Scand J Rheumatol 20: 91–97, 1991

Facs Analysis of Myeloid Differentiation Stages in Epiphyseal Bone Marrow, Adjacent to Joints Affected with Rheumatoid Arthritis

H. OWAKI[1], K. YUKAWA[2], T. OCHI[1], Y. SHIMAOKA[1] and K. ONO[1]

[1]*Department of Orthopaedic Surgery, and* [2]*Institute for Molecular and Cellular Biology, Osaka University Medical School, Osaka, Japan*

Owaki H, Yukawa K, Ochi T, Shimaoka Y and Ono K. Facs Analysis of Myeloid Differentiation Stages in Epiphyseal Bone Marrow, Adjacent to Joints Affected with Rheumatoid Arthritis. Scand J Rheumatol 1990; 20: 91–97.

To analyze the differentiation stages of myeloids statistically, we adopted a two-color FACS system and used appropriate monoclonal antibodies belonging to CD15, CD16 and CD11b. By using HL60 treated with DMSO or human bone marrow MNCs from patients with rheumatoid arthritis, it was proved that with this system, myeloids could be clearly separated according to differentiation stages. Furthermore, the number of myeloids at certain stages of differentiation in the epiphyseal bone marrow of patients with RA or OA was measured. Nine of 15 samples from RA patients showed immature and relatively mature myeloids, while none of the 8 OA samples did. When the proportions of myeloids in epiphyseal bone marrow MNCs were compared with the clinical features, disease subsets in RA and the degree of synovitis, seemed to be important factors for abnormal myelopoiesis.

Key words: rheumatoid arthritis, bone marrow, myeloids

Hajime Owaki, 8650 Southwestern Blvd. #2811, Dallas, Texas 75206, USA

We have previously reported the presence of myeloid lineage cells in the epiphyseal bone marrow, adjacent to joints affected with rheumatoid arthritis, and the absence of these cells in normal or non-RA joints(1). Following this report, we demonstrated a highly active myeloid growth factor in corresponding sites in RA(2) and indicated this could be an important factor for abnormal myelopoiesis. To study this phenomenon in more detail, it is necessary to determine the spectrum of the differentiation stages of these abnormally accumulated myeloids. Some specific cytoplasmic markers for differentiation of myeloids are known, such as lactoferrin(3), but these are not useful for quantitative analysis. On the other hand, Fluorescence Activated Cell Sorter (FACS) analysis, using monoclonal antibodies for the cell surface marker, is a powerful tool for quantitative study, even though the technique is very simple. In this study, we analyzed the differentiation stages of myeloids with FACS. For this purpose, we first used HL60, a well known myeloblastic leukemia cell line, differentiated into myelo-granulocytic lineage cells, in reaction to stimulation with chemical such as dimethyl-sulfoxide (DMSO)(4). We next analyzed the quantities of epiphyseal bone marrow cells in rheumatoid arthritis (RA) and osteoarthritis (OA) cases and compared the results with clinical findings.

MATERIALS AND METHODS

Patients

Fifteen joints from 13 patients diagnosed as RA according to the criteria of the American Rheumatism Association(5) were studied. There were 2 men and 11 women with an age range of 30–71 (mean 52). According to our classification of disease severity for RA(6),

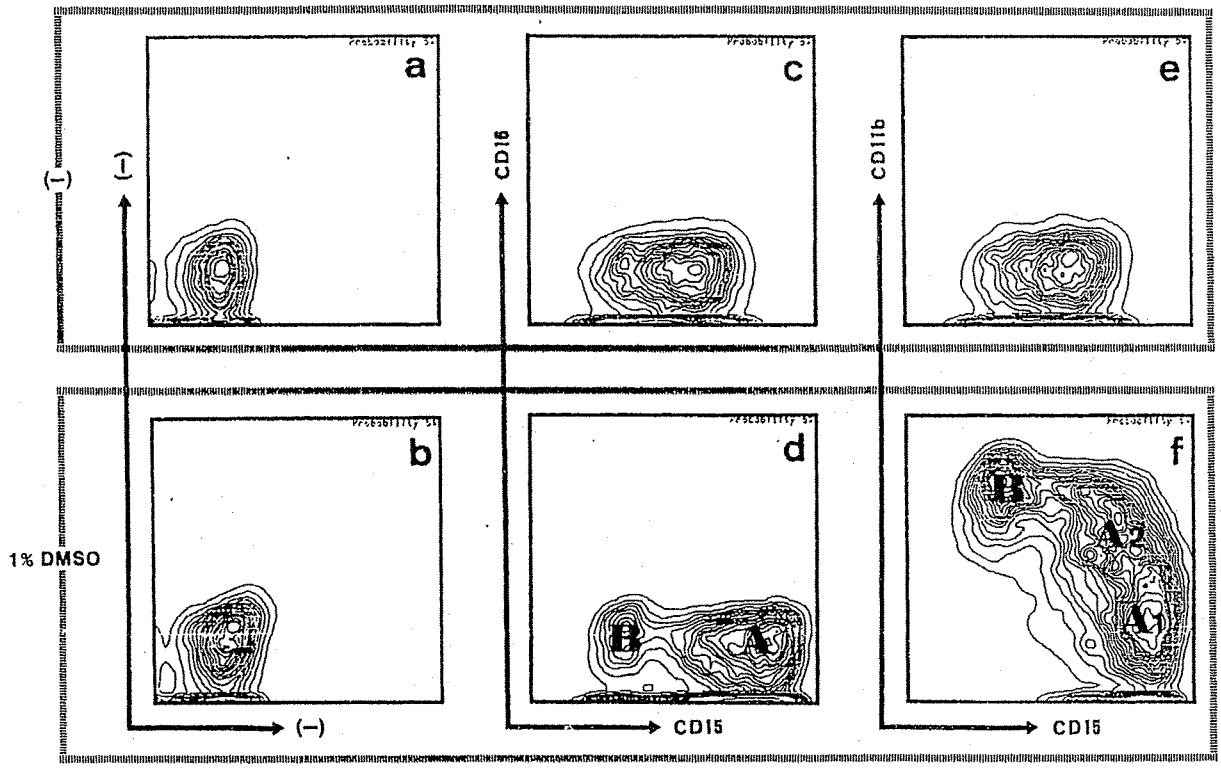


Fig. 1. Two-color FACS analysis of HL60 treated with (b, d, f) or without (a, c, e) 1% DMSO. Two-color staining was performed as follows: a and b: unstained; c and d: CD15(MX–GA) vs CD16(MG38); e and f: CD15(MX–GA) vs CD11b(Mol).

there were two cases of mutilating disease (MUD), six of the more erosive subset (MES), three of the least erosive subset (LES), and two that could not be classified because of the short duration of the disease. The controls were 8 joints from 8 patients with OA, one man and 7 women aged 48–70 (mean 58). All patients were diagnosed and treated at Oaska University Hospital.

Samples

Heparinized bone marrow blood was aspirated from the tibial proximal epiphysis during knee joint surgery, and mononuclear cell (MNC) fractions were separated by Ficoll-Paque

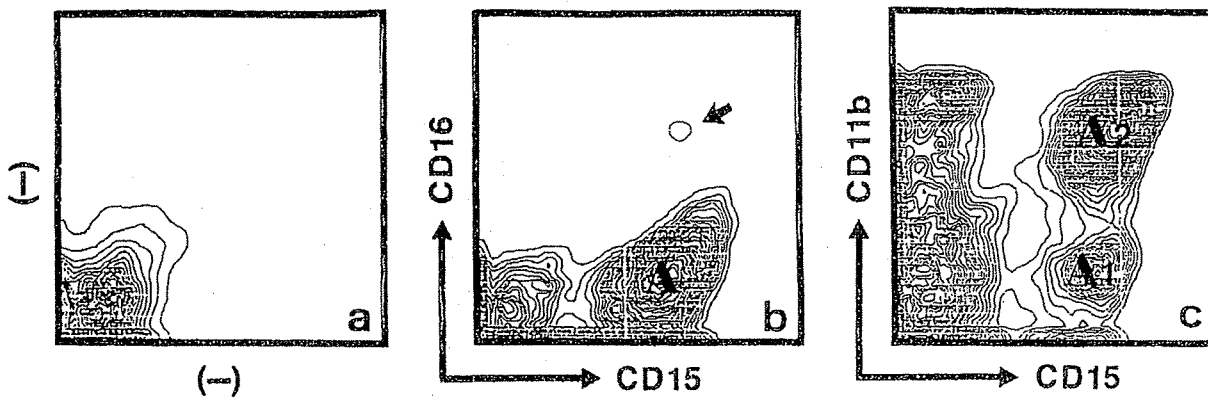


Fig. 2. Two-color FACS analysis of epiphyseal bone marrow MNCs from RA patients. a: unstained; b: CD15(MX–GA) vs CD16(MG38); c: CD15(MX–GA) vs CD11b(Mol).

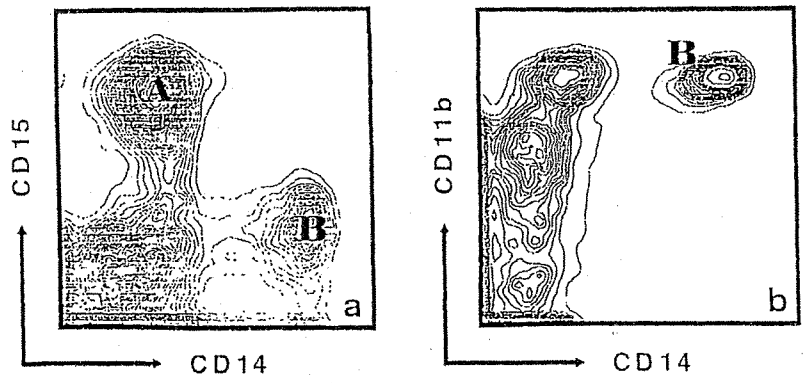


Fig. 3. Two-color FACS analysis of epiphyseal bone marrow MNCs from RA patients. a: CD14(Mo2) vs CD15(MX-GA); b: CD14(Mo2) vs CD11b(Mol).

(Pharmacia Fine Chemical, Sweden) discontinuous density gradient centrifugation (700 g, 20 min).

Cell line

HL60, a human myeloblastic leukemia cell line, was donated by Prof. Kishimoto (Institute for Molecular and Cellular Biology, Osaka University Medical School) and cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM of L-glutamine, 5×10^{-5} M of 2-mercaptoethanol, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Some cells were cultured in the same medium, but supplemented with 1% DMSO during 7 days before FACS analysis.

Monoclonal antibodies

MX–GA antibody (CD15, clone HL5), which recognizes pan-myeloids, from myeloblasts to polymorphonuclear leukocytes (PMN)(7), and also reacts with HL60(8), was purchased from Kyowa Medex (Japan). MG38 antibody (CD16), which reacts with FCγ receptors on PMN, but not on natural killer (NK)(9), was purchased from Seikagaku-Kogyo (Japan). Mol antibody (CD11b), which reacts with relatively mature myeloids, from myelocytes to PMN, and also monocytes-macrophages(10), was purchased from Coulter Clone (USA). Mo2 antibody (CD14), which is a specific marker of monocytes-macrophages(10), was purchased from Coulter Clone. If fluorescence-isothiocyanate(FITC)- or biotin-labelled antibodies were not available, labelling was performed as previously described(11).

Two-color FACS analysis

Bone marrow MNCs were washed and suspended in a staining buffer (RPMI1640 deficient biotin, riboflavin and phenol red/2% FCS/10 mM HEPES/0.02% NaN_3) at a concentration of 10^6 cells/20 μl. Appropriately diluted FITC- or biotin-labelled antibodies were simultaneously added to the cell suspension in quantities of 10 μl each, and incubated for 20 min at 4°C. After washing with the staining buffer, 20 μl of Texas-Red avidin solution was added to the cell pellets which were then suspended. Incubated for 20 min at 4°C, propidium iodide (10 μg/ml) was also incubated during the last 5 min to label dead cells. Washed 3 times, cells were applied to a FACS440 (Becton Dickinson) equipped with a dual-laser system.

