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Gamma/Delta T Cells Are the Predominant Source of Interleukin-17 in Affected Joints in Collagen-Induced Arthritis, but Not in Rheumatoid Arthritis

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Objective. **Although interleukin-17 (IL-17)–producing γ/δ T cells were reported to play pathogenic roles in collagen-induced arthritis (CIA), their characteristics remain unknown. The aim of this study was to clarify whether γ/δ T cells or CD4+ T cells are the predominant IL-17–producing cells, and to determine what stimulates γ/δ T cells to secret IL-17 in mice with CIA. The involvement of IL-17–producing γ/δ T cells in SKG mice with autoimmune arthritis and patients with rheumatoid arthritis (RA) was also investigated.**

Methods. **IL-17–producing cells in the affected joints of mice with CIA were counted by intracellular cytokine staining during 6 distinct disease phases, and these cells were stimulated with various combinations of cytokines or specific antigens to determine the signaling requirements. Similar studies were performed using SKG mice with arthritis and patients with RA.**

Results. **Gamma/delta T cells were the predominant population in IL-17–producing cells in the swollen joints of mice with CIA, and the absolute numbers of these cells increased in parallel with disease activity. IL-17–producing γ/δ T cells expressed CC chemokine receptor 6, were maintained by IL-23 but not by type II collagen in vitro, and were induced antigen independently in vivo. Furthermore, IL-17 production by γ/δ T cells was induced by IL-1β plus IL-23 independently of T cell receptor. In contrast to what was observed in mice with CIA, IL-17–producing γ/δ T cells were nearly absent in the affected joints of SKG mice and patients with RA, and Th1 cells were predominant in the joints of patients with RA.**

Conclusion. **Gamma/delta T cells were antigen independently stimulated by inflammation at affected joints and produced enhanced amounts of IL-17 to exacerbate arthritis in mice with CIA but not in SKG mice with arthritis or patients with RA.**

Rheumatoid arthritis (RA) is a chronic autoimmune disease that results in the destruction of cartilage and bone in joints. Collagen-induced arthritis (CIA) is a well-established murine model of this disease and shares many features with RA (1,2). Specifically, susceptibility to both CIA and RA is associated with the specific class II major histocompatibility complex allele (3,4). In addition, autoantibodies to type II collagen have been detected in the synovial fluid of patients with RA, and these autoantibodies have an aggravating effect on CIA in mice (5–7). In addition, pathogenic contributions of CD4+ T helper cells have been reported in both CIA and RA (8,9).

Interleukin-17 (IL-17) is a cytokine secreted by T cells, natural killer (NK) cells, and neutrophils (10),

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and it induces IL-6, IL-8, chemokine, and metalloproteinase production by target cells (11). Central pathogenic roles of IL-17 in CIA have been reported recently. For example, systemic or local IL-17 gene transfer aggravated CIA, whereas administration of an IL-17–blocking antibody ameliorated CIA even after the onset of arthritis (12,13), and IL-17–deficient mice also showed reduced severity of CIA (14). Furthermore, IL-23–deficient mice, which show an impaired Th17 response, do not exhibit CIA, because IL-23 is an essential factor for the maintenance of Th17 cells (15).

Although Roark et al recently reported the infiltration of IL-17–producing γ/δ T cells together with IL-17–producing CD4+ T (Th17) cells in inflamed joints of mice with CIA (16), the precise predominance, distribution, kinetics, cytokine-production requirements, and characteristics of these cells, especially in the context of IL-17–producing γ/δ T versus Th17 cells, remain unclear. Elucidation of these factors will be critical in terms of understanding the pathogenesis of CIA, finding novel therapeutic targets associated with IL-17, and determining the optimal timing and site for therapeutic intervention in CIA.

In the current study, we performed spatiotemporal analysis of IL-17–producing cells in CIA and demonstrated that γ/δ T cells are the predominant source of IL-17 in swollen joints of mice with CIA. IL-17–producing γ/δ T cells were maintained by IL-23 but not by type II collagen in vitro. Furthermore, IL-17 production by γ/δ T cells was efficiently stimulated by inflammatory cytokines independently of T cell receptor (TCR). Contrary to the results observed in mice with CIA, IL-17–producing γ/δ T cells could not be detected in the affected joints of patients with RA.

MATERIALS AND METHODS

Animals. DBA1/J mice and BALB/c mice were purchased from Charles River (Wilmington, MA). Eight-week-old male mice were used for induction of CIA, and 2-week-old mice were used to analyze thymocytes. The procedures for the induction of arthritis in SKG mice were described previously (17). Mice were maintained in our animal facility under specific pathogen–free conditions, and all animal procedures were approved by the Ethics Committee of Kyoto University.

Induction of CIA. Immunization-grade bovine type II collagen was purchased from Chondrex (Redmond, WA) and reconstituted at 2 mg/ml in 0.05*M* acetic acid and then emulsified with an equal volume of Freund's complete adjuvant (CFA) containing 4 mg/ml of heat-killed *Mycobacterium tuberculosis* (Arthrogen-CIA; Chondrex). In order to examine the immune process at the immunized site, CIA was initiated by subcutaneous injection with 100 μl of emulsified type II collagen into the left footpad rather than the tail base. This altered method of immunization did not result in skewed disease kinetics, severity, or cytokine profiles of cells in swollen joints (data not shown). A booster immunization was not given. Each joint was designated as follows: immunized joint = left hind paw that received immunization; swollen joint = a fore paw in which arthritis developed; nonswollen joint = right hind paw that was not immunized and in which arthritis did not develop macroscopically (Figure 1A). SKG and BALB/c mice were also immunized with CFA plus type II collagen into the left hind paw to analyze locally infiltrated cells 10 days later. In some experiments, control mice were treated with type II collagen emulsified in Freund's incomplete adjuvant (IFA; Difco, Detroit, MI) or 0.05*M* acetic acid emulsified in IFA or phosphate buffered saline (PBS) alone.

