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

Chiaki Wakasa-Morimoto¹, Tomoko Toyosaki-Maeda¹, Takaji Matsutani², Ryu Yoshida¹, Shino Nakamura-Kikuoka¹, Miki Maeda-Tanimura¹, Hiroyuki Yoshitomi³, Keiji Hirota³, Motomu Hashimoto³, Hideyuki Masaki⁴, Yoshiki Fujii⁵, Tsuneaki Sakata¹, Yuji Tsuruta¹, Ryuji Suzuki⁶, Noriko Sakaguchi³ and Shimon Sakaguchi³

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

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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...
T cell clones mediating arthritis and pneumonitis

T cell clones mediating 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 arthrogenic 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 synovocytes, 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 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 adaptively 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)F1; 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, 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 × 10³) as feeder cells. Synovial cells were prepared as previously described (16). Briefly, synovial tissues from wrist and ankle joints were digested with 400 ManiHi U/ml of Liberase Blendzyme II (Roche) in RPMI-1640 medium for 1 h at 37°C; digested cells were filtered through a nylon mesh to prepare single-cell suspensions. A typical composition of the synovocyte preparation was ~10% CD11b+ monocyte/macrophages, ~20% Gr-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% CO₂ 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 × 10⁶ 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).

51Cr release cytotoxicity assay
BALB/3T3 fibroblast line (H-2b), J774 macrophage line (H-2d), p815 mastocytoma line (H-2d), EL-4 lymphoma line (H-2b), L929 fibroblast line (H-2b) obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and synovial cells of SKG mice (H-2b) were used as target cells. Synovial cells (1 × 106) 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 Na51CrO4 (Daichi Radioisotope Laboratories, Ltd, Tokyo, Japan) for 2 h. Other target cells (3 × 105) were radiolabeled with 20 μCi of Na51CrO4 for 2 h and seeded in 96-well round-bottom plates at 1 × 104 cells per well. Effector cells (4 × 105) 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)F1 mice and each SKG T cell clones (1 × 106) 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
Histological evaluation of interstitial pneumonitis
Histological assessment of 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 diaminobenzidine (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 (Ci: 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 3SS 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 35P-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 3SS and dengue 2F7 clones, diluted to 1% of concentration with RNA of L9 cells, were used as positive controls. The detection limits of 3SS 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; 3SS: first PCR (BV8S3-1: 5′-ATA TGG TGC TGG CAA CCT TC-3′ and MCB1: 5′-AGG ATT GTG CCA GAA GTG AG-3′), second PCR (BV8S3-2: 5′-ACC ACA AGA ACA AGC GAA GAC T-3′ and MCB2: 5′-TTG TAG GCC TGA GGG TCC-3′), third PCR (BV8S3-3: 5′-TTC CTC CTG GAA TGG GC-3′ and BJ1.5: 5′-TAG AAC AGA GAT CGA GCC TCC-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′-TTG 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

<table>
<thead>
<tr>
<th>Spleen cell donor</th>
<th>Recipients</th>
<th>Arthritis</th>
<th>Interstitial pneumonitis</th>
<th>Colitis</th>
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<tr>
<td>SKG</td>
<td>1 ++ (4.6)</td>
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<td>2 ++ (4.0)</td>
<td>++</td>
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<td></td>
<td>3 ++ (4.0)</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>(SKG × BALB/c)F1</td>
<td>1 ++ (3.0)</td>
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Cells (1 × 10⁷) of T cells prepared from spleens of indicated mice were intravenously transferred to 8-week-old SCID mice. The severity of arthritis, interstitial pneumonitis and colitis in these mice was histologically assessed 2 months later.

Autoreactivity of T cell clones

In ⁵¹Cr 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-2d)-derived P815 cells, whereas the clone failed to lyse allogenic EL-4 (H-2k) lymphoid or L929 (H-2k) fibroblast cell line (Fig. 3B). Thus, 35S appears to recognize a ubiquitous self-peptide in an MHC-restricted manner. These

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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)F1 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 36S 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.

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+ T cell 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 stimulation. In one mouse out of seven recipients of the 35S clone and in none of the recipients of other clones (data not shown).

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 51Cr 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-2d 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 peribronchial 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 peribronchial 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.

### 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$\beta$ 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

<table>
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<tr>
<th>Clone</th>
<th>Individual recipients</th>
<th>Macroscopically evident arthritis</th>
<th>Histological analysis</th>
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<td></td>
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<td>Onset (months)</td>
<td>Sacrifice (months)</td>
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<tr>
<td>35S</td>
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Six-week-old BALB/c nude mice were intravenously injected with $1 \times 10^7$ 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).
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.
Discussion