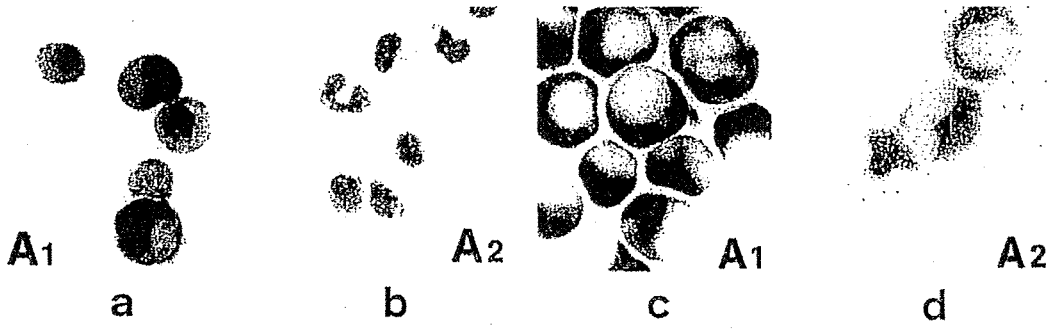


Fig. 4. Cytochemical staining of sorted cells. a and b: May-Grunwald; c and d: peroxidase staining. a and c: CD15-positive and CD11b-negative (A1) cells; b and d: CD15-positive and CD11b-positive (A2) cells.

Quantitative analysis

The gating in FACS was made on the plane of forward scatter and side scatter in each case, so as to remove the contaminated red blood cells. These could be clearly identified as small and no granular cells. The proportions of the number of each group of cells to that of whole MNCs were calculated by dividing the former by the latter.

Cytochemical staining

Sorted cells were attached to the glass slides with Cytospin (Shandon, England); some were stained with May-Grunwald and Giemsa solutions, and the others with peroxidase.

Classification of synovitis severity

The degree of synovitis was classified as follows, according to the findings at the surgical operations. (++): joint capsule was entirely covered with proliferated synovial cells; (+): about half of the capsular surface was covered with synovial proliferation; (±): synovitis was found only around the capsule-bone junction or bone-ligament junction; (−): there was no synovitis and the capsule was covered with fibrous tissue.

RESULTS

Two-color FACS analysis of HL60 cell line

The results for HL60 without chemical stimulation are shown in the upper row, and for HL60 treated with DMSO in the lower row, in Figure 1. HL60 cultured without DMSO reacted with CD15, but was not stained by CD16 nor CD11b (Figure 1 c and e). Cultured with DMSO, HL60 was not stained by CD16 either, but some cells (A) stained more brightly with CD15 (Figure 1 d). When stained with CD11b (Figure 1 f), these CD15-bright cells(A) were separated into two groups, CD11b-negative (A1) and -positive (A2).

Two-color FACS analysis of RA bone marrow MNC

One of the RA severe cases showed a FACS pattern similar to that of HL60 cultured with DMSO. There was a large number of cells (A) which reacted with CD15 (Figure 2 b), while the number of contaminated PMN was small (Figure 2 b, arrow). These CD15-positive cells

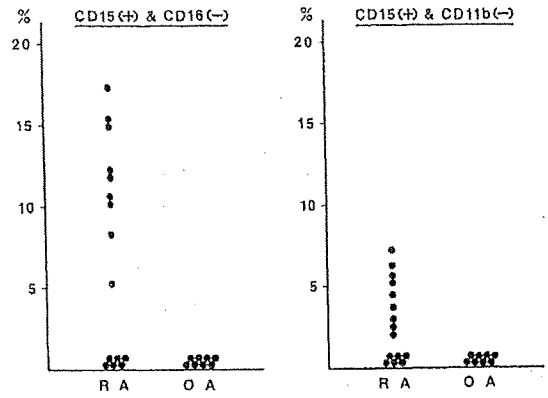


Fig. 5. Quantitative analysis of epiphyseal bone marrow myeloids from patients with RA or OA. The proportions of (A) or (A1) cells in MNCs are shown on the ordinate. (a): (A) cells = myeloids minus contaminated PMN; (b): (A1) cells = from myeloblasts to promyelocytes.

(A) could then be divided into CD11b-negative (A1) and CD11b-positive cells (A2) (Figure 2). Stained with CD14 and CD15, cells were clearly separated into three groups: CD15-positive cells (A), CD14-positive cells (B) and double-negative cells, but there existed no double-positive cells (Figure 3 a). All of the (B) cells reacted also with CD11b (Figure 3 b).

Cytochemical staining of sorted cells

The (A1) cells and (A2) cells seen in Figure 2 c were separately sorted by FACS. After being attached to glass slides, they were stained with May-Grunwald solution (Figure 4, a and b) or peroxidase (Figure 4, c and d). CD15-positive and CD11b-negative cells (A1) had a round nucleus and relatively large cytopasm (Figure 4 a). Their cytoplasmic granules were stained by peroxidase (Figure 4 c). CD15-positive and CD11b-positive cells (A2) showed a kidney-shaped nucleus (Figure 4 b), and their cytoplasmic large granules also stained with peroxidase. Consequently, (A1) cells showed the characteristics of myeloblasts-promyelocytes and (A2) cells those of myelocyte-metamyelocytes.

Quantitative analysis of myeloids in patients with RA or OA

Epiphyseal bone marrow MNCs from 15 joints with RA and 8 joints with OA were analyzed by FACS with results similar to those shown in Figure 2. The proportions of (A) or (A1) cells in epiphyseal bone marrow MNCs were calculated and are shown in Figure 5 on the ordinate. Nine of 15 joints with RA showed the existence of (A) cells (Myeloids minus contaminated PMN), with a range from 5.2% to 17.3% (mean: 11.8%) (Figure 5 a), while the others showed no myeloids (< 1%). The mean value for all RA joints was 7.1%. None of the patients with OA showed either (A) or (A1) cells. In RA patients, 9 joints with (A) Cells showed also (A1) cells (Myeloblasts-promyelocytes), with a range from 2.3% to 7.2% (mean 4.4%) (Figure 5 b). For all RA joints, the mean was 2.6%. Compared to (A) cells (Myeloids), the proportions of (B) cells (Macrophages) were more constant. In cases with RA (B) cells showed with a range from 8.7% to 16.3% (mean 12.7%), and OA showed with a range from 6.6% to 14.8% (mean 11.9%). There were no significant difference bitween RA and OA statistically.

Clinical features of RA joints

Profiles of patients with RA are listed in Table I, arranged according to the proportion of (A) cells. It was found that the group of joints with no (< 1%) (A) cells showed no synovitis, while none of the RA patients in subset LES had any (A) cells. Patients in subset MES who

Table I. Clinical features of RA joints and proportions of myeloids in bone marrow.

case	age	sex	disease period	disease subset	operat.	synovitis	(A): CD 15 (+) & CD 16 (−)	(A1): CD 15 (+) & CD 11b (−)
M.M	71	F	2 y	(ORA)	TKR(L)	(++)	17.3%	4.4%
M.Y	30	F	12 y	MUD	TKR(R)	(±)	15.3%	7.2%
K.K	57	F	13 y	MUD	TKR(L)	(−)	15.1%	5.6%
K.O	55	F	12 y	MES	TKR(R)	(+)	12.1%	6.2%
H.M	56	M	15 y	MES	TKR(R)	(+)	12.0%	5.2%
R.K	51	F	14 y	MES	TKR(R)	(+)	10.5%	3.6%
Y.D	47	F	7 y	(N.D.)	synovectomy	(++)	10.3%	2.6%
K.M	43	F	10 y	MES	TKR(R)	(+)	8.2%	2.4%
K.O	55	F	12 y	MES	TKR(L)	(+)	5.2%	2.3%
M.Y	34	F	12 y	MES	TKR(L)	(−)	<1.0%	<1.0%
S.Y	61	F	18 y	MES	TKR(R)	(−)	<1.0%	<1.0%
N.M	53	M	11 y	LES	TKR(R)	(±)	<1.0%	<1.0%
U.O	68	F	10 y	LES	TKR(R)	(−)	<1.0%	<1.0%
U.O	68	F	10 y	LES	TKR(L)	(−)	<1.0%	<1.0%
J.H	52	F	16 y	LES	TKR(L)	(−)	<1.0%	<1.0%

ORA: Old onset RA; N. D.: Not determined; TKR: Total knee replacement; MUD: Mutilating disease; MES; More erosive subset; LES: Least erosive subset.

had (A) cells showed (+) or (++) synovitis, but patients with MUD had many (A) cells although there was no evidence of synovitis.

DISCUSSION

Myeloid cells differentiate from bi-potential stem cells to PMN finally, and they have been traditionally characterized with cytochemical staining such as May-Grunwald, Giemsa and peroxidase. Later, some cytoplasmic markers for differentiation were found, such as lactoferrin(3) and inosine mono-phosphate(12), but they are not convenient for statistical or quantitative analysis. On the other hand, some cell surface antigens are known as differentiation markers of myeloids, namely, the C3bi and Fcγ receptors(13). The monoclonal antibodies for those receptors have been produced and are classified as CD11b and CD16, respectively. However, CD11b- or CD16- positive cells are not only myeloids, but also other lineage cells, so ti is necessary to purify the myeloids with cell-biological techniques or on the image of a FACS monitor. For the latter purpose, some monoclonal antibodies belonging to CD15 are useful, that is, they recognize only myeloid lineage cells, from myeloblasts to PMN. Double staining with CD15 monoclonal and CD11b or CD16 monoclonal, showed the differentiation stages of myeloids clearly, both in HL60 cell lines treated with DMSO and human bone marrow cells. Because of the simple staining method and quantitative analysis, this method could by useful for identifying the existence of immature myeloids.

We previously reported the elevated titers of myeloid growth activity in epiphyseal bone marrow adjacent to RA joints(2), and that this could be an important factor for abnormal myelopoiesis. It remains however unclear whether relatively mature myeloids invade in this site and proliferate or immature myeloids (or stem cells) differentiate and proliferate at this site. The results of the present study show a considerable number of immature myeloids besides relative mature cells, so that the latter hypothesis should be considered more likely.

The existence of myeloids in epiphyseal bone marrow in RA, is closely related to the clinical features of RA. Furthermore, disease subsets in RA and the degree of synovitis seem

to be important factors for this phenomenon. As for MES, synovial proliferation and the existence of myeloids are well correlated, so they may have a close pathological relationship. On the other hand, in the MUD subset, many myeloids exist in epiphyseal bone marrow without being accompanied by synovitis, so it is possible that myeloids appear independently of synovial lesions in this disease subset.

The mechanism of joint destruction in MUD seems to be different from that of the other types, where, the bone destruction begins as erosion at the capsule-bone junction. The destruction in MUD is so fast that it looks like a collapse of the bone structure. In view of these findings, the epiphyseal bone marrow in MUD could be the site of a severe reaction followed by osteoporosis and myelopoiesis, although these two may not directly correlate.

The functional abnormality of myeloids accumulating in epiphyseal bone marrow adjacent to joints with RA, is not clearly understood yet. However, PMN (the final differentiation stage of myeloids) in such lesions, are functionally different from those in other lesions in RA(14), and this function is most markedly shown in MUD cases. More detailed studies are needed to determine whether myeloids in such lessions also have an abnormal function as PMN do, and to understand the relation between myeloids and joint destruction in RA.