Preparation of mononuclear cells from joints. To prepare cells from the joints, the previously described technique (3) was used. Although a previous report confirmed that contamination of bone marrow cells had not occurred using this procedure (18), we compared the absolute counts of γ/δ T cells and CD19+ cells collected by this procedure and collected from the remaining tissues of the normal joints of naive DBA1/J mice. Cells in the remaining tissues were collected by mincing the remaining tissues, including bone marrow. The cells were stained with biotinylated anti-CD19 monoclonal antibody (mAb) (1D3; BD Biosciences, San Jose, CA) or anti-γ/δ TCR mAb (UC7-13D5), detected with streptavidin–allophycocyanin, and analyzed using fluorescence-activated cell sorting. Human synovial tissue or synovial fluid was obtained from patients with RA who were undergoing joint replacement surgery or subcutaneous puncture of the knee joints. Synovial tissue was dissected into small pieces with scissors, and lymphocytes were collected by density-gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Amersham, UK). All human procedures were approved by the Ethics Committee of Kyoto University and were performed after receiving informed consent.

Intracellular cytokine staining. Cell stimulation with phorbol myristate acetate (PMA) and ionomycin and intracellular cytokine staining were performed as described previously (16). When IL-17 production requirements were analyzed, 2×10^4 cells/well in a 96-well plate were stimulated with 10 μg/ml of plate-bound anti-γ/δ TCR (UC7-13D5), 2 μg/ml of soluble anti-CD28 (37. 51; BD Biosciences), 5 ng/ml of recombinant mouse IL-23 (1887-ML-010; R&D Systems, Minneapolis, MN), 50 ng/ml of recombinant mouse IL-1β (094-04681; Wako, Osaka, Japan), or 50 ng/ml of recombinant human transforming growth factor β1 (TGFβ1) (240-B; R&D Systems) for 24 hours, in the presence of 15 μ*M* monensin for the last 4 hours. Other stimulants were not included in the analysis of IL-17 production requirements.

To analyze surface antigens, the following antibodies were used: fluorescein isothiocyanate (FITC)–labeled anti-CD8 (53-6.7), FITC-conjugated anti-CD3e (145-2C11), FITC-conjugated anti-mouse CC chemokine receptor 6 (CCR6) (140706; R&D Systems), peridinin chlorophyll protein complex–labeled anti-CD4 (L3T4; BD Biosciences), biotinylated anti-γ/δ TCR (UC7-13D5), and biotinylated anti-CD49b (DX5) mAb detected using streptavidin–allophycocyanin or streptavidin–Cy-Chrome (BD Biosciences). Cytokines were detected using FITC- or allophycocyanin-labeled anti–

interferon-γ (IFNγ) (XMG1.2), phycoerythrin (PE)–labeled anti–IL-17 mAb (TC11-18H10; BD Biosciences), or an isotype control. When human synoviocytes were analyzed, the cells were stained using FITC-conjugated anti-human IL-17A (eBio64DEC17), allophycocyanin-conjugated anti-human IFNγ (4S. B3), Cy-Chrome–conjugated anti-human CD4 (PM-30158X; BD Biosciences), and PE-conjugated anti-human γ/δ TCR mAb (B1.1). Unless specified otherwise, all antibodies were purchased from eBioscience (San Diego, CA).

Flow cytometry analysis. The absolute numbers of cytokine-producing cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Lymphocytes were gated based on their forward and side scatter. The cytokine-positive subsets were determined by a comparison with isotype control staining. By applying cells from a whole joint, the absolute numbers of cytokine-positive cells in each joint were counted, and the data were analyzed using CellQuest software (BD Biosciences).

Sorting of γ/δ T cells. To analyze the IL-17 production requirements, cells were collected from peripheral lymph nodes of naive DBA1/J mice or from the draining lymph nodes (DLNs) of the swollen joints of mice with CIA. Cells were stained with FITC-conjugated anti-mouse γ/δ TCR mAb (UC7-13D5) and anti-FITC microbeads, and then γ/δ T cells were prepared by positive selection using an MS column (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell culture in the presence of IL-23 or type II collagen. Cells were prepared from the DLNs of swollen joints of mice with CIA. Then, 5×10^5 cells/well were cultured in 200 μl of RPMI 1640 complete medium in the presence or absence of 1 ng/ml of IL-23. For type II collagen, cells were cultured in the presence or absence of 15 μg/ml of type II collagen. After 7 days, the cells were stimulated with PMA and ionomycin for 4 hours. IL-17–producing cells were detected by intracellular cytokine staining. The ratios of the numbers of IL-17–producing cells in the presence of IL-23 or type II collagen to those in medium alone were calculated.

Analysis of the γ/δ TCR repertoire of IL-17–producing γ/δ T cells (CCR6+ γ/δ T cells). Cells from the DLNs of swollen joints were stained with FITC-conjugated anti-mouse CCR6 mAb (140706; R&D Systems) and anti-FITC microbeads, and then CCR6+ cells were prepared by positive selection using an MS column (Miltenyi Biotec). The purity of CCR6+ cells among γ/δ T cells was >99%. RNA isolation, complementary DNA synthesis, and TCR repertoire analysis with polymerase chain reaction (PCR) were performed as described previously (19,20) with the same PCR primer sets.

Adoptive transfer experiments with CCR6+ γ/δ T cells. Cells from the DLNs of swollen joints of mice with CIA were prepared. To enrich CCR6+ γ/δ T cells, single-cell suspensions were depleted of CD4+, CD8a+, CD45R+, CD49b+, CD11b+, and Ter-119+ cells by negative selection with a biotin antibody cocktail and antibiotin microbeads of a CD4+ T Cell Isolation Kit, CD4+ microbeads, and an LS column (Miltenyi Biotec). The remaining γ/δ TCR–positive–enriched cells were stained with FITC-conjugated anti-mouse CCR6 mAb (140706; R&D Systems) and anti-FITC microbeads, and the CCR6+ γ/δ T cells were prepared by positive selection using an MS column (Miltenyi Biotec). Control naive CD4+ T cells were purified using the CD4+CD62L+ T Cell Isolation Kit II (Miltenyi Biotec) in

