Enhancement of lymphocyte migration and cytokine production by ephrinB1 system in rheumatoid arthritis

Takuya Kitamura, Yukihito Kabuyama, Akihisa Kamataki, Miwako K. Homma, Hideo Kobayashi, Shigeo Aota, Shin-ichi Kikuchi, and Yoshimi Homma

Department of Biomolecular Science and Orthopedics, Fukushima Medical University School of Medicine, Fukushima, Japan

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RHEUMATOID ARTHRITIS (RA) is a systemic, chronic inflammatory disease characterized by abnormal immune responses and synovial proliferation that ultimately leads to the progressive destruction of tendons, cartilage, and bone (13). Although the etiology of the early events in RA remains undefined, an anomaly in T cell homeostasis and hyperproliferation of synovial-lining cells are involved in the disease process. Since it has been reported that the ephrin/Eph receptor system plays important signaling roles in inflammation processes, we attempted to examine ephrinB molecules in T cells and synovial cells derived from RA in this study. The expression level of ephrinB1 was significantly high in synovial fibroblasts and CD3-positive exudate lymphocytes in synovial tissues derived from patients with RA compared with those in osteoarthritis (OA). Protein and mRNA levels of ephrinB1 were also higher in peripheral blood lymphocytes (PBLs) prepared from patients with RA than those from normal controls. Similar results were obtained from an animal model of human RA, collagen antibody-induced arthritis mice. Moreover, a recombinant ephrinB1/Fc fusion protein stimulated normal PBLs to exhibit enhanced migration and production of TNF-α. EphrinB1/Fc also activated synovial cells established from patients with RA to produce IL-6. Tyrosine phosphorylation of EphB1 was induced in these cells by ephrinB1/Fc. The CpG islands in the 5′ upstream regulatory region of the ephrinB1 gene were hypomethylated in RA patients compared with those of normal donors. These results suggest that ephrinB1 and EphB1 receptors play an important role in the inflammatory states of RA, especially by affecting the population and function of T cells. Inhibition of the ephrinB/EphB system might be a novel target for the treatment of RA.

Address for reprint requests and other correspondence: Y. Homma, Dept. of Biomolecular Science, Fukushima Medical Univ. School of Medicine, Fukushima 960-1295, Japan (e-mail: yoshihom@fmu.ac.jp).

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patients fulfilled the American Rheumatism Association 1987 revised
criteria for the classification of RA (2) and classified into functional
classes II or III (28). RA patients underwent surgery during remission.
Synovium samples were obtained from three patients with OA (3
women, mean age: 70.7 yr, range: 67–73 yr) at the time of knee
arthroplasty, and blood samples were from eight healthy volunteers
(2 men and 6 women, mean age: 50.8 yr, range: 40–79 yr).

Histological analysis. Synovial tissues were fixed with 4% para-
formaldehyde in PBS for 24 h at 4°C. After being blocked with 3% normal
good serum, 6-μm-thick sections were incubated with rabbit anti-ephrinB1 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight
in a moist chamber at 4°C. After being rinsed with PBS, sections were
incubated with biotin-labeled goat anti-rabbit IgG followed by avidin-
biotin peroxidase complex. Positive signals were visualized using a
3,3’-diaminobenzidine substrate kit (Vectastain Elite ABC kit, Vector
Laboratories, Burlingame, CA). For immunofluorohistochemistry,
sections were incubated with rabbit anti-ephrinB1 and mouse anti-
CD3 (Santa Cruz Biotechnology) antibodies overnight at 4°C. After
being washed thoroughly, sections were incubated with Alexa fluor 488-labeled anti-rabbit IgG and Alexa fluor 555-labeled mouse-IgG antibodies (Invitrogen, Carlsbad, CA) for 1 h.