REFERENCES

1. Ochi T, Hakomori S, Adachi M, et al. 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 1989; 15: 1609–15.
2. Owaki H, Ochi T, Yamasaki K, et al. Elevated activity of myeloid growth factor in bone marrow adjacent to joints affected by rheumatoid arthritis. J Rheumatol 1989; 16: 572–7.
3. Tado TA, Wei X, Benz EJ. Isolation of lactoferrin cDNA from a human myeloid library and expression of mRNA during normal and leukemic myelopoiesis. Blood 1987; 70: 989–93.
4. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds, Proc Natl Acad Sci USA 1987; 75: 2458–62.
5. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988; 31: 315–24.
6. Ochi T, Iwase R, Yonemasu K, et al. Natural course of joint destruction and fluctuation of serum Clq levels in patients with rheumatoid arthritis. Arthritis Rheum 1988; 31: 37–43.
7. Hamasima N, Uede R, Takahashi T. Characterization of three monoclonal antibodies that reach with high-molecular mass glycopeptides isolated from F9 mouse teratocarcinoma cells. Differentiation 1986; 31: 174–82.
8. Namikawa R, Ogata S, Uede R, et al. Serological analysis of cell surface antigens of HL-60 cells before and after treatment with a phorbor ester tumor promorter. Leuk Res 1983; 7: 375–87.
9. Tetteroo PAT, Van Der Schoot CE, Visser FJ, Bos MJE, Von Dem Borne AEGKR. Three different types of Fcγ Rlow of neutrophils, Fcγ Rlow of K/NK lymphocytes, and Fcγ RII. In: McMichael AJ eds. Leukocyte Typing III: White Cell Differentiation Antigens. Oxford/New York/Tokyo: Oxford University Press, 1987: 702–6.
10. Hogg N, Horton MA. Myeloid antigens: new and previously defined clusters. In: McMichael AJ eds. Leukocyte Typing III: Withe Cell Differentiation Antigens. Oxford/New York/Tokyo: Oxford University Press, 1987: 576–663.
11. Goding JW. Conjugation of antibodies with fluorochrome: modification of the standard methods. J Immunol Methods 1976; 13: 215–26.
12. Knight RD, Mangum J, Lucas DL, Cooney DA, Khan EC, Wright DG. Inosine monophosphate dehydrogenase and myeloid cell maturation. Blood 1987; 69: 634–9.
13. Glasser L, Fiederlein RL. Functional differentiation of normal human neutrophils. Blood 1987; 69: 937–44.
14. Wakitani S, Sakamuro D, Ochi T, Owaki H, Fujimoto M, Ono K. Polymorphonuclear cell factor found in patients with rheumatoid arthritis. Biomedical Research 1988; 9: 395–9.

Received 6 June 1990
Received revised 27 September 1990

Review

Mesenchymal stromal cells

Nurse-like cells reside in the synovial tissue and bone marrow in rheumatoid arthritis

Takahiro Ochi[1], Hideki Yoshikawa[2], Tomoko Toyosaki-Maeda[3] and Peter E Lipsky[4]

[1]Sagamihara National Hospital, Sagamihara, Kanagawa, Japan
[2]Department of Orthopaedic Surgery, Osaka University Medical School, Suita, Osaka, Japan
[3]Department of Immunology, Shionogi Research Laboratories, Shionogi & Co. Ltd, Osaka, Japan
[4]National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892, USA

Corresponding author: Takahiro Ochi, t-ochi@sagamihara-hosp.gr.jp

Published: 12 February 2007
This article is online at http://arthritis-research.com/content/9/1/201

Arthritis Research & Therapy 2007, **9**:201 (doi:10.1186/ar2105)

Abstract

A major question concerning the immunopathology of rheumatoid arthritis is why the disease is localized to particular joints. A possible explanation could be the presence within the synovium of cells that foster inflammation or easy accessibility of the synovium to migratory disease enhancing cells. Within both the bone marrow and the synovium, fibroblastic stromal cells play an important role in supporting the differentiation and survival of normal cells, and also contribute to the pathologic processes. Among fibroblastic stromal cells in synovial tissue and bone marrow, nurse-like cells are a unique population having the specific capacity to promote pseudoemperipolesis (adhesion and holding beneath) of lymphocytes, and also the ability to promote the growth and function of some populations of lymphocytes and monocytes. Nurse-like cells could therefore contribute to the immunopathogenesis of rheumatoid arthritis, and may contribute to the localization of inflammation within specific joints. The present review considers the evidence that supports these possibilities.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by immunologically enhanced inflammation and damage to articular structures [1,2]. Rheumatoid synovium is a site of intense inflammation, with active involvement by various populations of infiltrating lymphocytes, myeloid cells, and resident synovial fibroblasts or synoviocytes [1]. One question that has not been addressed is why RA preferentially affects certain joints. Although the explanation for the localization of rheumatoid inflammation to particular joints is not clear, one possibility relates to the presence within the synovium of resident cells that can promote inflammation. In addition, cells that can be induced to migrate from adjacent bone marrow structures may contribute to the local facilitation and propagation of inflammation and bone damage. The present review will focus on one such population, the nurse-like cells (NLCs) that populate the rheumatoid synovium and bone marrow.

Fibroblastic stromal cells in bone marrow and synovial tissue

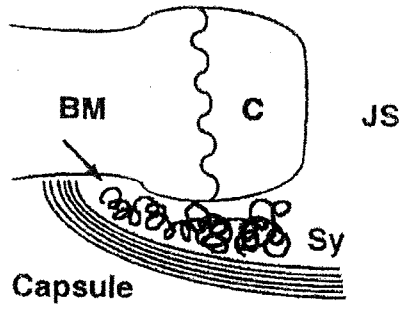
Initially, to examine the relationship between the epiphyseal bone marrow and synovial tissue, we employed the animal model of collagen-induced arthritis [3]. Fibroblastic stromal cells (FSCs) in the bone marrow of Lewis rats were labeled with a fluorescent probe or ^{3}HTdr and were examined for their migration at the onset of arthritis [4]. Accompanying the induction of polyarthritis, a large number of labeled FSCs in bone marrow were found to migrate into the joint cavity through canals observed in the bare zone of the joint (Figure 1), and then to proliferate in the synovial tissue. This observation suggested the hypothesis that pathophysiological cells of RA could be produced in bone marrow, from which some of these cells could migrate into the joint space and potentially play roles in inflammation or tissue damage in and around articular structures. Based on these findings, we have studied FSCs of RA patients, comparing the characteristics of FSCs from bone marrow and FSCs from synovial tissue [5-7].

Nurse-like cells found in bone marrow and synovial tissue

Among the FSCs derived from the bone marrow and synovium of RA patients, a population of NLCs was identified by the capacity to carry out pseudoemperipolesis. The

BST-1 = bone marrow fibroblastic stromal cell antigen 1; FSC = fibroblastic stromal cell; GM-CSF = granulocyte/macrophage colony-stimulating factor; HLA = human major histocompatibility antigen; IFN = interferon; IL = interleukin; mAb = monoclonal antibody; NLC = nurse-like cell; RA = rheumatoid arthritis; RANKL = receptor activator of NF-κB ligand; TNF = tumor necrosis factor; TRAP = tartarate-resistant acid phosphatase.

Figure 1



Migration of fibroblastic stromal cells from epiphyseal bone marrow (BM) into the joint space (JS) forming synovial (Sy) tissue in collagen-induced arthritis. C, cartilage.

function of the NLCs was reminiscent of thymic nurse cells [8,9], which have the capacity to interact with populations of thymic cells and gather them beneath their cell bodies in a process known as pseudoemperipolesis (adhesion and holding beneath). *In vivo*, such thymic nurse cells were thought to support the development and expansion of thymocytes and to also play a role in positive/negative selection of T cells in mouse and rat thymus. A very similar capacity to interact and support the maturation of some population of lymphocytes and monocytes was noted for FSCs of bone marrow [5,7] and for FSCs of synovial tissue [6,7] of RA patients, suggesting that the NLC function of FSCs could contribute to the pathophysiology of RA [7].

We established RA-NLC clones with the ability to promote pseudoemperipolesis from bone marrow [5] and synovial tissue [6] of RA patients. These RA-NLC clones were determined to be of mesenchymal origin, given that they expressed vimentin but not cytokeratin. They did not exhibit desmosomes or classical junctional complexes, both of which are characteristic features of epithelial cells. Elongated and branching mitochondria were present in the cytoplasm of the clones, and caveolae, which are unique to cells of mesenchymal origin, were present on the surface [5,6].

NLCs have a number of unique functional activities that could contribute to rheumatoid inflammation. Among these activities are their ability to promote antibody production by B cells, the capacity to protect lymphocytes from apoptosis, the ability to secrete large amounts of cytokines and chemokines that could promote the accumulation and activation of lymphocytes and monocytes, and their unique capacity to promote the differentiation of osteoclasts from myeloid precursors in a receptor activator of NF-κB/receptor activator of NF-κB ligand (RANKL)-independent manner [10].

Multipotent mesenchymal stem cells from bone marrow were also found to exist in the synovial membrane [11-14]. Those cells were shown to have multipotency to develop into various cells such as cartilage, bone, fat, and muscle. Although it is currently unknown whether these cells can differentiate into NLCs, RA-NLCs are a more differentiated population. Multipotential mesenchymal stem cells from the synovial fluid and bone marrow of patients with inflammatory and degenerative arthritis were reported to be negative for CD45 and to be positive for D7-FIB, CD13, CD105, CD55, and CD10 [13]; these mesenchymal stem cells therefore have a very different phenotype from that of RA-NLCs mentioned in the following.

Surface phenotype of rheumatoid arthritis nurse-like cells

RA-NLC clones from bone marrow and synovial tissue [5-7] expressed CD29, CD44, CD49c, CD54, CD106, and HLA-A, HLA-B, and HLA-C (class I major histocompatibility complex), but did not express CD1a, CD18 (LFA-1), CD35, CD40, CD154, or CD56. RA-NLCs constitutively expressed CD106 after long-term culture in the absence of cytokine stimulation. Constitutive expression of CD106 appears to be a characteristic appearance of nurse cell lines, permitting them to be distinguished from fibroblasts [7]. Human dermal fibroblast also expressed CD29, CD49c, CD54, and class I major histocompatibility complex, whereas constitutive expression of CD106 was minimal. IFNγ (100 U/ml) stimulation of RA-NLCs induced expression of CD40 and HLA-DR (class II major histocompatibility complex), but not expression of CD35 or CD154. The surface phenotype of RA-NLCs was therefore similar to that of FSCs derived from synovial tissue and bone marrow cells from non-RA controls. Namely, the phenotype of NLCs derived from osteoarthritis patients and human skin nurse cells was similar to that of RA-NLCs. Enhanced expression of CD106 and CD157 by IFNγ (mentioned below) was the characteristic observation in RA-NLCs and was different from human dermal fibroblasts [7].

Expression of CD106 by RA-NLCs was modestly enhanced by culture with normal peripheral B cells, and was markedly enhanced by IFNγ. In contrast, expression of CD106 by human dermal fibroblasts was much less marked after stimulation with IFNγ or by culture with peripheral B cells. One of the features of NLCs is their capacity to promote the survival of B lymphocytes [5-7]. Such B-cell survival was reduced by a blocking anti-CD106 mAb to the same level as B cells cultured in medium alone.

One notable product of NLCs is human bone marrow fibroblastic stromal cell antigen 1 (BST-1). This product was originally cloned from a human bone marrow FSC cell line by surveying for any unknown factors [15], supporting the FSC-dependent growth of the murine pre-B-cell line DW34. A new growth factor was identified, having the ability to enhance DW34 cell growth, and it was designated BST-1 [16]. Human BST-1 is expressed in various tissues and cell

lines, such as umbilical vein endothelial cells, myeloid cells, as well as FSCs of bone marrow and also synovial cells in RA, but is not expressed in lymphoid cell lines. Notably, serum levels of BST-1 were higher (30-fold to 50-fold) in 7% of RA patients than in non-RA samples [17]. Human BST-1 was later designed as CD157, and the human *Bst-1* gene was assigned to chromosome 4q15, regulating humoral immune responses *in vivo* [18]. Expression of CD157 (BST-1) was detected on all RA-NLCs, as well as on human dermal fibroblasts. Expression of CD157 by RA-NLCs, but not by dermal fibroblasts, was enhanced by IFNγ. This enhancement was much more marked with bone marrow-derived RA-NLCs compared with synovium-derived RA-NLCs. It should be noted that expression of CD106 and CD157 mRNA was found in all RA-NLC clones. Soluble CD157 together with RA-NLCs further increased the survival of B cells, which was reduced by a blocking anti-CD157 polyclonal antibody [7].