In this study, we have established two distinct CD8+ T cell clones from arthritic lesions of SKG mice. Interestingly, both exhibited \textit{in vitro} autoreactivity against not only synoviocytes but also a variety of MHC-matched cell lines and elicited both arthritis and interstitial pneumonitis when transferred to...
Fig. 7. Interstitial pneumonitis induced by the transfer of SKG T cell clones. (A–D) H&E-stained sections of the lungs of the recipients of control dengue 2F7 clone (A) or 73S clone (B–D) (A–B, ×100). Lower (C, ×10) and higher (D, ×400) magnification of the lung of 73S clone recipient show thickening of alveolar walls diffusely in the lung. (E–J) Serial sections of a lung of a 73S recipient mouse were stained for Ly-6G (Gr-1) (E), F4/80 (F), B220/CD45R (G), CD8a (H) or CD4 (I), with staining control (J) (×400). Typically positive cells in these stainings are arrowed. (A–J) 6 months after transfer.
histocompatible T cell-deficient mice. Furthermore, the arthritic and pulmonary lesions chronically progressed irrespective of the decline in the number of transferred T cell clones to hardly detectable levels in either lesion.

Our previous study showed that bulk CD4+ T cells alone from arthritic SKG mice were able to transfer the disease to athymic nude mice, whereas bulk CD8+ T cells alone were not and that abundant CD4+ T cells and only a small number of CD8+ T cells were found by immunohistochemistry in the arthritic subsynovial tissue of arthritic SKG mice (14). These apparently opposing results with CD8+ T cell clones versus bulk CD8+ T cells indicate that potentially arthritogenic CD8+ T cells are present in SKG mice and may usually need CD4+ T cell help for induction of arthritis; yet, they are potentially able to mediate arthritis without CD4+ T cell help if they are strongly activated, clonally expanded to a large number or possibly selected for stronger self-reactivity during *in vitro* culture. It remains to be determined how CD8+ T cells elicit proliferative synovitis rather than cytotoxic killing of certain cellular elements in the joint. One possibility is that these CD8+ clones, which exert *in vitro* killing activity at a high T cell/target cell ratio, might also be able to stimulate synoviocytes through secreting cytokines. It is of interest in this regard that the joints and the lungs with severe pneumonitis in some recipients of the CD8+ clones showed active transcription of IL-17 mRNA (Supplementary Figure 1, available at *International Immunology* Online). Although the CD8+ clones did not produce detectable amounts of IL-17 by *in vitro* stimulation, they might produce the cytokine in the joints or interact with nude mouse-derived αβ or γδ T cells and stimulate them to secrete IL-17 (33, 34). It is of note that a large number of Gr-1+ mature neutrophils exuded into the joint fluid and infiltrated into the subsynovial tissue of the recipient nude mice, as in the arthritic lesions of SKG mice (14). BM of the clone recipients also showed an increase in the number of Gr-1+ mature neutrophils. It remains to be determined how CD8+ T cells mediate arthritis and pneumonitis in SKG mice by recruiting other cellular elements including neutrophils, how they increase neutrophils in the BM and whether IL-17, which is capable of recruiting neutrophils, is involved in these processes (35, 36).

It also needs further investigation whether IFN-γ secreted by the transferred CD8+ clones or their killing activity could contribute to the development of synovitis. IFN-γ may activate synoviocytes directly or indirectly through activating macrophages, facilitating synoviocyte proliferation. It might up-regulate the expression of MHC class I in synovial cells, rendering them susceptible to cytotoxic activity of CD8+ T cells. With these apparently opposing activities of arthritogenic CD8+ T cells (i.e. killing versus proliferation of synoviocytes), they mediate proliferative synovitis rather than synoviocyte destruction presumably because synoviocytes might be more sensitive *in vivo* to the stimulatory effect than the cytotoxicity (see Discussion below).

The CD8+ clones exhibited *in vitro* cytotoxic activity against not only syngeneic synovial cells but also a variety of MHC-matched lymphoid and non-lymphoid cell lines. Although their precise antigen specificities need to be determined, this finding suggests that these clones may recognize a ubiquitous self-antigen (for example, ubiquitous cellular protein such as hsp complexed with MHC or the MHC molecule itself) expressed in the joint and lung and other tissues, rather than a common self-antigen exclusively expressed in the joint and lung. If this is the case, how are the joint and the lung selectively affected by these T cell clones? For the following reasons, one could attribute this to unique characteristics of the synoviocytes, and possibly the alveolar macrophage, as the target of this autoimmunity. Compared with other tissue cells, the synoviocytes are

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**Fig. 8.** Detection of a clone-specific TCR message of 35S clone in spleens by RT-PCR amplification and Southern blot analysis. After transfer of 1 × 10⁷ clone cells to BALB/c nude mice, RNA was extracted from spleens at indicated days. PC, positive control (RNA from 35S clone diluted to 1%); NC, negative control (RNA from a 6-month-old non-treated BALB/c athymic nude mouse). The separate lanes represent individual mice.