Analysis of mRNA levels. The level of ephrinB1 mRNA was
assessed by RT-PCR. Total RNA was extracted from PBL samples
derived from RA patients and normal donors using an ISOGEN kit
(Nippon Gene, Tokyo, Japan), and cDNA was prepared from total
RNA using the SuperScript III First Strand System (Invitrogen)
according to the manufacturer’s instructions. Aliquots (0.5 μl) of
the resultant mixtures were subjected to PCR amplification with 35 cycles
at 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min. This condition
resulted in that the amplification protocol. PCR products were electrophoresed
using 1.5% agarose gels and stained with ethidium bromide, and
intensities of the bands were quantified using NIH Image software.
The ratio between ephrinB1 and β-actin was calculated to normalize
for initial variations in the sample concentration. The primers used for
PCR amplifications were as follows: human ephrinB1 sense, 5’-
CGTGTGGTGTACCTGCAATAG-3’; human ephrinB1 antisense,
5’-GCTTCCATTTGGATGTTGAGGT-3’; human β-actin sense,
5’-CATGATGGTGGCTATCCAGGC-3’; and human β-actin anti-
sense, 5’-CTCTCTAGAGTCAAGCCAGAT-3’.

Western blot analysis. Protein fractions were prepared from the
PBL samples using an ISOGEN kit (Nippon Gene) according to the
manufacturer’s instructions. An aliquot (50 μg protein) was
subjected to SDS-PAGE and analyzed by Western blot analysis using
anti-ephrin B1 antibody (Santa Cruz Biotechnology) and anti-
GAPDH antibody (Chemicon, Temecula, CA). A horseradish per-
oxidase-conjugated antibody was used as the secondary antibody
(Bio-Rad Laboratories, Hercules, CA), and positive bands were
visualized by an enhanced chemiluminescence system (GE Health-
care, Buckinghamshire, UK). The intensity of each band was
quantified using NIH Image software. For immunoprecipitation,
cells were solubilized in a lysis buffer consisting of 20 mM
Tris·HCl (pH 7.5), 1% Nonidet P-40, 1 mM EDTA, 50 mM NaF,
50 mM β-glycerophosphate, 1 mM Na3VO4, 10 μg/ml leupeptin,
10 μg/ml aprotinin, and 1 mM PMSF. Cell lysates were incubated
for 2 h at 4°C with an anti-phosphotyrosine antibody 4G10 (Upstate,
Lake Placid, NY). Immune complexes were collected with protein
G-Sepharose (Zymed, San Francisco, CA). After being washed five
times, proteins were solubilized by heating in 30 μl SDS sample
buffer and subjected to SDS-PAGE.

Arthritis model. All animal experiments were carried out with the
approval of the Fukushima Medical University Review Board and in
concordance with Institutional Animal Care and Use Committee
regulations. Balb/c female mice were obtained from Charles River
Laboratories Japan and housed in a controlled environment with free
access to food and water. Collagen antibody-induced arthritis (CAIA)
was induced using arthrogen-collagen-induced arthritis antibody kits
(Chondrex, Redmond, WA) according to the manufacturer’s instruc-
tions. In brief, a cocktail of anti-collagen type II monoclonal antibody
mix (0.5 mg each) was injected intraperitoneally into 6-week-old mice
on day 0, followed by an intraperitoneal injection of 25 μg LPS on
day 3. Mice were killed for analyses by exsanguinations under anesthesia
with pentobarbital at day 10.

Bioassays for ephrinB1. For the preparation of the ephrinB1/Fc
fusion protein, the extracellular domain of ephrinB1 was subcloned into a human immunoglobulin Fc fusion protein expression vector
(kindly provided by Drs. Takashi Saito and Arata Takeuchi) and
transfected into COS7 cells. The protein was purified from the
culture supernatant using protein A-agarose (Upstate). PBL migra-
tion was determined in 24-well plates with 5-μm-pore size cell
culture inserts (Corning, Corning, NY). PBLs from normal donors suspended in RPMI-1640 at 2.5 x 105 cells/ml were treated with various amounts of ephrinB1/Fc or control Fc fragment for 1 h, and
100-μl aliquots of cell suspension were added to the upper inserts;
600-μl aliquots of medium containing stromal cell-derived factor
(SDF)-1α, a typical chemotactic factor for lymphocytes, were added
to the lower chambers. After an incubation for 3 h, the numbers of cells that had passed through the membrane into the lower chamber were quantified by counting five random fields. The production of TNF-α or IL-4 from normal PBLs and IL-6 from
fibroblast-like synovial cells were evaluated using human ELISA
kits (Pierce, Rockford, IL). PBLs from normal donors suspended in
RPMI-1640 at 5 x 105 cells/ml or synovial cells from RA patients
(18) seeded at 5 x 104 cells/well in 24-well plate were starved for
24 h and then treated with various amounts of ephrinB1/Fc or control Fc fragment for 48 h. Culture supernatants were used for
the evaluation.