Cytokine production by nurse-like cells of RA patients

RA-NLCs produced numerous cytokines [5-7]. RA-NLCs from both bone marrow and synovial tissue produced detectable levels of IL-6, IL-8, and granulocyte/macrophage colony-stimulating factor (GM-CSF), and the production of IL-6 and IL-8 was quite robust. RA-NLCs from bone marrow but not synovial tissue produced IL-7, whereas RA-NLCs from synovial tissue produced granulocyte colony-stimulating factor and a greater amount of IL-6. Regulation of the production of cytokines was examined by co-culture of RA-NLCs from synovial tissue in direct contact with B cells. Secretion of IL-6, IL-8, granulocyte colony-stimulating factor, and GM-CSF was markedly increased by co-culture with B cells. IL-1β and TNF were only detected in the culture supernatants after co-culture with B cells. The effect of co-culture with B lymphocytes on the secretion of cytokines and immunoglobulin production by the B cells were examined under various culture conditions [5-7] (Table 1). After co-culture with B cells, the levels of IL-6, IL-8, granulocyte colony-stimulating factor, GM-CSF, and the levels of IgM were increased, and IL-1β and TNF were detected. Direct contact with the B-cell clone was required for RA-NLCs to produce IL-1β and TNF and higher levels of the other cytokines.

Inhibition of spontaneous apoptosis of lymphocytes and the effect of adhesion molecules

RA-NLCs were found to promote lymphocyte viability. Although peripheral blood B cells cultured in medium alone rapidly died, culture of B cells with RA-NLCs markedly increased the B-cell viability. The loss of viability of B cells cultured alone related to the induction of apoptosis, whereas co-culture of B cells with RA-NLCs substantially blocked their apoptosis. The mechanism of the prevention of apoptosis of B cells involved the contact-dependent upregulation of Bcl-x_L by RA-NLCs [19].

The regulation of pseudoemperipolesis (adhesion and holding beneath) by RA-NLCs was examined using MC/car cells and a cloned RA-NLC line from synovial tissue [20]. Pretreatment with anti-CD29 (integrin β_1 chain) or anti-CD49d (integrin α_4 chain) reduced adhesion by MC/car cells by approximately 50%. This result indicated that integrin $\alpha_4\beta_1$ (very late antigen 4) on MC/car cells was involved, at least in part, in the cells' ability to participate in pseudoemperipolesis with RA-NLCs, although such interactions were not involved in IL-6 and IL-8 production by RA-NLCs. Pretreatment of MC/car cells with the Rho-specific inhibitor C3 transferase significantly inhibited the migration of MC/car cells underneath RA-NLCs in a concentration-dependent manner, whereas the same treatment did not inhibit the adhesion of the MC/car cells to RA-NLCs. In addition, RA-NLCs produced comparable levels of IL-6 and IL-8 when co-cultured with C3-treated transmigration-defective MC/car cells. The processes of pseudoemperipolesis, adhesion and holding beneath were therefore thought to be independent events [20]. Moreover, very late antigen 4 ($\alpha_4\beta_1$)-independent lymphocyte adhesion and not holding beneath induced the enhanced proinflammatory cytokine production by the RA-NLCs [20].

Regarding NLCs, another group reported that CD14(+) monocytes could differentiate into NLCs and support the viability of chronic lymphocytic leukemia B cells [21-23], and also support the viability of primary B cells in RA [24,25]. These effects were dependent on interactions between RA-NLC-expressed CD106 and B-cell-expressed very late antigen 4 [24], which were quite similar to the interactions between RA-NLCs and B cells we had previously reported [7]. Although the other group's NLCs were identified to be derived from CD14 myelomonocytic cells [22,23,25] we have not yet clarified the stem cell of our RA-NLCs, but it clearly appears to be of mesenchymal origin [5,6].

RANKL-independent differentiation of osteoclast-like cells supported by RA nurse-like cells

RA-NLCs also promoted a specific pathway of the differentiation of CD14(+) monocytes. After 3–4 weeks of co-culture, CD14(+) monocytes differentiated into tartarate-resistant acid phosphatase (TRAP)(+) mononuclear cells with abundant cytoplasm and an off-center nucleus without the involvement of RANKL. It was noted that RA-NLCs supported such differentiation of peripheral blood CD14(+) monocytes not only from RA patients, but also from normal control subjects [10]. The second step of differentiation from such TRAP(+) mononuclear cells into multinucleated bone-resorbing giant cells (osteoclast-like cells) could also be induced without RANKL in the presence of IL-3, IL-5, IL-7, or GM-CSF, and was inhibited by mAb to each cytokine [10]. Differentiation of these TRAP(+) mononuclear cells into multinucleated bone-resorbing giant cells could also be promoted by macrophage colony-stimulating factor and RANKL [26].

Table 1

Effects of co-culture on production of cytokines from rheumatoid arthritis nurse-like cells (RA-NLCs)

	Cytokines in cell culture supernatant (pg/ml)[a]									IgM (μg/ml)[a]	
	IL-1α	IL-1β	IL-6	IL-7	IL-8	G-CSF	GM-CSF	TNFα	TNFβ	Experiment 2	Experiment 3
Cytokine production from RA-NLCs derived from synovium and immunoglobulin from B cells[b] [6]											
RA-SNCs	<5.0	<10.0	2,200		4,300	460	40	<5.0	<5.0	<1.5	<1.5
B cells	<5.0	<10.0	<10.0		<10.0	<10.0	<2.5	<5.0	<5.0	1.8	2.7
B cells + RA-SNCs (separated)[c]	<5.0	<10.0	1,800		3,900	510	30	<5.0	<5.0	<1.5	<1.5
B cells + RA-SNCs	<5.0	153	15,900		34,500	2,400	740	690	<5.0	5.6	8.6
Cytokine production from RA-NLCs derived from bone marrow cells[d] [5]											
RA-BMNC-1 cell line	–	–	38,250	–	1,480	–	150	–			
+ MC/car cell line	–	320	89,015	–	33,510	755	915	275			
+ Molt-17 cell line	–	235	78,750	–	10,615	540	355	255			

RA-BMNCs, cytokine production from RA-NLCs derived from bone marrow cells; RA-SNCs, cytokine production from RA-NLCs derived from synovium; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; –, not detectable. [a]The amount of each cytokine and IgM in the culture supernatant was measured with an enzyme-linked immunosorbent assay kit. [b]B-cell clones (1×10^5) and RA-SNC3 (5×10^4) were cultured under the indicated conditions for 3 days in 24-well plates. [c]B-cell clones were cultured in a Millicell culture insert. [d]RA-BMNC cells (3×10^4 cells/well) were inoculated and cultured overnight, and 1×10^6 cells MC/car cells or Molt-17 cells were added to the culture. After 5 days of incubation, the culture supernatants were collected and the amount of each cytokine in the culture supernatant was measured with an enzyme-linked immunosorbent assay kit.

Expression of MMP-2, MMP-9, and MMP-12 was increased in both TRAP(+) mononuclear and multinucleated cells after differentiation by culture with RA-NLCs, and these cells could induce cartilage degeneration *in vitro* by a mechanism that was completely blocked by inhibitors of MMP-2 and MMP-9. Although MMP-2 expression was significantly increased in TRAP(+) mononuclear cells, expression of MMP-9 and MMP12 was also higher in TRAP(+) multinucleated cells [27]. Of note, both TRAP(+) mononuclear and multinucleated cells differentiated by culture with RA-NLCs specifically expressed MMP-12 [27], whereas multinucleated cells expressing MMP-12 were clearly found near the bone erosions (S Yamane, M Maeda-Tanimura, Y Shimaoka, M Yukioka, T Toyosaki-Maeda, S Ishida, N Yamane, Y Tsuruta, T Itoh, N Fukui, *et al.*, unpublished observation). RA-NLCs were therefore found to promote the differentiation of CD14(+) monocytes in a characteristic two-step differentiation process into multinucleated osteoclast-like cells with the capacity to degrade bone and cartilage.

Although TNF [28], IL-1 [29], macrophage colony-stimulating factor, and RANKL [30] are very important factors for developing osteoclasts, the RANKL-independent two-step differentiation of CD14(+) monocyte supported by RA-NLCs [10,26] may be an alternative pathway to develop multinucleated osteoclast-like cells specifically in RA. Beside the destruction of bone tissue by osteoclasts or osteoclast-like cells, we could confirm that FSCs from RA patients inoculated *in vivo* showed aggressive behavior, invading cartilage as reported previously [31-33], although we have not yet confirmed that pure RA-NLC lines have such function.

Comparison of the properties of RA nurse-like cells and fibroblast-like synoviocytes

A considerable amount of work has characterized another population of cells found in the rheumatoid synovium, namely fibroblast-like synoviocytes. The cells are thought to play a role in rheumatoid pathogenesis, especially because of their capacity to contribute to tissue damage [31-33]. RA-NLCs, however, have a number of specific attributes that suggest they may play a unique role in RA pathogenesis (Table 2).

Mechanisms of progressive proliferation of fibroblastic stromal cells specifically found in joint

To explain the remarkable proliferation of synovial tissue in the RA patient, various mechanisms have been reported such as the involvement of protooncogenes [34], inflammatory cytokines [35], and perturbations of Fas-mediated apoptosis [36]. As a mechanism specifically found in the synovial space but not in the bone marrow, we found that the interference with Fas-mediated apoptosis could upregulate specifically the growth of synovial FSCs [37,38]. In this regard, soluble Fas ligand was found to inhibit competitively the Fas–Fas ligand-mediated apoptosis [37] of FSCs bearing Fas. The levels of human soluble Fas ligand in synovial fluid from RA patients were found to be significantly higher than those from osteoarthritis patients.

Table 2

Comparison of the properties of rheumatoid arthritis nurse-like cells and fibroblast-like synoviocytes

Property	Rheumatoid arthritis nurse-like cells	Fibroblast-like synoviocytes
Pseudoemperipolesis	+	–
Constitutive expression of CD106	+	–
Enhanced expression of CD106 and CD157 by IFNγ	+	–
Promote B-cell differentiation	+	–
Promote differentiation of osteoclast-like cells from CD14(+) monocytes	+	–
Inhibit lymphocyte apoptosis	+	–

In contrast, soluble Fas ligand was not detected in the peripheral blood, and also not in bone marrow blood in RA patients [38]. This mechanism, therefore, could at least partially upregulate the FSC growth in synovial tissue, but not in bone marrow.

Conclusion

A specific population of FSCs, RA-NLCs reside in both the bone marrow and synovium of RA patients and have the functional capacity to interact with lymphocyte and monocyte populations, inducing cellular differentiation and biologic activities that mimic pathophysiologic features of rheumatoid inflammation. These findings suggest that RA-NLCs may play an essential role in the development of local immune and inflammatory responses in the synovium and the bone marrow. RA-NLCs could therefore be central elements in the pathologic events in RA and might be appropriate targets for therapeutic intervention in RA.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The work reported here has been supported in part by a grant-in-aid from the Health Science Research grant from the Ministry of Health and Welfare of Japan. The authors are grateful for the great collaboration and support of the people listed in each paper related to this review. Among them, we are especially grateful to Dr T Kishimoto, Dr T Hirano, Dr S Nagata, Dr T Suda, Dr M Miyasaka, Dr T Kaisho, and Dr K Ishihara of Osaka University Medical School, and to Dr R Suzuki and Miss T Uchida of the Research Center, Sagamihara National Hospital.

