone for 10 minutes. The sections were immunostained using a DakoCytomation Autostainer (DakoCytomation, Carpinteria, CA), with DakoCytomation LSAB+–horseradish peroxidase (DakoCytomation), and finally counterstained with hematoxylin (DakoCytomation).

Cell culture. RA FLS (from passages 4–6) were washed once in PBS and starved in an FCS-free RPMI 1640 medium for 24 hours. After the starvation period, RA FLS, at 1×10^5 cells per well, were seeded onto 24-well plates; as controls, OA FLS were evaluated in a similar manner. The FLS were cultured with 0.2% FCS in RPMI 1640 medium in the presence of 3–300 ng/ml recombinant human *Mega*APRIL (Alexis) or BAFF (PeproTech EC, London, UK), or in medium alone, for 24 hours. Thereafter, the adherent FLS were collected for further analysis. In some experiments, 1 μg/ml recombinant human BCMA-Fc fusion protein (R&D Systems), or control IgG1 (Ancell, Bayport, MN), was added to block the interaction of APRIL with BCMA.

Cell cycle analysis. After the 24-hour cell starvation period, RA FLS, at 1×10^5 cells per well, were seeded onto 24-well plates. Cells were cultured with 0.2% FCS in RPMI 1640 medium in the presence of 300 ng/ml of recombinant human APRIL. A recombinant human BCMA-Fc fusion protein (1 μg/ml), or control IgG1 (Ancell), was added to block the interaction of APRIL with BCMA. The adherent FLS were then collected and gently resuspended in 0.5 ml hypotonic fluorochrome solution (50 μg/ml propidium iodide [Sigma] in 0.1% sodium citrate plus 0.1% Triton X-100) (25) in 12 × 75–mm tubes. The tubes were placed in darkness at 4°C overnight, and the cells were then assessed by flow cytometry. The fluorescence of individual nuclei, detected by propidium iodide staining, was measured using a FACSCalibur cytometer (BD Biosciences) and FlowJo software (version 6.1.1; Tree Star).

Table 1. Characteristics of the patients with rheumatoid arthritis (RA) and patients with osteoarthritis (OA)

	RA (n = 16)	OA (n = 12)
No. male/female	3/13	3/9
Age, mean ± SD years	62.7 ± 11.7	77.5 ± 5.26
CRP, mean ± SD mg/dl*	2.64 ± 1.79	0.11 ± 0.07
Disease duration, mean ± SD years	25.2 ± 7.15	27.5 ± 5.27

* The level of C-reactive protein (CRP) in unaffected individuals is 0.00–0.30 mg/dl.

Statistical analysis. Results are expressed as the mean ± SEM. Statistical evaluation was performed with the Mann-Whitney U test. *P* values less than 0.05 were considered significant.

RESULTS

Characteristics of the patients. Synovial tissue specimens were obtained at the time of total joint replacement surgery from 16 patients with RA and from 12 patients with OA (as disease controls). As shown in Table 1, most of the RA patients showed a rather high disease activity as indicated by elevated serum C-reactive protein (CRP) levels, whereas the OA patients were generally observed to have normal levels of CRP.

Raised levels of soluble APRIL in the synovial fluid and serum of RA patients. We collected synovial fluid samples (n = 38) and serum samples (n = 31) from another series of RA patients to analyze the soluble

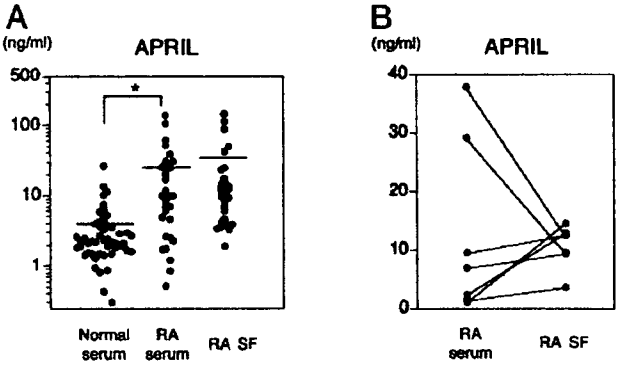

Figure 1. Concentrations of soluble APRIL in the serum and synovial fluid (SF) of patients with rheumatoid arthritis (RA). **A**, Levels of soluble APRIL were measured by sandwich enzyme-linked immunosorbent assay in RA serum samples (n = 31) and RA SF samples (n = 38); sera from 51 healthy individuals were used as normal controls. Circles indicate individual data; horizontal bars show the mean per group. * = $P < 0.05$. **B**, Levels of soluble APRIL were compared in paired samples of serum and SF, obtained on the same day, from 7 patients with RA.