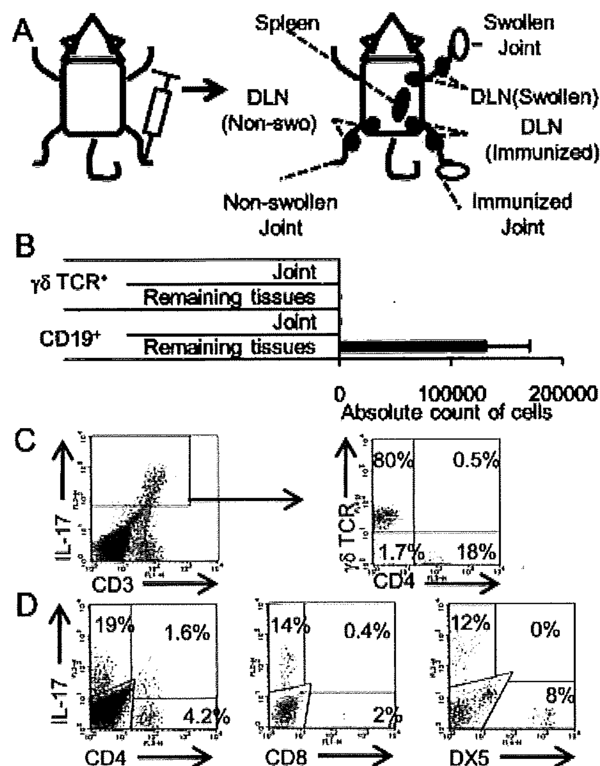

Figure 1. Predominance of interleukin-17 (IL-17)–producing γ/δ T cells in the swollen joints of mice with collagen-induced arthritis (CIA). **A,** Schematic of the analyzed joints and draining lymph nodes (DLNs) in mice with CIA. **B,** Comparison of the absolute counts of γ/δ T cells and CD19+ cells in the joints and remaining tissues of naive mice, as detected by fluorescence-activated cell sorting analysis. Values are the mean and SEM results from 3 different mice. **C** and **D,** Analysis of IL-17–producing γ/δ T cells in the swollen joints of mice. Cells were obtained from the swollen joints of mice with CIA at the peak of arthritis and stained with antibodies against CD3, CD4, CD8, DX5, and γ/δ T cell receptor (TCR). IL-17–producing cells were detected by intracellular cytokine staining. Live lymphocytes were gated based on their forward and side scatter. The percentage of cells in each region or quadrant is noted. One of 5 experiments with similar results is shown. In **C,** IL-17–producing cells were gated and plotted by their expression of CD4 and γ/δ TCR. Non-swo = nonswollen.

accordance with the manufacturer's instructions. Using a Hamilton microsyringe (Osaka Chemical, Osaka, Japan), 60,000 CCR6+ γ/δ T cells or naive CD4+ T cells in 10 μl of PBS, or PBS alone, were injected around each wrist or ankle of naive mice or mice immunized with type II collagen plus CFA 2 weeks previously (n = 79). Arthritis in each joint was examined every 3–4 days according to the scoring system described previously (21).

Patients with RA. Eleven female patients ages 37–81 years (mean ± SD 59 ± 12 years) with a diagnosis of RA based on the 1987 criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (22) were included. The duration of RA ranged from 4

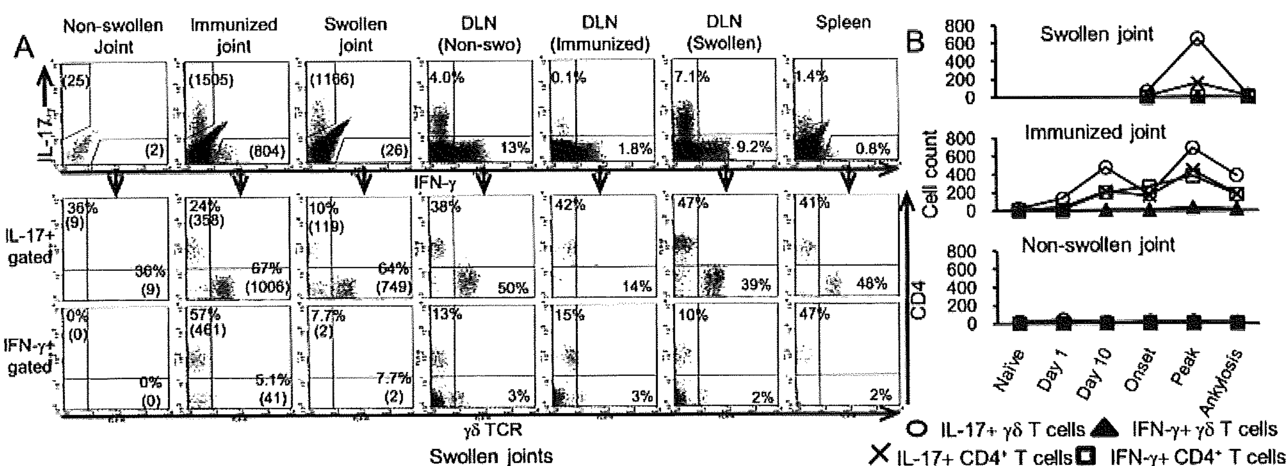

Figure 2. Distribution and kinetics of IL-17–producing γ/δ and CD4+ T cells in CIA. **A,** Cells were obtained from the joints, their DLNs, and the spleens of mice with CIA at the peak of arthritis. Viable lymphocytes were gated based on their forward and side scatter. Using intracellular cytokine staining, IL-17–producing cells and interferon-γ (IFNγ)-producing cells were detected (top row). IL-17–producing cells (second row) or IFNγ-producing cells (bottom row) were gated and plotted by their expression of γ/δ TCR and CD4. In the panels showing analysis of joints, the absolute number of IL-17–producing cells and the percentage of CD4+ cells and γ/δ TCR+ cells among them are indicated. In the panels showing analysis of DLNs and spleen, the percentage of cells in each quadrant is noted. One of 3 experiments with similar results is shown. **B,** Cells were recovered from the swollen joints, immunized joints, and nonswollen joints of mice with CIA during the 6 distinct phases of arthritis described in Materials and Methods. IL-17–producing cells and IFNγ+ cells were detected by intracellular cytokine staining, and their absolute numbers were calculated using fluorescence-activated cell sorting analysis. Values for each phase represent the mean from at least 3 different mice. In **B,** only 3 phases after the onset of arthritis are applicable for the DLNs of swollen joints. See Figure 1 for other definitions.