This review is part of a series on
Mesenchymal stromal cells
edited by Steffen Gay.

Other articles in this series can be found at
http://arthritis-research.com/articles/review-series.asp?series=ar_Mesenchymal

References

1. Eisenberg RA, Cohen PL: **The role of immunologic mechanisms in the pathogenesis of rheumatic disease.** In *Primer on the Rheumatic Diseases*. 10th edition. Edited by Schumacher HR, Klippel JH, Koopman WJ. Atlanta: Arthritis Foundation; 1993:27-35.
2. Genant HK: **Radiology of rheumatic diseases.** In *Arthritis and Allied Conditions*. 9th edition. Edited by McCarty DJ. Philadelphia: Lea & Febiger; 1979:70-130.
3. Trentham D, Townes A, Kang A: **Autoimmunity to type II collagen: an experimental model of arthritis.** *J Exp Med* 1977, **146**: 857-868.
4. Nakagawa S, Toritsuka Y, Wakitani S, Denno K, Tomita T, Owaki H, Kimura T, Shino K, Ochi T: **Bone marrow stromal cells contribute to synovial cell proliferation in rats with collagen induced arthritis.** *J Rheumatol* 1996, **23**:2098-2103.
5. Tomita T, Takeuchi E, Toyosaki-Maeda T, Oku H, Kaneko M, Takano H, Sugamoto K, Ohzono K, Suzuki R, Ochi T: **Establishment of nurse-like stromal cells from bone marrow of patients with rheumatoid arthritis: indication of characteristic bone marrow microenvironment in patients with rheumatoid arthritis.** *Rheumatology* 1999, **38**:854-963.
6. Takeuchi E, Tomita T, Toyosaki-Maeda T, Kaneko M, Takano H, Hashimoto H, Sugamoto 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.
7. 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.
8. Wekerle H, Ketelsen UP: **Thymic nurse cells – Ia bearing epithelium involved in T-lymphocyte differentiation?** Nature 1980, **283**:402-404.
9. Wekerle H, Ketelsen UP, Ernst M: **Thymic nurse cells. Lymphoepitherial cell complexes in murine thymuses: morphological and serological characterization.** *J Exp Med* 1980, **161**: 925-944.
10. Toyosaki-Maeda T, Takano H, Tomita T, Tsuruta Y, Maeda-Tanimura M, Shimaoka Y, Takahashi T, Iton T, Suzuki R, Ochi T: **Differentiation of monocytes into multinucleated giant bone-resorbing cells: two-step differentitaion induced by nurse-like cells and cytokines.** *Arthritis Res* 2001, **3**:306-310.
11. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP: **Multipotent mesenchymal stem cells from adult human synovial membrane.** *Arthritis Rheum* 2001, **44**:1928-1942.
12. De Bari C, Dell'Accio F, Vanlauwe J, Eyckmans J, Khan IM, Archer CW, Jones EA, McGonagle D, Mitsiadis TA, Pitzalis C, Luyten FP: **Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis.** *Arthritis Rheum* 2006, **54**:209-1221.
13. Jones EA, English A, Henshaw K, Kinsey SE, Markham AF, Emery P, McGonagle D: **Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor**

cells in inflammatory and degenerative arthritis. *Arthritis Rheum* 2004, **50**:817-827.
14. Jones EA, English A, Kinsey SE, Straszynski L, Emery P, Ponchel F, McGonagle D: **Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow.** *Cytom Part B (Clin Cytom)* 2006, **70B**:391-399.
15. Kaisho T, Oritani K, Ishikawa J, Tanabe M, Muraoka O, Ochi T, Hirano T: **Human bone marrow stromal cell lines from myeloma and rheumatoid arthritis that can support murine pre-B cell growth.** *J Immunol* 1992, **149**:4088-4095.
16. Kaisho T, Ishikawa J, Oritani K, Inazawa J, Tomizawa H, Muraoka O, Ochi T, Hirano T: **BST-1, a surface molecule of bone marrow stromal cell lines that facilitates pre-B-cell growth.** *Proc Natl Acad Sci USA* 1994, **91**:5325-5329.
17. Lee BO, Ishikawa K, Denno K, Kobune Y, Itoh M, Muraoka O, Kaisho T, Sasaki T, Ochi T, Hirano T: **Elevated levels of the soluble form of bone marrow stromal cell antigen 1 in the sera of patients with severe rheumatoid arthritis.** *Arthritis Rheum* 1996, **39**:629-637.
18. Ishihara K, Hirano T: **BST-1/CD157 regulates the humoral immune responses in vivo.** *Chem Immunol* 2000, **75**:235-255.
19. Hayashida K, Shimaoka Y, Ochi T, Lipsky PE: **Rheumatoid arthritis synovial stromal cells inhibit apoptosis and up-regulate Bcl-xL expression by B cells in a CD49/CD29-CD106-dependent mechanism.** *J Immunol* 2000, **164**:1110-1116.
20. Takeuchi E, Tanaka T, Umemoto E, Tomita T, Shi K, Takahi K, Suzuki R, Ochi T, Miyasaka M: **VLA-4-dependent and -independent pathways in cell contact-induced proinflammatory cytokine production by synovial nurse-like cells from rheumatoid arthritis patients.** *Arthritis Res* 2002, **4**:1-8.
21. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ: **Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1.** *Blood* 2000, **96**:2655-2663.
22. Tsukada N, Burger JA, Zvaifler NJ, Kipps TJ: **Distinctive features of 'nurselike' cells that differentiate in the context of chronic lymphocytic leukemia.** *Blood* 2002, **99**:1030-1037.
23. Nishio M, Endo T, Tsukada N, Ohata J, Kitada S, Reed JC, Zvaifler NJ, Kipps TJ: **Nurselike cells express BAFF and APRIL,which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1α.** *Blood* 2005, **106**:1012-1020.
24. Burger JA, Zvaifler NJ, Tsukada N, Firestein GS, Kipps TJ: **Fibroblast-like synoviocytes support B-cell pseudoemperipolesis via a stromal cell-derived factor-1-and CD106 (VCAM-1)-dependent mechanism.** *J Clin Invest* 2001, **107**:305-315.
25. Ohata J, Zvaifler NJ, Nishio M, Boyle DL, Kalled SL, Carson DA, Kipps TJ: **Fibroblast-like synoviocytes of mesenchymal origin express functional B functional B cell-activating factor of the TNF family in response to proinflammatory cytokines.** *J Immunol* 2005, **174**:864-870.
26. Tsuboi H, Udagawa N, Hashimoto J, Yoshikawa H, Takahashi N, Ochi T: **Nurse-like cells from patients with rheumatoid arthritis support the survival of osteoclast precursors via macrophage colony-stimulating factor production.** *Arthritis Rheum* 2005, **52**:3819-3828.
27. Tsuboi H, Matsui Y, Hayashida K, Yamane S, Maeda-Tanimura M, Nampei A, Hashimoto J, Suzuki R, Yoshikawa H, Ochi T: **Tartrate resistant acid phosphatase (TRAP) positive cells in rheumatoid synovium may induce the destruction of articular cartilage.** *Ann Rheum Dis* 2003, **62**:196-203.
28. Boyce BF, Li P, Yao Z, Zhang Q, Badell IR, Schwartz EM, O'Keefe RJ, and Xing L: **TNFα and pathologic bone resorption.** *Keio J Med* 2005, **54**:127-131.
29. Wei S, Kitaura H, Zhou P, Ross P, Teitelbaum SL: **IL-1 madiates TNF-induced osteoclastogenesis.** *J Clin Invest* 2005, **115**:282-290.
30. Saidenberg-Kermanac'h N, Cohen-Solal M, Bessis N, De Vernejoul MC, Boissier MC: **Role for osteoprotegerin in rheumatoid inflammation.** *Joint Bone Spine* 2004, **71**:9-13.
31. Gay S, Gay RE, Koopman WJ: **Molecular and cellular mechanism of joint destruction in rheumatoid arthritis: two cellular mechanisms explain joint destruction?** *Ann Rheum Dis* 1993, **52**:39-47.
32. Firestein GS: **Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors?** *Arthritis Rheum* 1996, **39**:1781-1790.
33. Shigeyama Y, Pap T, Kunzler P, Rethage J, Simmen B, Gay RE, Gay S: **Rheumatoid arthritis (RA) synovial fibroblasts express osteoclast differentiating factor (ODF) mRNA at sites of joint destruction [abstract].** *Arthritis Rheum* 1999, **42**:283.
34. Gay S, Gay RE: **Cellular basis and oncogene expression of rheumatoid joint destruction.** *Rheumatol Int* 1989, **9**:105-113.
35. Farahat MN, Yanni G, Poston R, Panayi GS: **Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis.** *Ann Rheum Dis* 1993, **52**:870-875.
36. Nagata S, Suda T: **Fas and Fas ligand: lpr and gld mutations.** *Immunol Today* 1995, **16**:39-43.
37. Suda T, Hashimoto H, Tanaka M, Ochi T, Nagata S: **Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing.** *J Exp Med* 1997, **186**:2045-2050.
38. Hashimoto H, Tanaka M, Suda T, Tomita T, Hayashida K, Takeuchi E, Kaneko M, Takano H, Nagata S, Ochi T: **Soluble fas ligand in the joints of patients with rheumatoid arthritis and osteoarthritis.** *Arthritis Rheum* 1998, **41**:657-662.

International Immunology, Vol. 20, No. 10, pp. 1331–1342
doi:10.1093/intimm/dxn091

Arthritis and pneumonitis produced by the same T cell clones from mice with spontaneous autoimmune arthritis

Chiaki Wakasa-Morimoto[1], Tomoko Toyosaki-Maeda[1], Takaji Matsutani[2], Ryu Yoshida[1], Shino Nakamura-Kikuoka[1], Miki Maeda-Tanimura[1], Hiroyuki Yoshitomi[3], Keiji Hirota[3], Motomu Hashimoto[3], Hideyuki Masaki[4], Yoshiki Fujii[5], Tsuneaki Sakata[1], Yuji Tsuruta[1], Ryuji Suzuki[6], Noriko Sakaguchi[3] and **Shimon Sakaguchi[3]**

[1]Discovery Research Laboratories, Shionogi & Co., Ltd, 2-5-1 Mishima Settsu-shi, Osaka 566-0022, Japan
[2]Department of Cell Biology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Sendai 980-8575, Japan
[3]Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[4]Department of Biochemistry, Kinki University School of Medicine, 377-2 Ohno-higashi, Osakasayama-shi, Osaka 589-8511, Japan
[5]Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[6]Clinical Research Center for Rheumatology and Allergy National Sagamihara Hospital, 18-1 Sakuradai, Sagamihara-shi, Kanagawa 228-8522, Japan

Keywords: animal model, interstitial lung disease, rheumatoid arthritis, T cell clone

Abstract

SKG mice, a newly established model of rheumatoid arthritis (RA), spontaneously develop autoimmune arthritis accompanying extra-articular manifestations, such as interstitial pneumonitis. To examine possible roles of T cells for mediating this systemic autoimmunity, we generated T cell clones from arthritic joints of SKG mice. Two distinct CD8$^+$ clones were established and both showed *in vitro* autoreactivity by killing syngeneic synovial cells and a variety of MHC-matched cell lines. Transfer of each clone to histocompatible athymic nude mice elicited joint swelling and histologically evident synovitis accompanying the destruction of adjacent cartilage and bone. Notably, the transfer also produced diffuse severe interstitial pneumonitis. Clone-specific TCR gene messages in the inflamed joints and lungs of the recipients gradually diminished, becoming hardly detectable in 6–11 months; yet, arthritis and pneumonitis continued to progress. Thus, the same CD8$^+$ T cell clones from arthritic lesions of SKG mice can elicit both synovitis and pneumonitis, which chronically progress and apparently become less T cell dependent in a later phase. The results provide clues to our understanding of how self-reactive T cells cause both articular and extra-articular lesions in RA as a systemic autoimmune disease.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology that primarily affects the synovial membranes of multiple joints (1, 2). A cardinal feature of joint inflammation in RA is proliferative inflammation of the synovium, i.e. synovitis, which leads to the destruction of adjacent cartilage and bone. In addition, RA frequently accompanies extra-articular manifestations, for example the development of rheumatoid factors, rheumatic nodules, vasculitis and interstitial lung disease (ILD). Recent studies with high-resolution imaging have indeed revealed a high prevalence of ILD in patients with RA (3–6). RA is thus a systemic disease; yet, the immunological basis of this systemic autoimmunity is poorly understood.