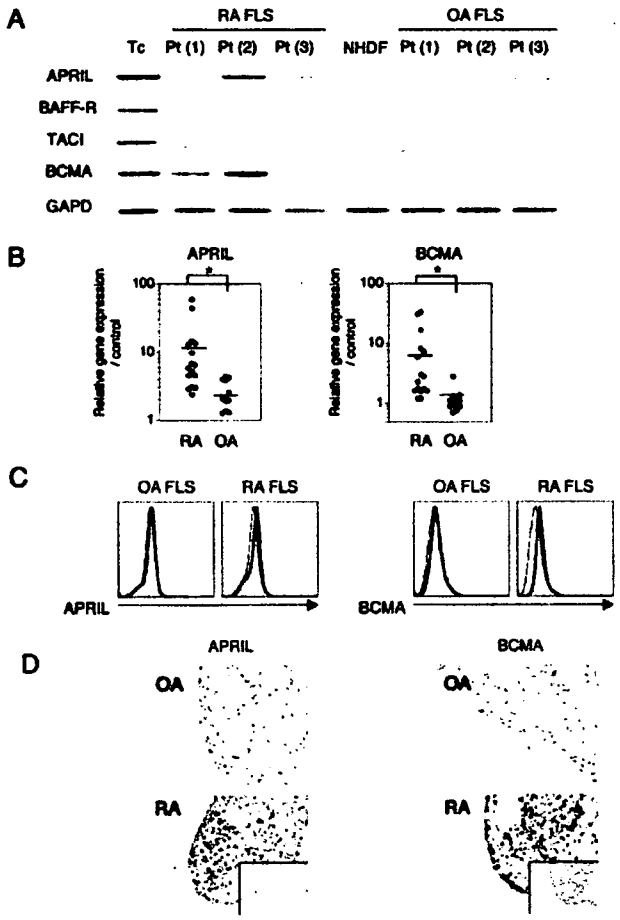

Figure 2. Expression of APRIL and its receptors in fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA FLS). **A,** Levels of mRNA for APRIL, BAFF receptor (BAFF-R), TACI, and BCMA, relative to GAPDH, were assessed in RA FLS and in FLS from patients with osteoarthritis (OA FLS) by reverse transcription–polymerase chain reaction (PCR); tonsil cells (Tc) were the positive control, and normal human dermal fibroblasts (NHDFs) were the normal control. Representative results, obtained from 3 patients (Pt) per group, are shown. **B,** Relative gene expression levels of APRIL and BCMA were assessed in RA and OA FLS by real-time PCR; NHDFs were used as the control. Horizontal bars show the mean of 16 RA FLS and 12 OA FLS samples. * = $P < 0.05$. **C,** Expression levels of APRIL and BCMA in RA and OA FLS were assessed by flow cytometry. Cells were stained with monoclonal antibodies (mAb) specific for APRIL and BCMA (shaded areas) or an isotype control mAb (open areas). Fluorescence histograms show results representative of 16 RA FLS and 12 OA FLS samples. **D,** Expression patterns of APRIL and BCMA in RA and OA synovial tissue were assessed by immunohistochemical analysis; insets show the isotype controls (original magnification × 400). Results are representative of 8 RA synovial tissue samples and 2 OA synovial tissue samples.

APRIL concentration. Serum samples from healthy individuals (n = 51) were used as a control. As shown in Figure 1A, the level of soluble APRIL in RA serum was significantly higher than that in normal serum (mean 21.09 ng/ml versus 3.49 ng/ml; $P < 0.05$ by Mann-Whitney U test). Unexpectedly, the level of soluble APRIL in RA synovial fluid did not exceed that in RA serum (mean 23.73 ng/ml versus 21.09 ng/ml), whereas the level of soluble BAFF has been shown to be higher in the synovial fluid than the serum of RA patients (14–16). A comparison of the soluble APRIL concentrations in 7 pairs of RA serum and synovial fluid samples that were obtained at the same time revealed no correlation between the soluble APRIL concentrations in the 2 specimens (Figure 1B).

Expression of APRIL and its receptors in RA FLS. We recently examined the expression profiles of BAFF and BAFF-R in affected joints from patients with RA and in isolated RA FLS (26). We found that RA FLS expressed BAFF, but did not express BAFF-R, the specific receptor of BAFF. This suggests that BAFF might not be the signal that targets RA FLS.

We therefore determined the expression profiles of messenger RNA (mRNA) for APRIL and its receptors in RA and OA FLS, using RT-PCR; tonsil cells were used as positive controls for the expression of these molecules. As shown in Figure 2A, RA FLS spontaneously expressed a large amount of APRIL mRNA after 4–6 passages, whereas OA FLS expressed only a small amount of APRIL mRNA after 4–6 passages. Of interest, RA FLS expressed BCMA, but not BAFF-R or TACI. OA FLS expressed almost no mRNA for any of these receptors. Moreover, the results of quantitative real-time PCR analysis demonstrated that both APRIL mRNA and BCMA mRNA were expressed at significantly higher levels in RA FLS than in OA FLS (Figure 2B). Flow cytometry revealed that APRIL and BCMA proteins were expressed in RA FLS, but not in OA FLS (Figure 2C).

We also examined the localization of expression of APRIL and BCMA in the RA synovium by immunohistochemical analysis. As shown in Figure 2D, APRIL was expressed in the hyperplasic synovial lining cells, mononuclear cells, and lymphocytes infiltrating the synovial sublining area. In contrast, BCMA was expressed on the synovial lining cells and plasma cells in the lymphoid aggregation. All 8 samples of RA synovium analyzed showed positive staining for APRIL and BCMA. Neither APRIL nor BCMA was expressed in the 2 OA synovium samples analyzed. APRIL, but not BAFF, has been shown to bind to BCMA with high affinity (23,27). These findings suggest that APRIL might be one of the mediators that can target RA FLS, in both an autocrine and a paracrine manner.

APRIL-induced production of proinflammatory cytokines on RA FLS. Our finding that RA FLS express both APRIL and one of its receptors, BCMA (Figure 1A), suggests that secreted APRIL from RA FLS might affect, either positively or negatively, the RA FLS in an autocrine manner. We therefore determined the effect of APRIL on the expression of APRIL itself in RA FLS. As shown in Figure 3A, 300 ng/ml of recombinant APRIL significantly enhanced the mRNA expression of APRIL itself in RA FLS, but not in OA FLS.

We then compared the effects of APRIL on the production of the proinflammatory cytokines IL-6, TNFα, and IL-1β between RA FLS and OA FLS. RA FLS showed enhanced mRNA expression of these proinflammatory cytokines after treatment with APRIL, whereas OA FLS did not respond to treatment with APRIL (Figure 3B). In contrast, BAFF did not stimulate the expression of any of these proinflammatory cytokines on either RA FLS or OA FLS (results not shown). The addition of the BCMA-Fc fusion protein to the