years to 33 years (mean ± SD 15 ± 9 years). Eight patients were receiving disease-modifying antirheumatic drugs (6 methotrexate, 2 bucillamine, and 2 sulfasalazopyridine) either as monotherapy or in combination, 9 patients were receiving prednisolone (2–10 mg/day), and 1 patient was being treated with an anti–tumor necrosis factor α biologic (etanercept). The 28-joint Disease Activity Score (23) ranged from 2.22 to 6.49 (mean ± SD 4.3 ± 1.6). The C-reactive protein level and the erythrocyte sedimentation rate ranged from 0 to 5.9 mg/dl (mean ± SD 1.9 ± 2.1) and from 9 to 83 mm/hour (mean ± SD 41.9 ± 22.5), respectively. According to the Steinbrocker criteria (24), 27% of the patients had stage III disease, and 73% had stage IV disease. According to the ACR 1991 revised criteria for the classification of global functional status in RA, 50% of the patients had stage II disease, and 50% had stage III disease (25).

Statistical analysis. All statistical analyses were performed using the Mann-Whitney U test with Microsoft Excel software (Microsoft, Redmond, WA) and Statcel2 add-in software (Hisae Yanai, Department of Mathematics, Faculty of Science, Saitama University, Japan). P values less than 0.05 were considered significant.

RESULTS

Predominance of IL-17–producing γ/δ T cells in swollen joints of mice with CIA. In the present study, it was first confirmed that cells from the joints were not contaminated by bone marrow cells. The number of CD19+ cells in the joints was negligible (Figure 1B). Next, IL-17–producing γ/δ T cells in the swollen joints of mice were analyzed at the peak of CIA. Interestingly, the percentage of IL-17–producing γ/δ T cells was 4.4-fold higher than that of Th17 cells (Figure 1C). Almost all of the IL-17–producing cells in swollen joints were either γ/δ T cells or CD4+ T cells, and neither CD8+ cells nor DX5+ NK cells produced IL-17 (Figures 1C and D).

Distribution and kinetics of IL-17–producing γ/δ and CD4+ T cells in CIA. To analyze the distribution and kinetics of IL-17–producing γ/δ T cells and Th17 cells in mice with CIA, intracellular cytokine staining was performed using cells obtained from the joints of mice with CIA during 6 distinct phases, as follows: before immunization (naive mice), 1 day after immunization (day 1), before onset of arthritis (day 10), onset of arthritis (day 32), peak of arthritis (day 42), and ankylosing phase of arthritis (day 70). At each phase, cells were collected from the swollen joint, an immunized joint, a nonswollen joint, the DLNs of each joint, and the spleen (Figure 1A).

In swollen joints, the absolute numbers of IL-17–producing γ/δ T cells were higher than the absolute numbers of Th17 cells, with the maximal counts ob-

tained at the peak of arthritis (Figures 2A and B). Surprisingly, neither IFNγ-producing CD4+ (Th1) cells nor IFNγ-producing γ/δ T cells were detected in the swollen joints at any of the time points analyzed. In contrast, Th1 cells were detected in the DLNs of swollen joints (Figure 2A). In immunized joints, IL-17–producing γ/δ T cells and Th17 cells were already observed on day 1, reached the first peak on day 10 after immunization, and then reached their highest counts at the peak of arthritis. The absolute numbers of IL-17–producing γ/δ T cells were consistently higher than the numbers of Th17 cells at most time points analyzed. In contrast to what was observed in swollen joints, Th1 cells were detected in immunized joints after immunization (Figures 2A and B). In both swollen and immunized joints, the percentages of IL-17–producing γ/δ T cells among IL-17–producing cells were higher than those in DLNs of swollen and immunized joints (Figure 2A). In nonswollen joints, both IL-17–producing T cells and IFNγ-producing T cells were rarely observed. In addition, IFNγ-producing γ/δ T cells were a minor population at the sites of CIA (Figure 2A).

Efficient stimulation of IL-17 production from γ/δ T cells by IL-1β and IL-23. A recent study showed that a subset of γ/δ T cells already differentiate to acquire an IL-17–producing function in the thymus (26). In other studies, specific expression of CCR6 on Th17 has been suggested (27–30). Therefore, the expression of CCR6 on IL-17–producing γ/δ T cells in the thymus of naive DBA1/J mice was evaluated. IL-17–producing, but not IFNγ-producing, γ/δ T cells preferentially expressed CCR6 (Figure 3A). A small number of γ/δ T cells are present in the normal joints of mice (18). To elucidate whether de novo CCR6+ IL-17–producing γ/δ T cells are present in the normal joints of naive DBA1/J mice, cells were collected from the normal joints of naive mice, and intracellular cytokine staining was performed. By analyzing cells from 2 normal paws and ankles at a time, CCR6+ IL-17–producing γ/δ T cells could be detected (Figure 3B). In addition, in mice with CIA, 92% of CCR6+ γ/δ T cells produced IL-17 (Figure 3C).

Next, the IL-17 production requirements for γ/δ T cells were analyzed. Gamma/delta T cells from naive DBA1/J mice were analyzed by stimulation with cytokines in the presence or absence of anti-γ/δ TCR–activating mAb (Figure 3D). IL-17–producing γ/δ T cells were detected with anti-γ/δ TCR mAb, IL-23, and IL-1β alone. In addition, additive stimulatory effects were observed when anti-γ/δ TCR mAb was combined with IL-23, IL-1β, or anti-CD28. Surprisingly, IL-23 plus IL-1β induced IL-17 production quite efficiently. These