T cells appear to play a key role in the development of RA as suggested by the infiltration of T cells, especially CD4$^+$ T cells, into the synovial tissue of RA (7–9) and the association of genetic susceptibility to RA with particular alleles of HLA-DR (10, 11). On the other hand, there is evidence in humans and animal models that stimulated synoviocytes, composed of macrophage-like and fibroblast-like synovial cells, can

Correspondence to: S. Sakaguchi; E-mail: shimon@frontier.kyoto-u.ac.jp

Transmitting editor: K. Yamamoto

Received 31 March 2008, *accepted* 17 July 2008

Advance Access publication 18 August 2008

themselves mediate joint destruction in a T cell-independent manner (12, 13). A key issue in elucidating the pathogenetic mechanism of RA is, therefore, to determine how self-reactive T cells contribute to the initiation and progression of synovitis and possibly extra-articular lesions such as ILD.

The SKG strain of mice spontaneously develops T cell-mediated chronic autoimmune arthritis (14–16). The strain possesses a mutation in the gene encoding a Src homology 2 domain of the ζ-associated protein of 70 kDa (ZAP-70), a key signal transduction molecule in T cells (17, 18). Impaired signal transduction through SKG ZAP-70 results in thymic positive selection and failure in negative selection of highly self-reactive T cells that include potentially arthritogenic T cells (14). The SKG arthritis progresses chronically, starting from small joints of the digits and symmetrically progressing to larger joints, such as the wrists and ankles. Histologically, affected joints show hyperplasia of synoviocytes, inflammatory cell infiltration, pannus formation and destruction of cartilage and bone, eventually leading to joint deformity. As extra-articular lesions, they develop interstitial pneumonitis, dermatitis, necrobiotic nodules akin to rheumatic nodules in RA and systemic vasculitides. Serologically, they spontaneously develop IgM-type rheumatoid factors, auto-antibodies against type II collagen and antibodies cross-reactive with *Mycobacterium tuberculosis* heat shock protein (hsp) 70. IL-1, tumor necrosis factor (TNF)-α, IL-6 or IL-17 deficiency inhibits the development of arthritis in SKG mice (15, 19), similar to the effects of anti-cytokine therapies in RA (20, 21). Thus, autoimmune disease in SKG mice closely resembles RA in clinical and immunopathological characteristics. In addition, considering recent findings that genetic polymorphism of a signaling molecule at a TCR proximal step involving ZAP-70 significantly contributes to the susceptibility to RA and other autoimmune diseases (22, 23), SKG mice can be a suitable model for elucidating how a T cell-intrinsic anomaly contributes to the development of RA as a systemic autoimmune disease.

In this study, we have attempted to determine the role of T cells in SKG autoimmune disease by establishing T cell clones from their arthritic lesions. We have established two distinct $CD8^+$ clones and show that both of them have the potential to induce not only arthritis but also pneumonitis. This indicates that inflammation in both the joints and the lung can be mediated, at least in part, by common autoreactive T cell clones in SKG mice. In addition, by adoptively transferring these T cell clones to normal mice, we show that autoreactive T cells are able to initiate arthritis; yet, the arthritis can progress apparently in a T cell-independent manner in a later phase. These findings contribute to our understanding of how T cells cause chronic arthritis and ILD in RA.

Materials and methods

Mice

SKG and (SKG × BALB/c)F_1 mice (14) were maintained in the animal facility of Kyoto University under a microbially conventional condition. Female C.B-17 SCID mice (Clea Japan, Tokyo, Japan), DBA/1J, BALB/c and BALB/c-nu/nu mice (Charles River Japan, Kanagawa, Japan) were maintained under specific pathogen-free conditions at Kyoto University or Discovery Research Laboratories of Shionogi & Co., Ltd. All experiments were approved by the Animal Care and Use Committee at Kyoto University and Shionogi & Co., Ltd.

Culture medium

The culture medium for SKG T cell lines and clones was AIM-V supplemented with 20% RPMI-1640, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol (ME), 2 mM L-glutamine, ×1 penicillin/streptomysin (Gibco BRL, Gaithersburg, MD, USA), 10% heat-inactivated FCS (Hyclone, Logan, UT, USA), 10% rat T-STIM™ with Con A (Becton Dickinson, Franklin Lakes, NJ, USA), 100 U/ml of recombinant mouse IL-2 (Genzyme, Cambridge, MA) and 5 μg/ml of Con A (Sigma, St Louis, MO, USA).

Establishment of T cell clones from arthritic joints of SKG mice

To establish T cell lines, severely swollen joints of SKG mice were aseptically excised, finely minced and cultured until clusters of mononuclear cells were confirmed in bulk culture. Outgrown T cells were cloned in 96-well microplates by using SKG synovial cells (1×10^3) as feeder cells. Synovial cells were prepared as previously described (16). Briefly, synovial tissues from wrist and ankle joints were digested with 400 Mandl U/ml of Liberase Brendzyme II (Roche) in RPMI-1640 medium for 1 h at 37°C; digested cells were filtrated through a nylon mesh to prepare single-cell suspensions. A typical composition of the synoviocyte preparation was ~10% $CD11b^+$ monocyte/macrophages, ~20% $Gr\text{-}1^+$ granulocytes, ~1% T cells and other cells. Several days later non-adherent cells were removed by washing the plates with culture medium. T cells that had outgrown from the bulk culture of synovial cells were dispensed at 1, 5, 20 or 50 cells per well and apparently single colonies were propagated in the culture medium described above. Clonality of each cell was confirmed by microplate hybridization assay (MHA) (24) and sequence analysis of TCR. Established T cell clones were maintained without feeder cells. Dengue 2F7 and 3F2 T cell clones, established by immunization of BALB/c mice with the NS3 peptide of dengue virus, were kindly provided by Dr H. Masaki (Kinki University). All cultures were performed in a humidified atmosphere of 7.5% CO_2 at 37°C.

Cytokine detection

Cytokine production by T cell clones were analyzed by ELISA. T cell clones were stimulated with 10 ng/ml of phorbol myristate acetate (PMA) (Wako Chemicals USA, Inc., Richmond, VA, USA) and 0.4 μg/ml of ionomycin (Calbiochem, Darmstadt, Germany) in culture medium at 1×10^6 cells/ml for 16 h. The supernatants were assayed for various cytokines using specific ELISA kits (Endogen, Woburn, MA, USA, and Axis-Shield, Oslo, Norway) according to the manufacturer's protocol. Cytokine mRNA levels in the joints and lungs of clone recipient mice were analyzed by quantitative PCR as described previously (25).

MHA for TCR AV and BV family and sequence analysis

MHA, cDNA synthesis and PCR amplifications of TCR of each T cell clone were performed as described previously (24). The

PCR products cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) were analyzed for TCR sequences using CEQ DTCS-Quick Start Kit according to the manufacturer's protocol (Beckman Coulter Inc., Fullerton, CA, USA).

^{51}Cr release cytotoxicity assay

BALB/3T3 fibroblast line (H-2^d), J774 macrophage line (H-2^d), p815 mastocytoma line (H-2^d), EL-4 lymphoma line (H-2^b), L929 fibroblast line (H-2^k) obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and synovial cells of SKG mice (H-2^d) were used as target cells. Synovial cells (1×10^4) were seeded in 96-well flat-bottom plates with 40 U/well of IFN-γ for 2 days and radiolabeled with 2.5 μCi/well of $Na_2^{51}CrO_4$ (Daiichi Radioisotope Laboratories, Ltd. Tokyo, Japan) for 2 h. Other target cells (3×10^5) were radiolabeled with 20 μCi of $Na_2^{51}CrO_4$ for 2 h and seeded in 96-well round-bottom plates at 1×10^4 cells per well. Effector cells (4×10^5) were added in each well in triplicate and incubated for 8 h. Relative cytotoxicity was calculated as follows from the radioactivity released in the culture supernatant; percent specific lysis = 100(experimental – spontaneous)/(maximal – spontaneous) counts per minute. Maximal lysis and spontaneous release were determined from target cells incubated with surfactant ×7 (Flow Laboratories, ICN Biomedicals, Inc., Aurora, OH, USA) or without effector cells, respectively.

Adoptive transfer

Spleen T cells from SKG mice or (SKG × BALB/c)F_1 mice and each SKG T cell clones (1×10^7) were intravenously transferred to C.B-17 SCID mice (8 weeks) or BALB/c-nu/nu mice (6 weeks), respectively. Control dengue 2F7 and 3F2 clone were collected 10–14 days after *in vitro* stimulation with specific peptide-pulsed irradiated (33 Gray) BALB/c spleen cells and transferred as described above. Severity of arthritis was scored weekly as previously described (14).

Clinical assessment of arthritis

Joint swelling was monitored by inspection and scored as follows: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist or ankle and 1.0, severe swelling of wrist or ankle. Scores for all fingers and toes, wrists and ankles were totalled for each mouse (14).

Histological assessment of interstitial pneumonitis

Interstitial pneumonitis was evaluated microscopically depending on diffusely affected area: –, normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis.

Histology and immunohistochemistry

Tissues were fixed in 10% neutral formalin, paraffin embedded and stained with Haematoxylin & Eosin (H&E). Joints were additionally decalcified for 3 weeks in 10% EDTA in PBS before staining. For immunohistochemistry of joints, deparaffinized sections were incubated with 20% normal rabbit serum (Dako, Hamburg, Germany) in PBS for 15 min to block non-specific binding, primary rat anti-Ly-6G mAb (Gr-1, RB6-8C5; BD PharMingen) with appropriate dilutions overnight at 4°C, biotinylated polyclonal rabbit anti-rat antibody (Dako) and HRP-conjugated streptavidin (Dako). The slides were developed using diaminobenzidene (Elite Kit; Vector, Burlingame, CA, USA) and counterstained with Mayer's hematoxylin.

For immunohistochemistry of lungs, tissues were fixed in 4% phosphate-buffered PFA (pH 7.4) and embedded in Tissue-Tek OCT compound (Ted Pella, Inc., Redding, CA, USA). Cryostat sections were stained with rat mAbs to mouse CD4 (H129.19), CD8a (53-6.7), CD45R/B220 (RA3-6B2), Ly-6G (RB6-8C5) (BD PharMingen) and F4/80 (Cl: A3-1) (CALTAG Laboratories, Burlingame, CA, USA) with appropriate dilutions followed by incubation with biotinylated secondary antibodies and HRP-conjugated streptavidin. The slides were developed as described above.

Southern blot analysis

The persistence of transferred clones in the recipients was assessed by Southern blot analysis. Two micrograms of total RNA of each tissue was treated with DNaseI and reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA). Nested PCRs were performed as described previously (24) to amplify TCR β chain of 35S or dengue 2F7 with the primers specific for V, J and C region. Ten microliters of the PCR products were separated on 2% agarose gel, transferred onto Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The membranes were prehybridized overnight with PerfectHyb (TOYOBO CO., Ltd, Osaka, Japan) at 54°C and hybridized with the third complementarity-determining region (CDR3)-specific probes labeled with ^{32}P-deoxyadenosine triphosphate for 3 h at 54°C. The membranes were washed in ×2 standard saline citrate (SSC) and 0.1% SDS at room temperature and ×0.2 SSC and 0.1% SDS at 37°C. RNA extracts of 35S and dengue 2F7 clones, diluted to 1% of concentration with RNA of L9 cells, were used as positive controls. The detection limits of 35S and dengue 2F7 were compared using the serial dilution of positive controls and both systems detected the RNA extract corresponding to the amount of one cell.