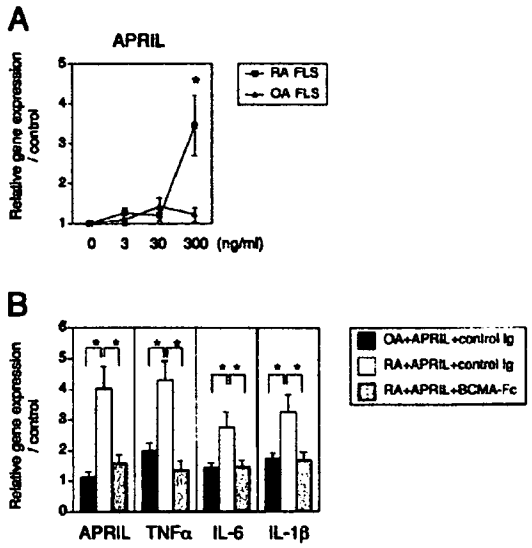

Figure 3. APRIL induction of APRIL itself and of proinflammatory cytokine production in RA FLS. **A**, RA and OA FLS were starved in fetal calf serum (FCS)–free RPMI 1640 medium for 24 hours, after which a total of 1×10^5 RA FLS per well were seeded onto 24-well plates. The FLS were then cultured in 0.2% FCS–RPMI 1640 medium with 3–300 ng/ml recombinant human APRIL for 24 hours; medium alone served as the control. The relative gene expression of APRIL was analyzed by real-time PCR. Results are the mean ± SD from 8 independent experiments. **B**, RA and OA FLS were cultured as described in A along with 300 ng/ml recombinant human APRIL plus control Ig or 300 ng/ml recombinant human APRIL plus 1 μg/ml recombinant human BCMA-Fc chimera (to block the interaction with APRIL); medium alone served as the control. The relative gene expression of APRIL, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and IL-1β was assessed by real-time PCR. Results are the mean and SD from 10 independent experiments. * = $P < 0.05$. See Figure 2 for other definitions.

cultures of RA FLS abrogated these effects of APRIL (Figure 3B). Thus, the results indicate that APRIL might be an upstream regulator of proinflammatory cytokine production in the rheumatoid synovium.

APRIL-induced production of RANKL on RA FLS. FLS act as main effector cells in RA-associated joint destruction through their ability to produce matrix metalloproteinases and their ability to induce osteoclastogenesis by expressing RANKL (28). Both RANKL mRNA and RANKL protein were expressed at a significantly higher level in RA FLS than in OA FLS (Figures 4A and B).

In addition, the effects of APRIL on RANKL expression in RA FLS and OA FLS were also determined. APRIL enhanced the expression of RANKL mRNA in RA FLS, but not in OA FLS (Figure 4C). Similar to its lack of effect on proinflammatory cytokine

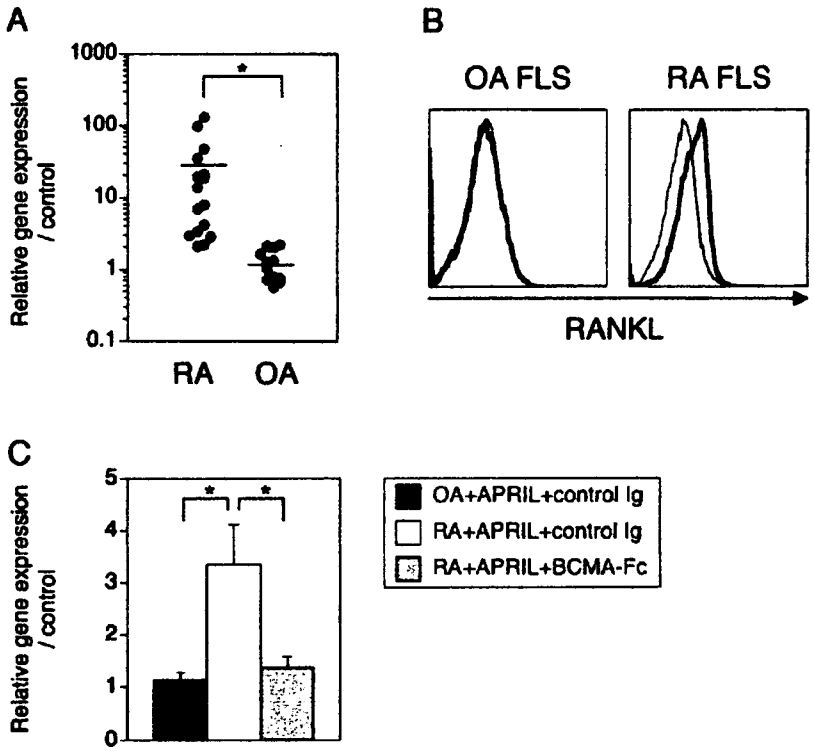

Figure 4. Induction of RANKL production by APRIL in RA FLS. **A**, The relative gene expression of RANKL was assessed in RA and OA FLS by real-time PCR; NHDFs served as the control. Horizontal bars show the mean of 16 RA FLS and 12 OA FLS samples. **B**, The relative gene expression of RANKL in RA and OA FLS was assessed by flow cytometry. Cells were stained with mAb specific for RANKL (shaded areas) or an isotype control mAb (open areas). Fluorescence histograms show results representative of 16 RA FLS and 12 OA FLS samples. **C**, RA and OA FLS were starved in fetal calf serum (FCS)–free RPMI 1640 medium for 24 hours, and then cultured in 0.2% FCS–RPMI 1640 medium with 300 ng/ml recombinant human APRIL plus control Ig or 1 μg/ml recombinant human BCMA-Fc chimera for 24 hours. The relative gene expression of RANKL was analyzed by real-time PCR; medium alone served as the control. Results are the mean and SD from 10 independent experiments. * = $P <$ 0.05. See Figure 2 for other definitions.

production, BAFF did not enhance the expression of RANKL on RA FLS (results not shown).

Furthermore, the BCMA-Fc fusion protein was again able to abrogate this stimulatory effect of APRIL on RANKL expression (Figure 4C). These findings suggest that APRIL might contribute to osteoclastogenesis in the RA synovium.

Enhancement of progression of the RA FLS cell cycle by APRIL. APRIL was originally identified as a TNF family member that induces tumor cell proliferation (21). RA FLS exhibit tumor-like properties of activation and proliferation, which are maintained in the absence of an environmental stimulus. We therefore examined the capacity of APRIL to maintain and enhance the proliferation of RA FLS. Addition of recombinant APRIL to the culture resulted in an enhancement of progression of the RA FLS cell cycle. Furthermore, addition of the BCMA-Fc fusion protein abrogated this effect of APRIL on RA FLS (Figure 5).