**Fig. 9.** Detection of TCR mRNA of the transferred clones in recipient BALB/c nude mice. 35S (A) or dengue 2F7 (B) in the recipients were detected by Southern blot analysis using primers and probes specific for TCR V and J region and CDR3 sequences of each clone. All mice with at least one positive signal out of four joints were considered to be positive. (A) n = 3 at 1 week; n = 6 at 1 month; n = 6 at 3 months; n = 2 at 6 months; n = 2 at 10–11 months. (B) n = 2 in every group. No signal was detected in control 6- or 11-month old BALB/c nude mice in each Southern blot analysis (data not shown).
highly sensitive to pro-inflammatory cytokines, for example systemic overproduction of transgenic TNF-α or IL-1 almost exclusively produces chronic arthritis even in mice deficient of both T and B cells (37–39); similarly, systemic deficiency of the IL-1R antagonist, and resulting overproduction of IL-1, or systemic alteration of signal transduction via IL-6 receptor results in predominant development of arthritis with no inflammatory damage to other tissues (40, 41). These findings collectively indicate that synoviocytes are much more sensitive to the SKG self-reactive T cell clones (at least to those secreting pro-inflammatory cytokines) than other tissue cells, even if the common self-antigens recognized by the clones are ubiquitously expressed. In addition, synoviocytes are unique in that they are the target cells and also the mediators of autoimmunity, i.e. upon stimulation (e.g. by cytokines or via cell contact stimulation by self-reactive T cells), they proliferate and secrete pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) and other inflammatory substances (matrix metalloproteinases and prostaglandins), mediating inflammation and tissue damage (42). It is likely that the cells composing the alveolar walls, in particular the alveolar macrophages, are sensitive and responsive to T cell self-reactivity in a similar manner as synoviocytes and that excessively and chronically activated macrophages might mediate alveolitis and interstitial inflammation. A similar mechanism might also be responsible for the development of colitis in SKG mice (Table 1).

We do not assert, however, that SKG arthritis and pneumonitis are solely mediated by T cells recognizing a ubiquitous common self-antigen. We have previously shown that SKG mice spontaneously produce IgG isotype auto-antibody specific for joint-rich type II collagen or IgG antibody cross-reactive with hsp-70 of Tuberculosis bacilli (14). This indicates that helper CD4⁺ T cells that specifically react with these self-antigens may also be induced in SKG mice either primarily or secondarily to joint damage. Moreover, we have recently shown that some self-reactive T cells in SKG mice may not be arthritogenic but can polyclonally stimulate antigen-presenting cells in the spleen and lymph nodes to secrete IL-6 and other cytokines, which in turn facilitate differentiation of potentially arthritogenic self-reactive T cells to Th17 effector T cells that mediate synovitis (19). In addition to our current approach to the characterization of antigen specificity of SKG autoimmune T cells by preparing T cell clones, efforts are being made to further characterize infiltrating T cells in situ at a single-cell level by amplifying their TCR message.

Tracing the fate of transferred T cell clones revealed that clone-specific TCR gene messages gradually diminished not only in the inflamed joints and the lungs but also in the regional lymph nodes and spleens of the recipients, becoming hardly detectable in 6–11 months; yet, inflammation in the joints and the lung continued to progress and severe arthritis and pneumonitis were apparent even 12 months after clone transfer. Thus, initial triggering of synovitis requires arthritogenic T cells; yet, synovitis apparently becomes less T cell dependent in a later phase, albeit it chronically progresses with the formation of pannus destroying adjacent cartilage and bone, as in human RA (2). This may correlate with the findings in humans that T cell-targeted mAb therapy is not much efficacious in the treatment of RA at a chronic stage (43). Further characterization of each stage of disease development in SKG mice will contribute to our understanding of the cellular and molecular basis of the T cell-dependent and -independent phases of disease progression in the joints and also in the lung in RA.

In conclusion, we have shown that CD8⁺ T cell clones established from arthritogenic lesions of SKG mice are capable of mediating not only arthritis but also interstitial pneumonitis immunopathologically resembling ILD in RA. This provides a possible common pathogenetic basis between arthritis and ILD in RA. The etiology of RA is largely obscure at present (1, 2). Yet, there are recent findings that genetic polymorphism of the PTPN22-encoded lymphoid tyrosine phosphatase, which alters signal transduction at a TCR proximal step involving ZAP-70, contributes significantly (second only to MHC polymorphism) to the susceptibility to RA and other autoimmune diseases (22, 23, 44, 45). The polymorphism might be responsible for thymic generation of arthritogenic and other self-reactive T cells. Further elucidation of the mechanism by which such autoreactive T cells are generated and activated in SKG mice, and characterization of putative ubiquitous self-antigen recognized by self-reactive T cells capable of mediating arthritis and pneumonitis, would facilitate our understanding of the etiology and the pathogenetic mechanism of RA as a systemic autoimmune disease. This should help devising preventive or curative measures for the disease.

Supplementary data
Supplementary figure is available at International Immunology Online.

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Disclosures
The authors declare no conflicting interests.

Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>CDR3</td>
<td>the third complementarity-determining region</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin &amp; eosin</td>
</tr>
<tr>
<td>hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>ILD</td>
<td>interstitial lung disease</td>
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<tr>
<td>MHA</td>
<td>microplate hybridization assay</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>ζ-associated protein of 70 kDa</td>
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