The sequences of PCR primers and probes are as follows; 35S: first PCR (BV8S3-1: 5′-ATA TGG TGC TGG CAA CCT TC-3′ and MCB1: 5′-AGG ATT GTG CCA GAA GGT AG-3′), second PCR (BV8S3-2: 5′-ACC AGA ACA ACG CAA GAA GAC T-3′ and MCB2: 5′-TTG TAG GCC TGA GGG TCC-3′), third PCR (BV8S3-3: 5′-TTC CTC CTG CTG GAA TTG GC-3′ and BJ1.5: 5′-TAG AAC AGA GAT CGA GTC CC-3′) and probe (5′-AGT GGG ACA GGG GGC AAC CA-3′). Dengue 2F7: first PCR (BV8S1-1: 5′-CCC AAA GTC CAA GAA GCA AG-3′ and MCB1), second PCR (BV8S1-2: 5′-GTA CAA GGC CTC CAG ACC AA-3′ and MCB2), third PCR (BV8S1-3: 5′-TGG CTT CCC TTT CTC AGA CA-3′ and BJ2.7: 5′-AAG GAG ACC TTG GGT GGA GT-3′) and probe (5′-TGC CAC CAA CGA CAA CTC CT-3′).

Results

Induction of arthritis and interstitial pneumonitis in SCID mice by the transfer of SKG splenic T cells

In our conventional housing environment, SKG mice started to develop arthritis around 2 months of age and

histologically evident mild interstitial pneumonitis around 6 months of age (14). To determine the role of T cells in SKG mouse autoimmunity, we transferred splenic T cells from 3-month-old arthritic SKG mice (without histologically evident pneumonitis or colitis) to T/B-cell-deficient C.B-17 SCID mice, which are histocompatible with SKG mice on the BALB/c background (14). Within 2 months after transfer, the recipient developed arthritis (14) and mild but histologically evident interstitial pneumonitis (Table 1, Fig. 1); they also developed mild colitis (data not shown). Similar cell transfer from non-arthritic heterozygotes of the SKG mutation failed to induce such lesions in the recipients. Age-matched SCID mice similarly maintained in our facility did not develop these lesions histologically (data not shown). The results thus indicate that SKG T cells are able to adoptively transfer arthritis and also have a potential to induce interstitial pneumonitis and colitis when transferred to SCID mice.

Establishment of T cell clones from arthritic joints

To analyze the mechanism of such T cell-mediated inflammatory tissue damage in multiple organs, we attempted to establish T cell clones from arthritic joints of SKG mice, as described in Materials and methods. Two T cell clones, designated 35S and 73S, were established in separate experiments. The clones were maintained and expanded with culture medium containing IL-2 and Con A (see Materials and methods). $CD8^+$ CTL clones specific for dengue virus NS3 protein were used as control.

Cytofluorometric analyses revealed that the 35S and 73S clones were $CD8^+$. Both expressed α and β chains of the TCR, and the expression level of the TCR on 35S was slightly lower than normal (Fig. 2). In response to *in vitro* PMA and ionomycin stimulation, 35S and 73S produced IFN-γ but no detectable amount of TNF-α, IL-4, IL-5, IL-6, IL-10 or IL-17 by ELISA (Table 2).

Clonality of each T cell line was confirmed by MHA (24) (data not shown) and sequence analysis of the TCR α and β chains with determination of the amino acid sequences of the TCRs (Table 3). Interestingly, these T cell clones shared in common the BV8S3 TCR Vβ subfamily; yet, the CDR3 sequences of the TCR β chains were different (26–29).

Table 1. Induction of arthritis, interstitial pneumonitis and colitis in SCID mice by the transfer of SKG splenic T cells

Spleen cell donor	Recipients	Arthritis	Interstitial pneumonitis	Colitis
SKG	1	++ (4.6)	++	+
	2	++ (4.0)	++	+
	3	++ (4.0)	+	+
	4	++ (3.0)	+	–
(SKG × BALB/c)F_1	1	–	–	–
	2	–	–	–
	3	–	–	–
	4	–	–	–

Cells (1×10^7) of T cells prepared from spleens of indicated mice were intravenously transferred to 8-week-old SCID mice. The severity of arthritis, interstital pneumonitis and colitis in these mice was histologically assessed 2 months later.

Autoreactivity of T cell clones

In ^{51}Cr release cytotoxicity assay to determine cytotoxic activity of the SKG clones against syngeneic synovial cells, 35S and 73S lysed SKG synovial cells prepared by crude collagenase digestion of inflamed synovium (44.0 and 16.3% of specific lysis, respectively, at a high 40:1 ratio), while control dengue 2F7 clone did not (Fig. 3A). 35S lysed not only syngeneic synovial cells but also MHC-matched cell lines, such as BALB/c-derived 3T3 cells, macrophage-like J774 cells and DBA/2 ($H\text{-}2^d$)-derived P815 cells, whereas the clone failed to lyse allogenic EL-4 ($H\text{-}2^b$) lymphoid or L929 ($H\text{-}2^k$) fibroblast cell line (Fig. 3B). Thus, 35S appears to recognize a ubiquitous self-peptide in an MHC-restricted manner. These

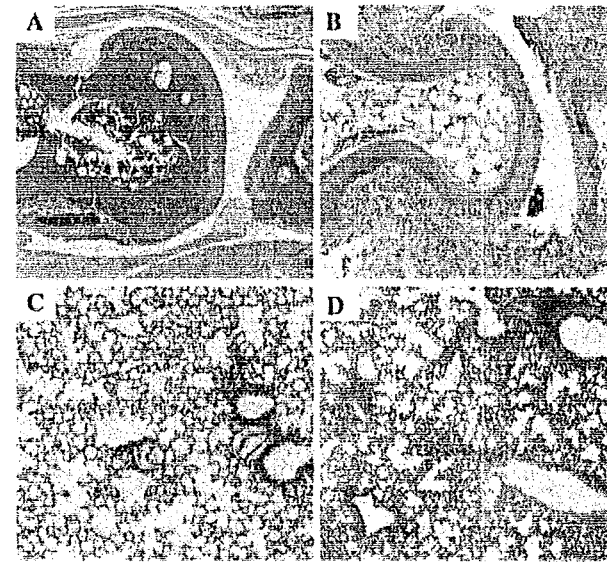


Fig. 1. Arthritis and pneumonitis in SCID mice transferred with T cells from SKG mice. Histology of a joint (A) and lung (C) of a SCID mouse T cell transferred from (SKG × BALB/c)F_1 mouse. Arthritis (B) and interstitial pneumonitis (D) in a SCID mouse T cell transferred from a SKG mouse. H&E staining (A and B, ×100; C and D, ×50).

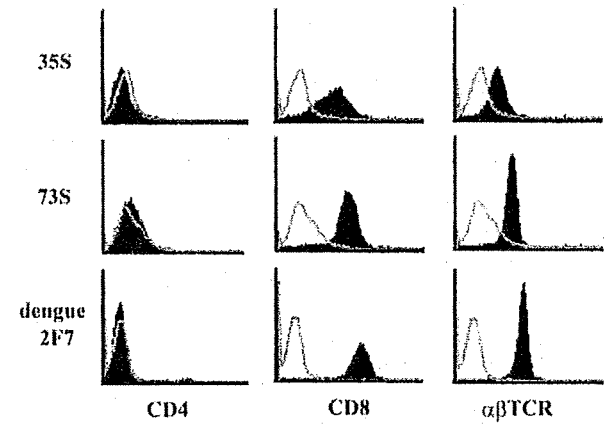


Fig. 2. Expression levels of CD4, CD8 and αβ TCR on 35S, 73S and dengue 2F7 clones.

functional characteristics, together with cell surface and cytokine-secreting profiles, indicate that 35S and 73S are CTL and that they bear self-reactive specificity.

Induction of synovitis in BALB/c nude mice by adoptive transfer of T cell clones

To examine possible arthritogenicity of the T cell clones, they were transferred to BALB/c nude mice once, and the degree of joint swelling of each recipient mouse was assessed once a week for 12 months (Fig. 4). Transfer of 35S and 73S clones induced joint swelling with incidences of 57.1% (4 out of 7 mice) and 42.9% (3 out of 7 mice), respectively, during the observation period; synovitis was histologically evident in 71.4% (5 out of 7 mice) in each transfer (Table 4, Fig. 5). Once joint swelling started in one joint following cell transfer, it slowly progressed with remissions and exacerbations, leading to swelling of other joints in a symmetrical fashion (Figs 4 and 5A–D). Two mice showed progressive debilitation to death without an apparent cause, although one of them showed dermatitis; with debilitation, joint swelling somehow remitted in these mice.

Table 2. Cytokine production (ng/ml) of T cell clones derived from SKG joints and control clones

	TNF-α	IFN-γ	IL-4	IL-5	IL-10	IL-6	IL-17
35S	0.02	180	0.03	<0.02	<0.04	0.2	<0.01
73S	0.02	80	0.03	<0.02	1.2	<0.05	<0.01
Dengue 2F7	0.2	10	ND	ND	<0.04	<0.05	<0.01
Dengue 3F2	0.02	20	ND	ND	<0.04	<0.05	<0.01

Culture supernatant of activated cells by PMA and ionomycin for 16 h were assayed by ELISA. ND, not done.

Table 3. CDR3 sequences of the TCR α and β chain used by the SKG T cell clones

TCR α chains	AV	V	N	J	AJ
35S	3S6	C A V T	S D	S G T Y Q R F	13
73S	3S1	C A A S M	R R	N S G T Y Q R F	13
Dengue 2F7	2S2/7	C A A		N Q G G R A L I F	15
Dengue 3F2	2S2/7	C A A	S G R D	Y A N K M I F	47
TCR β chains	**BV**	**V**	**N-D-N**	**J**	**BJ**
35S	8S3	C A S S G	T G G	N Q A P L F	1.5
73S	8S3	C A S S G	W G D	A E Q F F	2.1
Dengue 2F7	8S1	C A T	N D N	S Y E Q Y E	2.7
Dengue 3F2	8S2	C A S E	T R	E Q Y F	2.6

The amino acid sequences of the V, D and J regions of the TCR were determined according to the nucleotide sequences. AV and BV gene families were assigned according to Arden *et al.* (26). AJ genes were numbered according to Koop *et al.* (27). BJ genes were assigned according to Malissen *et al.* (28) and Gascoigne *et al.* (29).

Histologically, swollen joints showed marked synovial and peri-articular inflammation when examined 6–12 months after cell transfer (Fig. 5E and F). The inflammation accompanied a marked proliferation of synovial lining cells, infiltration of inflammatory cells into subsynovial tissue and joint cavity and active angiogenesis; pannus eroded the adjacent cartilage and bone (Fig. 5F). Gr-1-positive neutrophils were abundant among the infiltrating cells, as observed in the arthritic lesions of SKG mice (14, 15), whereas few T cells infiltrated into the inflammation sites (Fig. 5G and H).

In accordance with the appearance of multinuclear cells at the interface between proliferating synoviocytes and bone, many tartrate-resistant acid phosphatase-positive osteoclasts were observed in the inflamed joints (Fig. 6A–D). Safranin-O staining revealed a decrease in proteoglycan in the articular cartilage matrix of severely affected joints (Fig. 6E and F). Notably, Gr-1-positive cells, mainly neutrophils, also increased in the bone marrow (BM) of the affected recipients (Fig. 6G and H).