Bisulfite sequencing analysis. Bisulfite modification of genomic
dNA was performed as previously described (24). Briefly, 5 μg
 genomic DNA suspended in 50 μl water was incubated for 10 min
at 95°C and then for 30 min at 37°C with 1.5 μl of 10 M NaOH. The
solution was further incubated overnight at 55°C after the addition
of 310 μl of 5 M sodium bisulfite, 2.5 μl of 0.1 M hydroquinone,
and 136 μl water in a final volume of 500 μl. After modification, DNA
was purified using a Wizard clean-up kit (Promega, Madison, WI) and
desulfonated with 0.3 M NaOH for 15 min at 37°C. Bisulfite-treated
DNA was amplified using the following primers: human ephrinB1
sense (−205 to −181), 5’-GGATTGAGGAGGTATATTTATAT-
T-3’; human ephrinB1 antisense (−25 to −48), 5’-CAAAACAAC-
CCTACTCTTAAAC-3’; mouse ephrinB1 sense (−332 to −308)
5’-GTTGGTGTGTTTTAGTGTGTTGTGTT-3’; and mouse ephrinB1
antisense (−48 to −69), 5’-ACACACACACACACTTCACT-3’. PCR
amplification was performed for 35 cycles of 30 s at 95°C, 1 min
at 50°C, and 3 min at 72°C, with a final extension at 72°C for
10 min. PCR products were cloned into the pGEM-T-easy vector
(Promega), and at least eight clones for each independent sample
were sequenced.

Luciferase assay. The luciferase reporter plasmid containing the
human ephrinB1 promoter sequence from −1000 to +1 relative to the
start site of transcription was generated using pGL3-Basic (Promega).
COS7 cells (5 x 104) plated on 24-well plates were transfected with
0.4 μg of reporter construct and 5 μg of pRL-TK vector (Promega) as an internal control using Polyfect Transfection
Reagent (QIAGEN, Hilden, Germany). After an incubation for 48 h, relative luciferase activities were determined by a dual-
luciferase reporter assay system (Promega). For the preparation of
methlated and mock methylated vector, reporter vector (1.2 μg)
was incubated with or without 4 units of SsI methylase (New
England Biolabs, Ipswich, MA) and 160 μM S-adenosylmethi-
onine for 2 h at 37°C, followed by an incubation for 20 min at 65°C
to inactivate the methylase.
RESULTS

High expression of ephrinB1 in tissues and PBLs from RA patients. We examined the expression of ephrinB1 in RA and OA synovial tissues by immunohistochemistry. In the RA synovium, ephrinB1-positive mononuclear cells were observed in the sublining layer. Mononuclear cells that formed lymphoid follicles did not react with the anti-ephrinB1 antibody. In the OA synovium, some ephrinB1-positive cells were observed, but their numbers and expression levels in the OA synovium were quite low compared with those in RA tissues (Fig. 1A). To characterize ephrinB1-positive mononuclear cells, double immunofluorohistochemistry using antibodies against ephrinB1 and CD3 was carried out. The results shown in Fig. 1B clearly demonstrate that ephrinB1 and CD3 coexpress on mononuclear cells in the RA synovium, indicating an aberrant accumulation of ephrinB1-positive T cells in sublining layers of the RA synovium.

We examined the expression of ephrinB1 in PBLs of five RA patients and three age-matched normal controls. Protein levels of ephrinB1 in PBLs derived from RA patients were relatively high compared with those of controls (Fig. 2A); the mean level was 2.3 times more in RA than in control subjects. Enhanced mRNA levels were also observed in RA; the mean level of ephrinB1 mRNA was 1.4 times higher in RA patients than in healthy donors (Fig. 2B). Similar results were obtained from an animal model of human RA, CAIA mice, which were generated by injecting mice with a mixture of four kinds of anti-collagen type II monoclonal antibodies and LPS. The mean level of ephrinB1 protein in PBLs derived from four CAIA mice was 2.5 times higher than that of PBLs from three control mice (Fig. 2C).