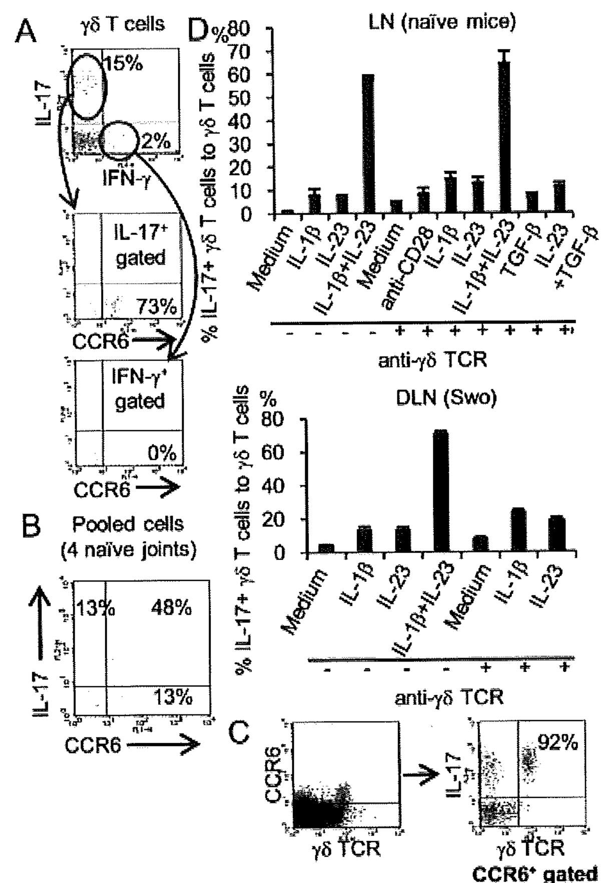

Figure 3. Efficient stimulation of IL-17 production from γ/δ T cells by IL-1β and IL-23. **A,** Thymocytes from naive mice were stimulated with phorbol myristate acetate and ionomycin for 4 hours. TCR+ cells were gated, and CCR6+ cells among IL-17–producing or interferon-γ (IFNγ)–producing γ/δ T cells were detected. **B,** Cells were collected from the paws and ankles of naive mice and stained for γ/δ TCR and CCR6. Gamma/delta TCR+ cells were gated, and CCR6+ IL-17–producing cells were detected by intracellular cytokine staining. In **A** and **B,** the percentages of cells in each quadrant are shown. **C,** Cells were collected from the DLNs of swollen joints, and IL-17–producing cells were detected by intracellular cytokine staining. CCR6+ cells were gated, and IL-17–producing cells were analyzed. The percentage of IL-17–producing cells among CCR+ γ/δ T cells is shown. **D,** Gamma/delta T cells were sorted from the peripheral lymph nodes of naive DBA1/J mice (upper panel) or from the DLNs of swollen joints of mice with CIA at the peak of arthritis (lower panel) and stimulated with cytokines, activating anti-γ/δ TCR antibodies, and anti-CD28 antibodies for 24 hours. The percentages of IL-17–producing cells among γ/δ T cells were determined by intracellular cytokine staining. Bars show the mean and SEM results from 3 different mice. TGFβ = transforming growth factor β (see Figure 1 for other definitions).

observations indicated that TCR signaling was not necessary to stimulate IL-17 production by γ/δ T cells. Furthermore, a combination of IL-23 and IL-1β was a much more potent stimulator than was TCR signaling.

Similar results were obtained with γ/δ T cells sorted from DLNs of swollen joints at the peak of CIA (Figure 3D, lower panel).

Type II collagen–independent induction and maintenance of IL-17–producing γ/δ T cells. Because IL-23 plays important roles in the maintenance of Th17 cells (31–36), we next addressed the maintaining effect of IL-23 or type II collagen on IL-17–producing γ/δ T cells. To this end, cells from the DLNs of swollen joints were cultured with IL-23, type II collagen, or medium alone (Figure 4A). Both IL-17–producing γ/δ T cells and Th17 cells were maintained in the presence of IL-23. In contrast, IL-17–producing γ/δ T cells were not type II collagen dependently maintained, whereas Th17 cells showed type II collagen dependency. To further investigate the factors that enhanced the accumulation of IL-17–producing γ/δ T cells in inflamed joints, the numbers of IL-17–producing γ/δ T cells in the differently immunized joints of mice were counted on day 10. Mice were immunized with PBS, IFA plus solution (0.05 m*M* acetic acid), IFA plus type II collagen, or CFA plus type II collagen (Figure 4B). The numbers of IL-17–producing γ/δ T cells were not significantly different between mice immunized with IFA plus solution, IFA plus type II collagen, or CFA plus type II collagen. In contrast, the numbers of IL-17–producing γ/δ T cells were significantly smaller in mice immunized with PBS compared with the 3 other treatments. The numbers of Th17 cells were significantly higher in mice immunized with IFA plus type II collagen than those in mice treated with IFA plus solution. These data indicate that IL-17–producing γ/δ T cells do not specifically respond to type II collagen and may only respond to adjuvant (IFA plus solution) or adjuvant-induced IL-23.

Next, the γ/δ TCR repertoire was analyzed (Figure 4C). The V_γ repertoire of IL-17–producing γ/δ T cells was composed of $V_{\gamma 1}$, $V_{\gamma 2}$, $V_{\gamma 4}$, and $V_{\gamma 6}$ rather than a single V_γ chain in CIA. In addition, the V_δ repertoire of IL-17–producing γ/δ T cells was composed of $V_{\delta 1}$ and $V_{\delta 5}$.

Exacerbation of arthritis by IL-17–producing γ/δ T cells. Next, the pathogenic roles of IL-17–producing γ/δ T cells in CIA were analyzed. When transferred to the joints of naive mice, CCR6+ γ/δ T cells did not induce arthritis. However, when transferred to the joints of mice immunized with type II collagen plus CFA, CCR6+ γ/δ T cells significantly worsened the arthritis score of joints with arthritis compared with the scores of joints treated with PBS (Figure 4D). The arthritis-exacerbating effect of CCR6+ γ/δ T cells from swollen joints was equivalent to that of CCR6+ γ/δ T cells from the DLNs of swollen joints (data not shown).