A high level of circulating rheumatoid factors was detected in one mouse out of seven recipients of the 35S clone and in none of the recipients of other clones (data not shown).

Some of the swollen joints following transfer of 35S $CD8^+$ clones exhibited higher expression levels of IL-17 mRNA assessed by quantitative reverse transcription (RT)–PCR than those from mice transferred with control $CD8^+$ clones (Supplementary Figure 1A, available at *International Immunology* Online), despite that 35S failed to produce IL-17 upon *in vitro* stimulation.

Taken together, the $CD8^+$ T cell clones prepared from arthritic lesions of SKG mice were able to induce arthritis in athymic nude recipients, leading to the destruction of the surrounding cartilage and the bone.

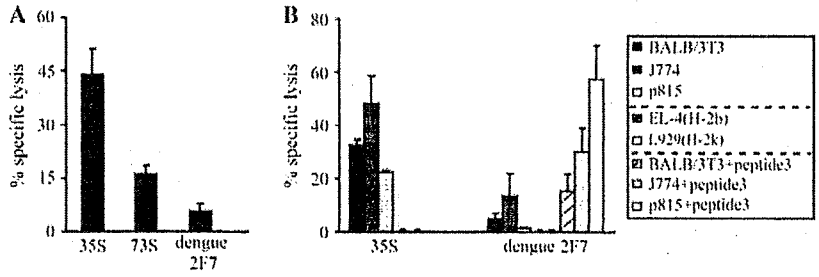


Fig. 3. *In vitro* self-reactivity of SKG T cell clones. (A) CTL activity of SKG T cell clones against SKG synovial cells. CTL clones specific for dengue virus NS3 protein, dengue 2F7, was used as control. IFN-γ-treated target cells were ^{51}Cr labeled in adherent condition and incubated with effector cells for 8 h (E:T ratio = 40). (B) CTL activity of SKG T cell clones against various types of cell lines (E:T ratio = 40). CTL activity of dengue 2F7 clone was also analyzed against $H\text{-}2^d$ cells pulsed with a specific peptide (E:T ratio = 10). All assays were conducted in triplicate with 8 h of incubation. The mean and standard deviation of three independent experiments are shown in each bar.

Induction of interstitial pneumonitis in BALB/c nude mice by the transfer of T cell clones

Notably, histologically evident severe alveolitis and diffuse interstitial pneumonitis also developed in all the recipients of 35S and 73S but not in those recipients of dengue 2F7 and 3F2 clones (Table 4 and Fig. 7A–D). Some recipients of 35S and 73S developed only pneumonitis without histologically evident synovitis. No histologically apparent inflammation was observed in other tissues/organs including the liver and the colon in any of these recipient mice (data not shown). The diffuse pulmonary lesions (Fig. 7A and B) comprised thickening of the alveolar walls, and perivascular and peribronchiolar infiltration by inflammatory cells (Fig. 7C and D). Immunohistochemical analysis of the 73S recipients 6 months after cell transfer revealed the infiltration of a large number of granulocytes as Gr-1$^+$ cells (Fig. 7E), macrophages as F4/80$^+$ cells (Fig. 7F) and B cells as B220$^+$ cells (Fig. 7G) into the alveolar walls and spaces and also the perivascular and peribronchiolar area where only a small number of CD8$^+$ T cells were detected, which might be transferred to CD8$^+$ clones or derived from nude mice (30) (Fig. 7H). CD4$^+$ T cells were occasionally found in the lesions and could be those derived from endogenous T cells that might develop extrathymically in aged nude mice (Fig. 7I) (30).

The pulmonary tissues with severe interstitial pneumonitis following CD8$^+$ clone transfer exhibited higher expression levels of IL-17 mRNA by quantitative RT-PCR compared with the mice transferred with control CD8$^+$ clones (Supplementary Figure 1B, available at *International Immunology* Online).

Thus, the SKG arthritogenic T cell clones are able to induce interstitial pneumonitis when transferred to athymic nude mice.

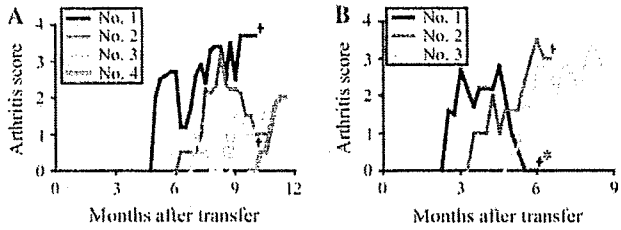


Fig. 4. Time course of joint swelling in the recipient mice of SKG T cell clones, 35S (A) and 73S (B). Score for all paws were totalized for each mouse. +, Sacrificed at the indicated time points; *, the mouse developed dermatitis at 5 months after transfer.

Detection of transferred clones in recipient mice

Since T cells were hardly detected by immunohistochemistry at the site of synovitis or pneumonitis 6 months after clone transfer (data not shown and see above), the persistence of transferred clones in the recipients was assessed by RT-PCR amplification of TCR β chain gene and Southern blot analysis of the products with a CDR3 sequence-specific probe. We adopted this method to avoid detecting nude mouse-derived oligoclonal endogenous T cells that may expand with aging (see above) (30–32). For example, a clone-specific TCR message of the 35S clone was detected in the majority of recipient spleens 1 month after transfer but not in the spleens examined 6 months later (Fig. 8). As shown in Fig. 9, the messages were

Table 4. Development of arthritis and interstitial pneumonitis in BALB/c nude mice transferred with T cell clones

Clone	Individual recipients	Macroscopically evident arthritis			Histological analysis	
		Onset (months)	Sacrifice (months)	Clinical score[a]	Synovitis[b]	Interstitial pneumonitis[c]
35S	1	5	10	3.7	++	+
	2	6	11	3.2	++	++
	3	7	11	1.6	++	+++
	4	10	12	2.0	++	+++
	5	–	9	0	+	±
	6	–	12	0	—	+++
	7	–	12	0	—	+++
73S	1	2.5	6	2.8	++	++
	2	3.5	6	3.5	++	+
	3	5	9	3.3	++	++
	4	–	8	0	+	++
	5	–	9	0	+	+++
	6	–	12	0	—	++
	7	–	12	0	—	++
Dengue 2F7	1	–	9	0	—	—
	2	–	9	0	—	—
	3	–	9	0	—	—
	4	–	9	0	—	—
	5	–	12	0	—	—
	6	–	12	0	—	—
	7	–	12	0	—	—
Dengue 3F2	1	–	12	0	—	—
	2	–	12	0	—	—

Six-week-old BALB/c nude mice were intravenously injected with 1×10^7 cells of each clone. The incidence of joint swelling of the recipient mice was examined weekly. Mice were sacrificed 6–12 months after cell transfer.

[a]Maximum clinical score of arthritis.

[b]–, Without change; +, microscopically observed synovitis without joint swelling; ++, macroscopically obvious joint swelling.

[c]–, Normal histology; +, 10–30%; ++, 30–60%; +++, >60% of the sections of the lungs showed pneumonitis (Fig. 7).

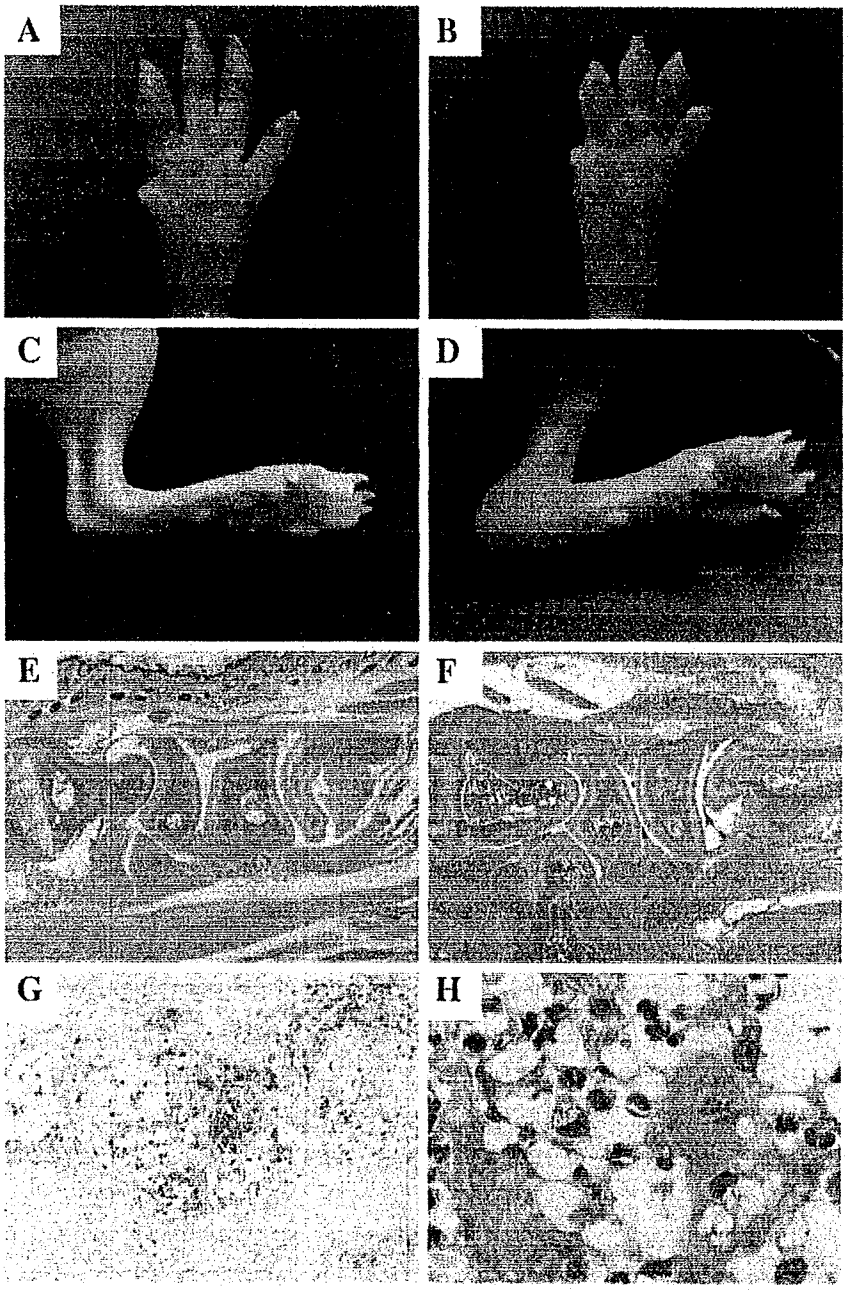


Fig. 5. Arthritis in athymic nude mice transferred with SKG T cell clones. (A–D) Macroscopic views of a forepaw (A) and a hind paw (C) of a recipient of control dengue 2F7 and a forepaw (B) and a hindpaw (D) of a recipient of 35S. (E–H) Histology of the joints of recipients of control dengue 2F7 (E) or 35S (F). Proliferation of the synovial lining cells, erosive destruction of cartilage and bone and infiltration of inflammatory cells is noted in a joint of a 35S recipient (F) (H&E staining, ×40). (G) Gr-1-positive cells were abundant among the infiltrating cells in a joint of 35S recipient mouse (×200). High-magnification view (×1000) of the synovial lesion in 35S transferred mouse, showing that most of the infiltrating cells are granulocytes or monocytes (H) (H&E staining). (A, C and E) 12 months after transfer. (B, D and F–H) 10 months after transfer.

detected in every tested tissue with high frequency for the first 3 months after cell transfer; the detection rate became lower with time; clone-specific TCR signals were not detected in most tissues examined at 6–11 months after transfer, irrespective of the swelling of the joints and the presence of interstitial pneumonitis by histological examination. These findings collectively indicate that the T cell clones initiate arthritis but the progression and persistence of the disease may not require the expansion of the clones even if a small number of them might persist in the joints and the lung.