DISCUSSION

According to the current concept of RA pathogenesis, FLS are among the principal effector cells in the joint destruction of RA (1,3–6). Recent research has provided much information about the signals that can target the FLS in the affected RA synovium, although the RA-specific mechanisms contributing to the condition have not been well described.

BAFF and its close homolog, APRIL, are known

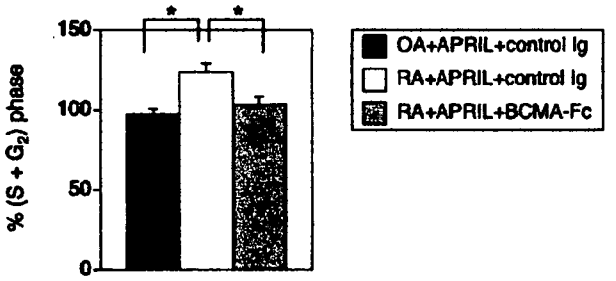

Figure 5. Enhancement of cell cycle progression in RA FLS by APRIL. RA and OA FLS were starved in fetal calf serum (FCS)–free RPMI 1640 medium for 24 hours, and then cultured in 0.2% FCS–RPMI 1640 medium with 300 ng/ml recombinant human APRIL plus control Ig or 300 ng/ml recombinant human APRIL plus 1 μg/ml recombinant human BCMA-Fc chimera for 24 hours. The adherent FLS were then stained with propidium iodide solution. Rates of cell cycle progression were determined as the percentage of cells in the S phase plus the G_2 phase compared with that in RA or OA FLS cultured in medium alone. Bars show the mean and SD from 10 independent experiments. * = $P < 0.05$. See Figure 2 for other definitions.

to contribute to autoimmune responses and have recently been shown to play roles in the process of inflammation-associated lymphoproliferation and germinal center formation in the rheumatoid synovium (2,17). The present study characterizes a variety of stimulating effects of APRIL, specifically on RA FLS. We found that APRIL induces RA FLS to express many of the known pathogenic phenomena observed in the RA synovium, such as the production of proinflammatory cytokines, enhancement of RA FLS proliferation, and induction of osteoclasts.

A significantly higher level of soluble APRIL was detectable in RA serum compared with that in normal serum. However, the soluble APRIL level in RA synovial fluid did not exceed that in RA serum, in contrast to previous reports in which the level of soluble BAFF was shown to be higher in RA synovial fluid than in RA serum (14–16). Tan et al previously reported that the level of soluble APRIL was higher in the synovial fluid than in the serum of patients with inflammatory arthritis; however, the APRIL level in the synovial fluid did not always correlate with that in the serum in individual patients (16). Those authors also showed that in some patients who underwent sequential arthrocenteses, changes in synovial fluid levels of APRIL were not parallel with changes in synovial fluid levels of BAFF, suggesting that these molecules are differentially regulated.

However, expression analyses have shown that BCMA is the receptor most likely to be relevant in the later stages of B cell maturation, such as in CD38-positive plasmablasts (29,30) and germinal center B cells (31). Signals through BCMA in B cells might be regulated by stage-specific expression of BCMA. Taken together, these findings suggest the possibility that the effects of APRIL on the RA synovium might be regulated locally by the expression level of one of its receptors, BCMA, in affected cells.

Our analysis also shows that, among the 3 receptors of BAFF and APRIL, RA FLS express only BCMA; however, OA FLS and normal human dermal fibroblasts express none of these receptors. As expected based on the receptor expression profile, APRIL induced a significant increase in the production of the proinflammatory cytokines IL-6, TNFα, and IL-1β in RA FLS, but not in OA FLS. BAFF did not induce the production of these cytokines in either RA or OA FLS (results not shown). High concentrations of these cytokines were detected in the affected rheumatoid joints, and they mediated inflammatory reactions between FLS and mononuclear cells infiltrating the RA synovium. Thus, APRIL might act as an upstream mediator of the cytokine network to facilitate inflammatory reactions, specifically in the RA synovium.

It should be noted that for stimulation of FLS in vitro, we used 300 ng/ml of APRIL, a concentration that was ~10 times higher than that observed in vivo. The active form of soluble APRIL is a 63-kd noncovalent trimeric protein. The recombinant human APRIL (R&D Systems) that was used in the present study was monomeric, and a higher concentration was required to show its biologic activities (32).

APRIL also could stimulate RA FLS to produce APRIL itself, suggesting that APRIL stimulates the production of proinflammatory cytokines in RA FLS in an autocrine manner. However, there might be another major source of APRIL, other than FLS, in RA, because the level of soluble APRIL in RA synovial fluid did not always exceed that in RA serum. We are still unable to fully understand the systemic regulation and functions of APRIL in RA. The as yet unrecognized functions of APRIL might contribute to the maintenance of a wide range of physiologic and/or pathologic reactions throughout the system.

This study also demonstrated that APRIL could enhance the progression of the cell cycle of RA FLS, and that this effect was dependent on the interaction with BCMA. RA FLS have been shown to proliferate spontaneously and to constitutively express cytokines, oncogenes, and cell cycle proteins, which is indicative of their role in systemic transformation (8,9). APRIL was originally identified as a ligand involved in the formation and

maintenance of tumors (21). High levels of APRIL mRNA have been detected in transformed cell lines and human cancers of the colon, thyroid, and lymphoid tissue in vivo (21). Moreover, APRIL is shown to have a remarkable capacity to stimulate both solid and lymphoid tumor growth in vitro (21,22). APRIL may be one of the important factors that maintain the tumor-like spontaneous proliferation of RA FLS both in an autocrine manner and in a paracrine manner.

One of the most exciting recent developments in understanding the pathogenetic mechanisms of bone erosion in RA relates to the discovery of osteoclast-mediated bone resorption that is regulated by RANKL. It has been shown that RANKL is essential for the development of monocyte/macrophages into mature osteoclasts (28). In patients with RA, both the T cells and FLS have been found to produce RANKL, and it has been proposed that this promotes osteoclast development (4,28). Several groups of investigators have demonstrated the production of RANKL by synovial fibroblasts from patients with RA (4,28). The results of the present study show that APRIL can enhance the expression of RANKL in RA FLS. This may lead to osteoclastogenesis at the site of pannus formation in the RA synovium.