Activation of lymphocytes and synovial cells by ephrinB1. Recent studies have demonstrated that the ephrin/Eph signaling system controls lymphocyte migration (1, 31). Based on this information, we examined the effect of ephrinB1 on lymphocyte migration using PBLs derived from healthy donors and a recombinant ephrinB1/Fc fusion protein. When PBLs treated with various amounts of ephrinB1/Fc were in the upper cham-

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Fig. 1. Immunohistochemical detection of ephrinB1 expression in synovial tissues. A: paraffin-embedded synovium sections derived from patients with rheumatoid arthritis (RA; \( n = 5 \)) and osteoarthritis (OA; \( n = 3 \)) were examined by staining with an anti-ephrinB1 antibody. Representative results are shown. Bar = 50 \( \mu \)m. B: sections derived from the same samples were double stained with rabbit anti-ephrinB1 and mouse anti-CD3 antibodies, followed by an incubation with Alexa fluor 488-labeled anti-rabbit IgG or Alexa fluor 555-labeled anti-mouse IgG. Bars = 100 \( \mu \)m for RA sections or 50 \( \mu \)m for OA sections.
EphrinB1 activated lymphocytes in a dose-dependent manner to respond to SDF-1α added to the lower chamber, whereas control Fc had no effect on the migration of PBLs (Fig. 3A). EphrinB1 itself possessed no chemotactic activity against PBLs, because PBLs in the upper chamber did not respond to ephrinB1/Fc added to the lower chamber. This activity of ephrinB1 was attenuated by genistein, a selective inhibitor of protein tyrosine kinases. It has been reported that EphB1 is a receptor of ephrinB1, so that tyrosine phosphorylation of EphB1 was analyzed using normal PBLs. As shown in Fig. 3C, tyrosine phosphorylation was enhanced by ephrinB1/Fc, and genistein inhibited this phosphorylation. In addition, we also detected a 60-kDa band of EphB1, which was produced upon stimulation with ephrinB1. These results are consistent with recent findings (6) suggesting that the ephrinB1/EphB1 signaling system might be involved in the stimulation of the lymphocytes.

We next examined the effect of ephrinB1 on the production of cytokines using PBLs derived from normal donors. As shown in Fig. 3B, ephrinB1/Fc stimulated PBLs to produce TNF-α in a dose-dependent manner. Production levels in ephrinB1/Fc-stimulated PBLs were significantly higher than those in Fc-treated PBLs. The stimulation of the TNF-α production was suppressed by genistein. A moderate stimulation was observed in PBLs treated with Fc at high concentrations, although the underlying mechanism was unclear. On the other hand, no stimulation in the production of IL-4 was observed by ephrinB1/Fc at any dose examined (data not shown). It is well known that the activation of T helper cell type 1 (Th1), rather than Th2-type T cells, is predominant in patients with RA. Since TNF-α is typical of Th1-type T cells, and IL-4 of Th2-type T cells, the ephrinB1/EphB1 system should play an important role in the activation of Th1-type T cells.

We also investigated the effect of ephrinB1 on the production of cytokines in synovial cells. As shown in Fig. 4A, ephrinB1/Fc, but not Fc, stimulated the production of IL-6 in synovial cells derived from RA patients in a dose-dependent manner. The production of IL-6 was significantly suppressed by genistein, suggesting an important role of receptor tyrosine kinase EphB1 for the production of IL-6. Indeed, EphB1 of synovial cells was phosphorylated at tyrosine residues by ephrinB1/Fc, and genistein inhibited the phosphorylation of EphB1 (Fig. 4B). These results suggest that ephrinB1 expressed on the T cell surface might be involved in the activation of synovial cells via EphB1 stimulation, which per se induces an expansion of inflammation in RA.

EphrinB1 gene is differentially methylated in RA. To understand genetic and epigenetic conditions underlying the high expression of ephrinB1 in RA, we analyzed the methylation status of the 5′ upstream region of the ephrinB1 gene in genome samples derived from seven patients with RA and five healthy donors. Although no disease-associated single-nucleotide polymorphism was detected in the 5′ upstream region of the ephrinB1 gene, a significant difference in methylation...
status of this region was detected between RA and control. There are 11 CpG sites between positions −179 and −21 in the promoter region of the ephrinB1 gene (Fig. 5A), and the methylation states of each clone are shown in Fig. 5B. The results indicate that the methylation levels of this region in the RA genome are lower than in the normal control. This tendency was more evident in the region from −79 to −140 compared with the downstream region (Fig. 5C); the methyl-C content was 25% in RA samples and 72% in control samples at position −148 and 21% in RA samples and 65% in control samples at position −142.