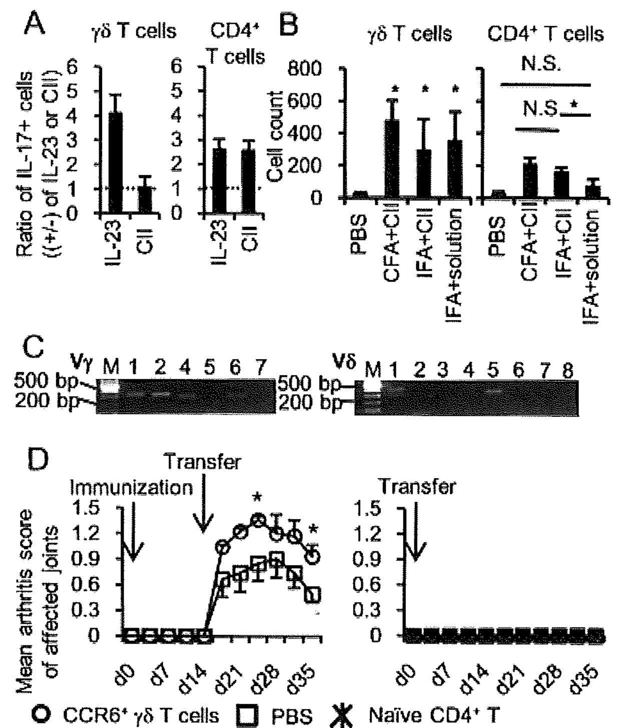

Figure 4. Exacerbation of arthritis by IL-17–producing γ/δ T cells. **A,** Cells were prepared from the DLNs of swollen joints and cultured for 7 days in the presence of IL-23, type II collagen (CII), or medium alone. IL-17–producing cells were detected by fluorescence-activated cell sorting (FACS) analysis. The ratio of the numbers of IL-17–producing cells in the presence of IL-23 or type II collagen to those in medium alone was calculated. Bars show the mean and SEM results from at least 3 different experiments. **B,** Various combinations of substances were administered into the footpads of DBA1/J mice. Ten days later, the absolute numbers of IL-17–producing cells were counted using FACS analysis. Bars show the mean and SEM results from at least 3 different mice. **C,** The use of γ/δ TCR by CCR6+ γ/δ T cells was analyzed by reverse transcription–polymerase chain reaction. **D,** CCR6+ γ/δ T cells from the DLNs of swollen joints were enriched. CCR6+ γ/δ T cells or phosphate buffered saline (PBS) alone was injected into nonimmunized wrists or ankles of mice that had been immunized with type II collagen plus Freund's complete adjuvant (CFA) 2 weeks previously. For naive mice, CCR6+ γ/δ T cells or PBS alone was injected. Values are the mean ± SEM arthritis scores in affected joints. * = $P < 0.05$ versus PBS. NS = not significant; IFA = Freund's incomplete adjuvant; M = marker (see Figure 1 for other definitions).

Absence of IL-17–producing γ/δ T cells in swollen joints of SKG mice or affected joints of patients with RA. To elucidate the pathologic differences from other arthritis models, the same analysis was performed using SKG mice (21). SKG mice carry a point mutation of the

gene encoding ZAP-70, and homozygous mice show IL-17–dependent arthritis resembling RA. Although the present study could detect only a few IL-17–producing γ/δ T cells in the DLNs of swollen joints, surprisingly, almost all of the IL-17–producing cells were Th17 cells, and the number of IL-17–producing γ/δ T cells was negligible in the swollen joints of SKG mice (Figure 5A).

SKG is a BALB/c background strain, and autoimmune arthritis in SKG mice is induced using zymosan as an adjuvant (17,21). To exclude the possibility that IL-17–producing γ/δ T cells are absent in the joints of SKG mice with arthritis because of the differences in strain and adjuvant compared with CIA, the absolute numbers of cell subsets from the joints of SKG or BALB/c mice immunized with CFA plus type II collagen were counted. Even with this protocol, IL-17–producing γ/δ T cells were not detected in SKG mice, whereas IL-17–producing γ/δ T cells were more abundant than Th17 cells in BALB/c mice (Figure 5B).

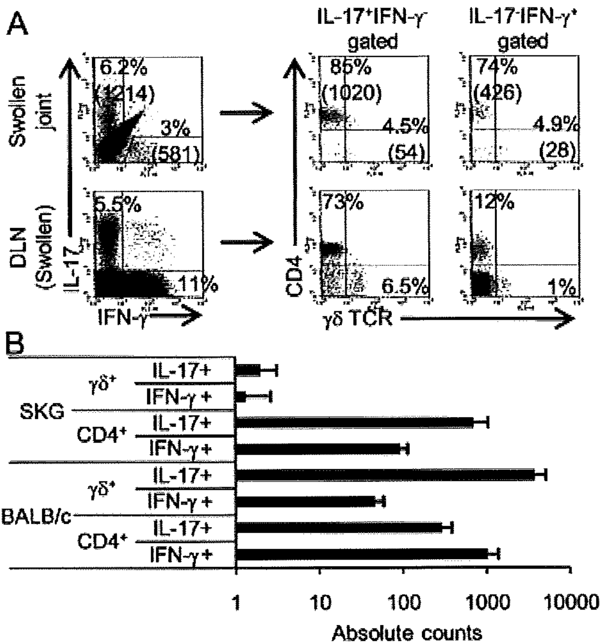

Figure 5. Absence of IL-17–producing γ/δ T cells in the swollen joints of SKG mice. **A,** Cells were collected from the ankles with maximum arthritis (and their DLNs) of SKG mice that had been treated with zymosan 7 weeks previously. Lymphocytes were gated based on their forward and side scatter. IL-17–producing cells and interferon-γ (IFNγ)-producing cells were detected by intracellular cytokine staining (left column). IL-17–producing IFNγ-negative cells (middle column) or IFNγ-producing IL-17–negative cells (right column) were gated and their expression of γ/δ TCR and CD4 was plotted. In the panels showing analysis of joints, the absolute numbers and percentages of CD4+ cells and γ/δ TCR+ cells are indicated. In the panels showing analysis of DLNs, the percentage of cells in each quadrant is noted. One experiment representative of 3 that were performed is shown. **B,** SKG or BALB/c mice were immunized with Freund's complete adjuvant plus type II collagen, and cells from the immunized joints were collected 10 days later. The absolute numbers of cells were counted using fluorescence-activated cell sorting analysis. Bars show the mean and SEM results for 3 different mice. See Figure 1 for other definitions.