IL-1β and TNFα have been shown to be involved in both the proliferation of RA FLS and the expression of RANKL by RA FLS. IL-1β is considered to play an especially critical role downstream of TNFα, based on a report that TNF-induced RANKL synthesis by bone marrow stromal cells was abolished by IL-1 receptor antagonist (IL-1Ra) and was absent in stromal cells derived from type I IL-1R–deficient mice (33). The present study showed that APRIL stimulated RA FLS to produce both TNFα and IL-1β. Enhancement of RANKL expression and cell cycle progression in APRIL-stimulated RA FLS could be the result of the APRIL-induced production of TNFα and IL-1β. However, the administration of IL-1Ra to RA patients produced only a modest effect as compared with that of a TNFα inhibitor (34). There might exist signals that bypass the TNFα–IL-1β pathway and directly enhance both the proliferation and RANKL expression of RA FLS.

Proinflammatory cytokine–induced activation of RA FLS has been reported to be dependent on the p38 and ERK MAPKs and NF-κB (35,36). Signals through BCMA have been shown to activate NF-κB, p38, and JNK, but not ERK, in the 293 T cell line and A20 B cell line (37,38). Nonetheless, the mechanisms of APRIL signaling in fibroblasts remain to be elucidated.

In contrast, fibroblast growth factor 2 (FGF-2) transfers to FGF receptor 1 through binding to heparan sulfate proteoglycan (HSPG), which is characteristically expressed on RA FLS, and results in RANKL- and intercellular adhesion molecule 1–mediated maturation of osteoclasts via ERK activation (5). In contrast to the signal pathway in lymphoid cells, APRIL signaling through BCMA in RA FLS might be able to activate ERK. Alternatively, APRIL may signal through HSPG on RA FLS and activate ERK, since APRIL has been shown to bind to cell surface HSPG and thus enhance tumor growth (39). We are currently investigating the details of the signal transduction pathways of APRIL in RA FLS, which would allow us to determine whether APRIL is the factor that bypasses the TNFα–IL-1β signals.

The regulatory mechanisms of BCMA expression have not been fully described. OBF.1 is a transcriptional coactivator that binds with OCT.1 or OCT.2 to the octamer DNA element in the regulatory regions of B cell–specific genes (40,41). OBF.1 has been shown to regulate the expression of PU.1, an essential transcription factor for the development of both myeloid and lymphoid cells (42), and also regulates the expression of BCMA (43–45). Previous studies have shown that the expression of PU.1 mRNA is up-regulated in the peripheral blood monocytes of RA patients (46). We recently demonstrated that BCMA expression in RA FLS was correlated with the expression of both PU.1 and OBF.1 (26). The mechanisms of the breakdown in the regulation of PU.1 and OBF.1 expression in RA FLS should be further investigated as a means to understanding the pathogenesis of RA.

Collectively, these results provide evidence that APRIL is one of the regulators in the pathogenesis of RA. Thus, both BCMA and APRIL could be considered as potential therapeutic targets in ameliorating damage to the affected joints of patients with RA.

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AUTHOR CONTRIBUTIONS

Dr. Itoh had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Itoh, Aotsuka, Mimori.

Acquisition of data. Nagatani, Itoh, Nakajima, Kuroki, Katsuragawa.

Analysis and interpretation of data. Nagatani, Itoh, Mochizuki, Mimori.

Manuscript preparation. Nagatani, Itoh.

Statistical analysis. Nagatani.

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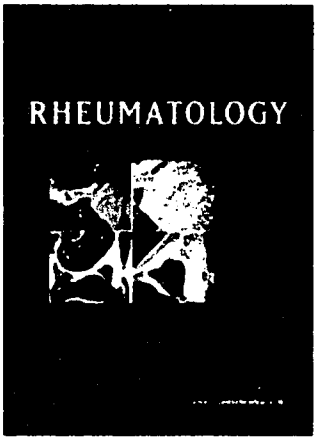

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Expression of BAFF and BAFF-R in the synovial tissue of patients with rheumatoid arthritis

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Objective: The elevated expression of B-cell-activating factor belonging to the TNF family (BAFF) is associated with systemic autoimmune disease, including rheumatoid arthritis (RA). The present study was undertaken to determine the distribution of BAFF and its receptor BAFF-R in the cells residing in the rheumatoid synovium.
Methods: The expression of BAFF and BAFF-R in synovial tissues obtained from 12 RA patients was examined by immunohistochemistry and flow cytometry. The mRNA expression of these molecules was determined by reverse transcriptase polymerase chain reaction (RT-PCR). Soluble BAFF levels were measured with an enzyme-linked immunosorbent assay (ELISA). Fibroblast-like synoviocytes (FLS) purified from the RA (RA-FLS) were co-cultured with peripheral B cells. The degree of apoptosis in the B cells was measured to assess the effects on the viability of the B cells.
Results: The RA synovium showed focal or diffuse infiltration of mononuclear cells (MNCs), and one specimen showed germinal centre (GC)-like structures. Synovial sublining cells, but not lining cells, expressed BAFF. These sublining cells were negative for BAFF-R. BAFF and BAFF-R were expressed in B and T cells extracted from the RA synovium. Notably, RA-FLS spontaneously expressed cytoplasmic BAFF after 4–6 passages; however, they did not express BAFF or BAFF-R on their cell surface. RA-FLS could support the survival of B cells by preventing their apoptosis, but its effect on B cells might not be BAFF dependent.
Conclusions: BAFF and BAFF-R are widely expressed in the RA synovium. The cells residing in the RA synovium might affect each other through BAFF.