To confirm the effect of the methylation of the detected CpG motif on ephrinB1 expression, we made luciferase reporter constructs harboring the 5′ upstream region (−1000 to +1) of the human ephrinB1 gene. Reporter constructs were incubated

Fig. 3. Stimulation of PBLs derived from normal donors by ephrinB1. A: PBLs from normal donors suspended in RPMI-1640 (2.5 × 105 cells/ml) were pretreated with the indicated doses of ephrinB1/Fc or control Fc in the absence or presence of genistein for 1 h. Migration was determined in 24-well plates with 5-μm-pore size cell culture inserts; a 100-μl aliquot of the cell suspension was added to the upper insert, and a 600-μl aliquot of medium containing stromal cell-derived factor-1α (100 ng/ml) was added to the lower chamber. After an incubation for 3 h, numbers of cells that had passed through the membrane into the lower chamber were quantified by counting 5 random fields (means and SE). The examination was repeated five times, and representative results are shown. Statistical significance of the differences between ephrinB1 and Fc was calculated by Student’s t-test (*P < 0.05; **P < 0.01). B: PBLs derived from RA patients were treated with ephrinB1/Fc or control Fc in the absence or presence of genistein (10 μg/ml) for 15 min. Lysates were incubated with 4G10, and immunoprecipitates were analyzed by immunoblot analysis with an anti-EphB1 antibody. GAPDH was used as an internal control.

Fig. 4. Stimulation of cytokine production by ephrinB1/Fc. A: synovial cells derived from RA patients (18) were treated with ephrinB1/Fc or control Fc in the absence or presence of genistein for 48 h. Levels of IL-6 produced in the culture supernatant were determined (means and SD). The examination was repeated three times, and representative results are shown. Statistical significance of the differences between normal and RA groups was calculated by Student’s t-test (*P < 0.05; **P < 0.01). B: synovial cells derived from RA patients were stimulated by the indicated doses of ephrinB1/Fc in the absence or presence of genistein (10 μg/ml) for 15 min. Lysates were incubated with 4G10, and immunoprecipitates were analyzed by immunoblot analysis with an anti-EphB1 antibody. GAPDH was used as an internal control.

To confirm the effect of the methylation of the detected CpG motif on ephrinB1 expression, we made luciferase reporter constructs harboring the 5′ upstream region (−1000 to +1) of the human ephrinB1 gene. Reporter constructs were incubated
in the presence or absence of SssI methylase, and their luciferase activities were measured. High levels of relative activity were observed in cells transfected with the unmethylated construct, whereas the activity was strongly diminished by methylation of the construct (Fig. 5D).

**DISCUSSION**

Ephrin ligands on the cell surface transmit signals through interactions with their receptors, Eph receptors, on adjacent cells, and there is growing evidence that ephrins and Eph receptors play an important role in the regulation of cell migration and activation in the neuronal system (10). EphrinB1 activates EphB receptors to generate adhesive or repulsive signals through the modulation of integrin activation and actin cytoskeleton rearrangement. In the immunological sphere, various types of ephrinB and EphB receptors have been detected in immune cells (31), and the activation of EphB receptors might be involved in the regulation of the immune system (12, 21, 25, 27, 29). In fact, we demonstrate here using an ephrinB1/Fc fusion protein that ephrinB1 itself has no chemotactic activity but effectively stimulates the SDF-1 receptor on PBLs and RA synovial cells. Tight adhesion and arrest are important steps in the targeting and transmigration of leukocytes, and surface densities of ephrinB1 correlated closely with EphB1-coupled cell attachment (15, 22). T cell-T cell or T cell-synovial cell interactions might be early and critical events in acute and chronic inflammation.