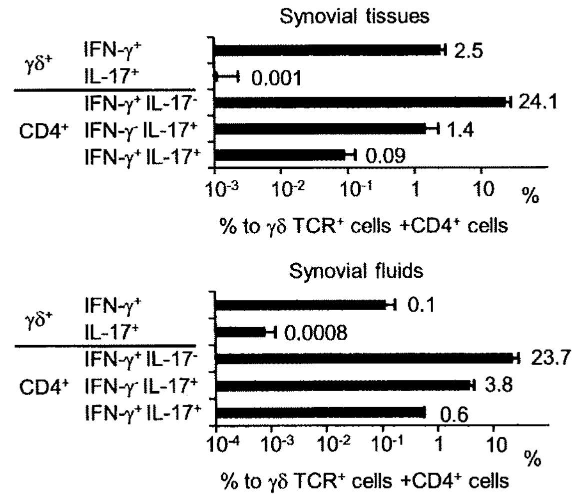

Figure 6. Absence of interleukin-17 (IL-17)–producing γ/δ T cells in the affected joints of patients with rheumatoid arthritis (RA). Cells in RA synovial tissue (n = 4) or synovial fluid (n = 7) were stained with antibodies against CD4 and γ/δ T cell receptor (TCR). IL-17–producing and interferon-γ (IFNγ)-producing cells were analyzed. The percentages of cells among total γ/δ T cells plus CD4+ T cells were determined. Bars show the mean and SEM.

Finally, cells in RA synovial tissue or fluid were analyzed to determine the presence of IL-17–producing γ/δ T cells and Th17 cells at the effector sites of arthritis. In contrast to what was observed in CIA, IL-17–producing γ/δ T cells could not be detected in the synovial tissue of affected joints, whereas IFNγ-producing γ/δ T cells were present in synovial tissue (Figure 6). Among the CD4+ T cells in synovial tissue, IL-17–producing cells were present. However, the proportions of Th1 cells among CD4+ T cells were much larger than those of Th17 cells in affected joints. Similar results were obtained in cells from synovial fluid.

DISCUSSION

The present study first focused on IL-17–producing T cells in the swollen joints of mice with CIA. It was observed that γ/δ T cells were the predominant source of IL-17 and were more abundant than Th17 cells. DX5+ NK cells did not secrete IL-17 in swollen

joints. A direct comparison of the absolute numbers of IL-17–producing γ/δ T cells with the absolute numbers of Th17 cells simultaneously in each joint of mice with CIA was performed for the first time. Although it is known that γ/δ T cells are not necessary for the induction of CIA, because γ/δ TCR–deficient mice can mount CIA (37), the present findings in the kinetics study and adoptive transfer experiments, together with previous reports (16,18,38), suggest that not only Th17 cells but also IL-17–producing γ/δ T cells contribute to the exacerbation of CIA. In contrast, α/β T cells, especially Th17 cells, are essential for the induction of CIA, because α/β TCR–deficient mice cannot mount CIA (37). In addition, IL-17–producing invariant NK T cells in CIA have been reported recently (37), but these cells were not analyzed in the current study.

The origin and functions of IL-17–producing γ/δ T cells in physiologic and pathologic conditions have been elucidated recently. It was reported that a subset of γ/δ T cells acquired an IL-17–producing function in the thymus (26) and produced cytokines immediately in response to initial stimulation. In various murine infectious disease models, these γ/δ T cells predominantly produce IL-17 and eradicate pathogens (40–43). However, the precise requirements of IL-17 production by γ/δ T cells especially in CIA are unknown, although IL-23 was known as a sufficient stimulant of IL-17 production by γ/δ T cells in naive mice (42). Here, it was demonstrated that the combination of IL-23 and IL-1β synergistically stimulated IL-17 production, but stimulation via γ/δ TCR had a limited effect. Given the enhanced expression of IL-1β and IL-23 in the inflamed joints of mice with CIA (44,45), these findings suggest that IL-17 production by γ/δ T cells in CIA might mainly be an inflammatory cytokine–driven process rather than a TCR signal–driven process.

The present study showed that IL-17–producing γ/δ T cells were CCR6 positive, and CCR6 was already expressed on IL-17–producing γ/δ T cells in the thymus of naive mice. CCL20, the only chemokine known to interact with CCR6, is physiologically expressed at epithelial surfaces (46) and fibroblast-like synoviocytes (29) and is up-regulated in inflammatory conditions (30,46). These findings suggest that CCR6 might have some roles in determining the physiologic distribution of IL-17–producing γ/δ T cells. In fact, it was found that a small number of CCR6+ IL-17–producing γ/δ T cells were present in the joints of naive mice.

Next, we focused on the differences between IL-17–producing γ/δ T cells and Th17 cells. IL-17–producing γ/δ T cells were maintained by IL-23 but not by a specific antigen (type II collagen, in this case). In contrast, Th17 cells responded to type II collagen and IL-23. Furthermore, IL-17–producing γ/δ T cells were induced equivalently in response to stimulation by IFA plus solution in the absence of type II collagen. Together with results from the previous study demonstrating that IL-17–producing γ/δ T cells are induced equally by CFA plus type II collagen and CFA (16), the present data suggest that IL-17–producing γ/δ T cells do not recognize the specific antigen (type II collagen) but rather proliferate in response to IL-23, which may be produced locally by synovial cells (44). The ligands of γ/δ T cells are largely unknown, and further analysis of possible antigens of IL-17–producing γ/δ T cells in CIA could be difficult (47). However, the present study confirmed the diverse usage of γ/δ TCR in IL-17–producing γ/δ T cells in CIA (Figure 4C), which supported the present conclusion that IL-17–producing γ/δ T cells are antigen independently induced by inflammatory cytokines.