Rheumatoid arthritis (RA) is characterized by joint destruction resulting from chronic inflammation in synovial tissue (1). The RA synovial tissue displays a heterogeneous pattern of chronic inflammation involving various resident and immigrant cell types (2, 3). The persistent chronicity of the disease is postulated to be maintained by interactions between infiltrated mononuclear cells (MNCs) and synovial cells, in addition to autocrine stimuli by proinflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6 (3). Histopathological studies have also demonstrated that about 10–23% of RA synovium show aggregation of T cells, B cells, and follicular dendritic cells that resemble germinal centres (GCs) regarded as 'ectopic lymphatic follicles', which possess at least some of the functions of typical GCs including antigen-specific B cell differentiation (2, 4, 5). Recent clinical studies on B-cell-targeted therapy using anti-CD20 antibodies (rituximab) reported an impressive symptomatic improvement in RA patients without a decrease in circulating immunoglobulin (6). This implies still unknown roles for B cells in RA pathogenesis.

B-cell-activating factor belonging to the TNF family (BAFF) is essential for B-cell generation (7–9). Three receptors have been identified that bind to BAFF: a transmembrane activator, calcium modulator and cyclophilin ligand interactor (TACI); B cell maturation Ag (BCMA); and BAFF-R. Another TNF family member, a proliferation-inducing ligand (APRIL), also binds to TACI and BCMA (9). High levels of BAFF are detectable in the sera of patients with autoimmune rheumatic diseases, particularly systemic lupus erythematosus (SLE) and Sjögren's syndrome (10–12). BAFF is also present at high levels in rheumatoid synovial fluid (12, 13) and in the salivary glands of patients with Sjögren's syndrome (10). In BAFF transgenic mice, increased numbers and sizes of GCs are seen (14). BAFF and APRIL were shown to be involved in the ectopic GC

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reaction in the RA synovium, and might contribute to the pathogenesis of RA (5).

Consequently, we were interested in examining the putative involvement of BAFF in RA pathophysiology. The present study was undertaken to determine the distribution and the function of BAFF and its receptor BAFF-R in the cells residing in the rheumatoid synovium.

Materials and methods

Patients and synovial specimens

Twelve patients who fulfilled the American College of Rheumatology (ACR) criteria for the diagnosis of RA (15) were enrolled. The profiles of the patients are listed in Table 1. Synovial tissue specimens from osteoarthritis (OA) patients, tonsillar cells, and lymph nodes from healthy adults were evaluated as controls. For analysing the soluble BAFF level, synovial fluids were collected from another series of 20 RA patients, who required a therapeutic arthrocentesis of the joint. Serum samples were obtained from 28 RA patients and 31 normal individuals. All patients who participated in this study provided informed consent according to the Declaration of Helsinki. The ethics committee of the International Medical Centre of Japan approved the study.

Cell preparation

Synovial tissues were obtained at the time of total joint replacement surgery, and were digested with deoxyribonuclease I (Worthington, Lakewood, NJ, USA), collagenase IV (Worthington), and hyaluronidase (Sigma, St Louis, MO, USA), to obtain single-cell suspensions. MNCs were isolated from these cell suspensions by density gradient centrifugation using Ficoll (Muto Pure Chemicals Co. Ltd., Tokyo, Japan).

Table 1. Characteristics of the 12 RA patients.

Age, mean (range), years	63.7 (35–83)
Sex	
Male	2 (16.7)
Female	10 (83.3)
CRP, mean (range), mg/dL	3.7 (0.12–12.45)
Treatment	
NSAIDs	5 (41.7)
DMARDs	
Methotrexate	7 (58.3)
Salazosulfapyridine	6 (50.0)
Bucillamine	3 (25.0)
Prednisolone	7 (58.3)
Dose of prednisolone, mean mg/day (range)	6.7 (3–10)

RA, rheumatoid arthritis; CRP, C-reactive protein; NSAIDs, non-steroidal anti-inflammatory drugs; DMARDs, disease-modifying anti-rheumatic drugs. Values are the number (%) of patients, except where indicated otherwise. Range of CRP in unaffected individuals is 0.00–0.30 mg/dL.

After overnight culture in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal calf serum (FCS; ICN Biomedicals Inc., OH, USA), penicillin–streptomycin (Invitrogen), and gentamycin (Sigma), adherent cells were cultivated as fibroblast-like synoviocytes (FLS). FLS from passages 4–6 were used for each experiment. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using Ficoll (Muto Pure Chemicals). CD19-positive B cells were purified using CD19-MicroBeads (Miltenyi Biotec, Auburn, CA, USA), following the manufacturer's instructions. The purity of the isolated B cells was >95%, as assessed by flow cytometry.

Immunohistochemistry

The following antibodies (Abs) were used for immunohistochemical visualization; rat anti-human BAFF (Buffy-2) monoclonal (m)Ab (1:500; Alexis Corporation, Switzerland), mouse anti-human BAFF-R (11C1) mAb (1:1000; Alexis), mouse anti-human CD3 (F7.2.38) mAb (1:200, DakoCytomation, Carpinteria, CA, USA) and mouse anti-human CD20 (L26) mAb (1:200, DakoCytomation). Tissue sections fixed and embedded in paraffin wax were pretreated by autoclave heating for 10 min at 121°C in citrate buffer solution (pH 6.0). The sections were immunostained using a DakoCytomation Autostainer (DakoCytomation), with those antibodies listed above, and Mouse EnVision HRP (DakoCytomation). Antibody bindings were visualized using liquid diaminobenzidine (DakoCytomation) and finally counterstained with haematoxylin.

Flow cytometry

Synovial MNCs and FLS were stained with a mAb tomouse anti-human CD3-FITC (SK7, BD Biosciences, San Diego, CA, USA), CD19-FITC (4G7, Beckton Dickinson, San Jose, CA), CD14-FITC (MϕPQ, BD Biosciences), BAFF-PE (137314, R&D Systems, Minneapolis, MN, USA), and BAFF-R-PE (11C1, BD Biosciences). Cells were incubated on ice for 30 min in phosphate-buffered saline (PBS) containing 2% FCS and 50 μg/mL of Abs. Before the staining of cytoplasmic BAFF, cells were incubated in cold 4% paraformaldehyde (PFA) fixative in PBS at room temperature for 10 min, and then washed with 0.05% saponin (ICN) in Hanks' Balanced Salt Solution (HBSS; Sigma) for permeabilization (16). Analysis was performed using BD FACSCalibur (BD Immunocytometry Systems) and FlowJo 6.1.1 software (Tree Star, Inc., Ashland, OR, USA). Cytoplasmic BAFF, membrane-bound BAFF, and BAFF-R in RA-FLS were stained before and after the stimulation with 10 ng/mL of recombinant TNFα (RELIATech GmbH, Germany).