RA is the most common form of inflammatory arthritis and is characterized by a disordered synovial microenvironment in which there is hyperplasia of resident stromal cells and a heavy infiltration of hematopoietic cells (5). The inflammatory process is usually tightly regulated, involving both mediators that initiate and maintain inflammation and mediators that shut the process down. In the states of chronic inflammation, an imbalance between the two mediators leaves inflammation unchecked, resulting in cellular damage and synovial tissue destruction. Thus, ephrinB1/EphB signaling might be involved in the imbalance of immune regulation and the aberrant accumulation of inflammatory lymphocytes in synovial tissues of patients with RA (25). In this context, it is quite interesting that RA synovial cells were also stimulated by ephrinB1/Fc (Fig. 4, A and B). These biological effects, namely, enhanced migration and cytokine production, were suppressed by genistein. EphB1 receptor on PBLs and RA synovial cells was actually phosphorylated by ephrinB1/Fc, and this phosphorylation was inhibited by genistein (Figs. 3B and 4B). Since EphB1 is highly expressed on some T cells and synovial cells in RA synovial tissues (data not shown), it is conceivable that ephrinB1 expressed on T cells derived from patients with RA interacts with EphB1 on neighboring T cells or other types of cells, such as synovial cells. Tight adhesion and arrest are important steps in the targeting and transmigration of leukocytes, and surface densities of ephrinB1 correlated closely with EphB1-coupled cell attachment (15, 22). T cell-T cell or T cell-synovial cell interactions might be early and critical events in acute and chronic inflammation.
in the pathogenesis of RA, and anti-TNF-α antibodies strongly suppress inflammatory reactions in RA joints. Taken together, it is possible that ephrinB1-EphB interactions occur in RA synovial tissues, resulting in situ in activating Th1-type T cells to exhibit enhanced production of inflammatory cytokines such as TNF-α.

It is noteworthy that DNA hypomethylation is involved in the high expression of ephrinB1 in PBLs derived from patients with RA. Both human (Fig. 5A) and mouse (Suppl. Fig. A) ephrinB1 genes possess a CpG island 200–400 bp upstream of the transcription start site. The results clearly demonstrate that the methylation status of the CpG island of ephrinB1 promoters was lower in RA patients (Fig. 5, B and C) and CAIA mice (Suppl. Figs. B and C) than in normal controls. DNA methylation of this promoter region affects transcription activity (Fig. 5D). Indeed, high expression levels of ephrinB1 were observed in PBL samples derived from RA patients (Fig. 2, A and B), and ephrinB1-positive CD3 cells were abundant in the synovial tissues of RA patients (Fig. 1B). These changes were detected in all PBL and tissue samples from patients with RA examined and were significant compared with samples derived from normal controls and patients with OA. In addition, similar results were also obtained using an experimental RA model, CAIA mice. These findings suggest that methylation states are closely associated with expression levels of Th1-type T cells.

There are several binding sites in this CpG region for methyl-CpG-sensitive transcription factors such as activator protein-2, STAT, c-Myb, and Ets. Methylation of CpG in binding sites for these factors prevents transcription activity (4, 8). A cluster of Sp1-binding sites is also located in this region. Sp1 family members act as positive regulators of TATA-less promoters (3). The Sp1 protein can bind methylated CpG sites but do not activate transcription when the sites are covered with methyl-CpG-binding proteins (20). Thus, methylation of the promoter region of the ephrinB1 gene affects the expression level of ephrinB1, which might be associated with controlling the mechanism of inflammation and, per se, an imbalance in T cell function. The mechanism underlying the hypomethylation of the ephrinB1 gene in RA or CAIA lymphocytes is not clearly understood. Our results reveal that the stimulation of PBLs prepared from normal donors with ephrinB1/Fc did not affect the methylation status (unpublished data). On the other hand, it has been reported that some ephrins in rats are induced in circulating lymphocytes by the administration of LPS (17). Thus, it is quite natural to hypothesize that some environmental factors, such as LPS, induce an ephrinB1-positive subset of T cells, which frequently interact with EphB receptor-positive stroma or Th1-type T cells. This interaction activates EphB receptor-expressing cells to produce excess amounts of inflammatory cytokines. Further careful studies are required to identify ephrinB1-positive T cells and to gain an understanding of their function in the pathogenesis of RA.

In conclusion, ephrinB1-positive T cells are abundant in synovial tissues of patients with RA, and high expression levels of ephrinB1 were observed in blood lymphocytes derived from RA patients, probably due to hypomethylation in the promoter region of the ephrinB1 gene. EphrinB1 is involved in enhancing the migration and production of TNF-α of lymphocytes and production of IL-6 of synovial cells. These results suggest that ephrinB1-positive T cells play an important role in the pathogenesis of RA. Further studies on the function and regulation of ephrinB1 may lead to an understanding of the etiology of RA and to the development of effective therapies for RA.

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