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from synovial MNCs, FLS, and control cells using TRI REAGENT (Molecular Research Center, Cincinnati, OH, USA). One microgram of total RNA was then used for cDNA synthesis, with 200 U Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA), 25 mM dNTPs (Applied Biosystems, Foster City, CA, USA), and 50 μg oligo(dT)12–18 primer (Invitrogen). PCR reactions were carried out in a GeneAmp PCR system 9700 (Applied Biosystems) with the specific oligonucleotide primers: BAFF, 5′-CCCCAACCTTCAAAGTTCAAGTAG-3′ (forward), 5′-TGAGTGACTGTTTCTTCTGGACCC-3′ (reverse); BAFF-R, 5′-GGTCCTGGTGGGTCTGGTGAG-3′ (forward), 5′-GGCTGAATGCTGTGGTCTG-TAGTG-3′ (reverse); GAPD, 5′-GAAATCCCATC-ACCATCTTCCAG-3′ (forward), 5′-ATGAGTCC-TTCCACGATACCAAAG-3′ (reverse).

Enzyme-linked immunosorbent assay (ELISA)

Soluble BAFF in serum and synovial fluids was assayed with a sandwich ELISA using a Quantikine Human BAFF Immunoassay kit (R&D Systems) following the manufacture's instructions. Optical density was measured with an ImmunoMini NJ-2300 plate reader (InterMed, Tokyo, Japan).

Detection of apoptotic B cells

Normal B cells 3×10^5 were cultured alone, co-cultured with RA-FLS 1×10^4, or co-cultured with FLS and 1 μg/mL of BAFF-R: Fc fusion protein (R&D Systems) in a 96-well plate. After 5 days of incubation, B cells were incubated with 50 μg/mL of 7-amino-actinomycin D (7-AAD; BD Bioscience) in PBS containing 2% FCS for 20 min at 4°C. Samples were analysed using FACSCalibur, and the cells were determined as 7AAD-negative (live cells), 7AAD-dim (apoptotic cells), and 7AAD-bright (late-apoptotic or dead cells) (17). Apoptotic B cells were also detected with propidium iodide (PI; Sigma, 100 μg/mL in PBS) staining (18).

Statistical analysis

Analyses were performed using StatView 5.0 software (SAS, Cary, NC, USA). Comparisons were based on the paired t-test or the Wilcoxon signed-ranks test between two groups and on the Kruskal–Wallis test among three groups. The Scheffé test was used for multiple comparisons.

Results

The present study investigated the expression of BAFF and its receptor BAFF-R in the synovial tissues of 12 patients with RA who had undergone total joint replacement surgery. As shown in Table 1, most of the patients showed a fairly high disease activity with elevated serum C-reactive protein level, even though they were taking prednisolone and disease-modifying anti-rheumatic drugs (DMARDs) including methotrexate. The total synovitis score of the synovial tissue specimens obtained from these patients showed more than 11 points, confirming the histological diagnosis of RA (19).

Expression and distribution of BAFF and BAFF-R in RA synovial tissue

Supporting previous reports on the pathohistology of the synovial tissues of RA patients (2, 4, 5), the RA synovial tissues that were analysed in this study could be classified into three categories according to the infiltration patterns of the MNCs. Most of the tissue specimens showed diffuse infiltration of MNC and formed many GC-negative lymphoid aggregations (Figure 1B, C). One of the specimens showed lymphatic follicles with GC-like structures (so-called 'ectopic GC') (Figure 1A). Distribution of T cells and B cells was also the same as in normal lymph nodes. As has been observed in normal lymphoid organs (20), both T cells and B cells infiltrated and formed cell aggregations in the rheumatoid synovium expressed BAFF and BAFF-R (Figure 1). The expression characteristics of BAFF and BAFF-R in synovial ectopic GCs were the same as has been observed in normal lymph nodes (in this study; data not shown) (20). Of note, synovial sublining cells, but not initial lining cells, expressed BAFF. The major population of the cells expressing BAFF in the synovial sublining was negative for CD3 or CD20. These cells had large pale nuclei containing prominent nucleoli and an abundant cytoplasm, and were characterized as synovial cells. Moreover, these synovial sublining non-lymphoid cells did not express BAFF-R (Figure 1C, D, arrows).

Expression of BAFF and its receptors in synovial MNC and FLS

To examine the expression of BAFF and BAFF-R in each cell population in the RA synovium, the synovial tissues from RA were digested to obtain the residing MNCs as explained in the Material and Methods section. Flow cytometric analysis on these MNCs showed that the BAFF protein was expressed significantly higher in the cytoplasm of T cells [mean fluorescence intensity (MFI)=8.4; $p<0.01$, paired t-test; $n=5$], B cells (MFI=17.1; $p<0.01$, paired

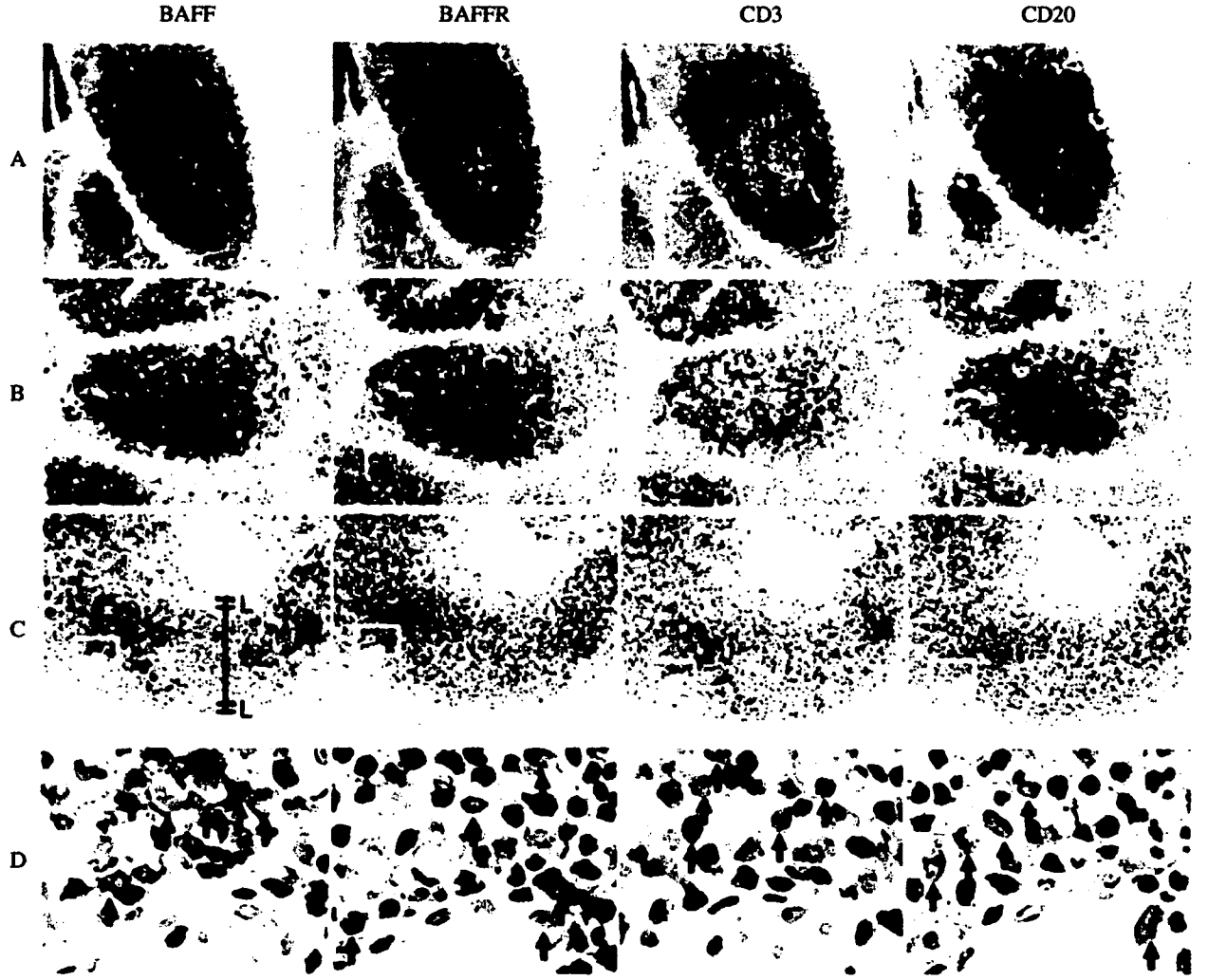

Figure 1. Immunohistochemical analysis of BAFF and BAFF-R expression in RA synovial tissue presenting three different infiltration patterns of MNCs. (A) Lymphatic follicles with ectopic GCs. (B) Lymphoid aggregates. (C, D) Diffuse infiltrations of MNCs. Synovial lining layer and sublining area are indicated as L and SL, respectively. The areas indicated by white squares in C are shown at higher magnification in D. Original magnification: ×20 (A–C), ×400 (D).

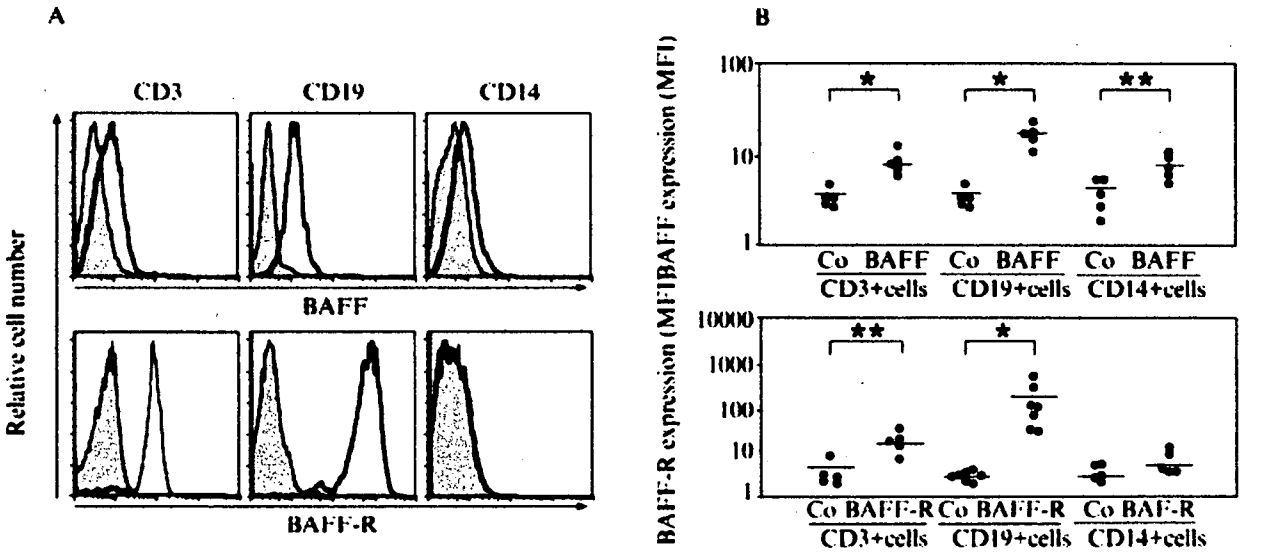

Figure 2. Expression of BAFF and BAFF-R proteins in MNCs infiltrating RA synovial tissue (RA-ST). (A) Representative histograms of MNCs infiltrating RA-ST stained with mAbs specific for cytoplasmic BAFF and membrane-bound BAFF-R (performed by gating on CD3, CD19, and CD14 positive cells). Shaded histograms indicate isotype-matched controls. (B) Distribution of BAFF and BAFF-R protein expression level in MNCs from RA-ST. The solid bar represents the mean value for the group. *$p<0.01$. **$p<0.05$. MFI, mean fluorescence intensity.