In summary, it is speculated that the sequence of pathology of CIA is as follows. First, type II collagen–specific Th17 cells are induced by type II collagen plus CFA, which then infiltrate into the joints and cause primary inflammation. Although antigen-independent IL-17–producing γ/δ T cells could be induced simultaneously by CFA, they are not essential for the induction of arthritis. Next, primary inflammation induces local production of IL-23 from synoviocytes and increases the expression of IL-1β in joint cartilage and pannus (45). Locally produced IL-23 induces the proliferation of resident IL-17–producing γ/δ T cells. These γ/δ T cells, stimulated by IL-1β and IL-23, produce enhanced amounts of IL-17 and exacerbate the arthritis of CIA. Another, but not mutually exclusive, possibility is that primary inflammation enhances CCL20 expression in vascular endothelial cells and fibroblast-like synoviocytes (30) in inflamed joints and recruits CCR6+ IL-17–producing cells. In the ankylosing phase, the burned-out tissue does not produce inflammatory cytokines, and the activities and the number of IL-17–producing γ/δ T cells decrease to the basal level.

Finally, the cytokine profiles of T cells in the inflamed joints of SKG mice and patients with RA were compared with those in mice with CIA. In contrast to what was observed in mice with CIA, IL-17–producing γ/δ T cells were not detected in the swollen joints of SKG mice. A lack of IL-17–producing γ/δ T cells in SKG mice was not caused by the differences in strain or adjuvant. It was also observed that IL-17–producing γ/δ T cells are hardly induced in immunized joints, their DLNs, non-DLNs, and spleens of SKG mice (data not

shown) 10 days after immunization with CFA plus type II collagen. Given that TCR signals in SKG mice are attenuated because of a point mutation in ZAP-70 (21), and differentiation of γ/δ T cells needs a strong signal via the TCR (48,49), there may be some defects in γ/δ T cell differentiation in SKG mice. This speculation was supported by data showing impaired development of specific subsets of γ/δ T cells in ZAP-70–knockout mice (50). Furthermore, IL-17 production from γ/δ T cells in the synovial tissue of patients with RA has not yet been detected. In contrast to IL-17–producing γ/δ T cells, IFNγ-producing γ/δ T cells were present. In addition, among CD4+ T cells, Th1 cells were predominant; this finding was consistent with a previous report (51).

These results suggest that IFNγ-producing cells, but not IL-17–producing cells including γ/δ T cells, play predominant pathogenic roles in RA. These distinct pathogenic cell populations may result from differences between CIA and RA such as species and age-related susceptibility. Alternatively, IL-17–producing γ/δ T cells may play an important role in RA as well but are suppressed by the effects of medical treatment. It should be noted that in the present study, we could access joint materials only from patients with progressed stages of RA. Therefore, further studies with patients with recent-onset RA who have not received medical treatment are necessary to determine whether IL-17–producing γ/δ T cells are present.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Usui had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Analysis and interpretation of data. Y. Ito, Usui.

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Clinical and Immunogenetic Features of Patients With Autoantibodies to Asparaginyl–Transfer RNA Synthetase

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Objective. **We have previously described anti-KS autoantibodies and provided evidence that they are directed against asparaginyl–transfer RNA (tRNA) synthetase (AsnRS). The aim of the present study was to identify patients with anti-AsnRS autoantibodies and elucidate the clinical significance of this sixth antisynthetase antibody. In particular, we studied whether it was associated with the syndrome of myositis (polymyositis or dermatomyositis [DM]), interstitial lung disease (ILD), arthritis, and other features that had been previously associated with the 5 other anti–aminoacyl–tRNA synthetase autoantibodies.**

Methods. **More than 2,500 sera from patients with connective tissue disease (including myositis and ILD) and controls were examined for anti-AsnRS autoantibodies by immunoprecipitation (IP). Positive and control sera were tested for the ability to inhibit AsnRS by preincubation of the enzyme source with the serum. The HLA class II (DRB1, DQA1, DQB1, DPB1) alleles were identified from restriction fragment length polymorphism of polymerase chain reaction–amplified genomic DNA.**

Results. **Anti-AsnRS antibodies were identified in the sera of 8 patients (5 Japanese, 1 American, 1 German, and 1 Korean) by IP of the same distinctive set of tRNA and protein that differed from those precipitated by the other 5 antisynthetases, and these antibodies showed specific inhibition of AsnRS activity. Two of these patients had DM, but 7 of 8 (88%) had ILD. Four patients (50%) had arthritis, and 1 had Raynaud's phenomenon. This antisynthetase was very rare among myositis patients (present in 0% of Japanese myositis patients), but it was found in 3% of Japanese ILD patients. Thus, most patients with anti-AsnRS had chronic ILD with or without features of connective tissue disease. Interestingly, all 4 Japanese patients tested had DR2 (DRB1*1501/1502), compared with 33% of healthy controls.**

Conclusion. **These results indicate that anti-AsnRS autoantibodies, like anti-alanyl-tRNA synthetase autoantibodies, have a stronger association with ILD than with myositis and may be associated with the DR2 phenotype.**

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The aminoacyl–transfer RNA (aminoacyl-tRNA) synthetases are a family of cytoplasmic enzymes that catalyze the formation of aminoacyl-tRNA from a specific amino acid and its cognate tRNA and play a crucial role in protein synthesis. Autoantibodies to certain of these synthetases (histidyl–, threonyl–, alanyl–, isoleucyl–, and glycyl–tRNA synthetases) have been identified in patients with inflammatory myopathies (1–6). Among these "antisynthetase autoantibodies," the most common is anti–Jo-1 (anti–histidyl–tRNA synthetase [anti-HisRS]), found in 20% of patients with polymyositis/dermatomyositis (PM/DM) (7–11). Anti–PL-7 (anti–threonyl–tRNA synthetase [anti-ThrRS])

and anti–PL-12 (anti–alanyl–tRNA synthetase [anti-AlaRS]) autoantibodies are less common, found in 3–4% of all patients with PM/DM (4,5,11–13), while anti-OJ (anti–isoleucyl–tRNA synthetase [anti-IleRS]) and anti-EJ (anti–glycyl–tRNA synthetase [anti-GlyRS]) autoantibodies are the least common, occurring in <2% (6,14,15), although the frequency may vary in different populations (16).

Characteristic clinical features have been found in patients with anti-HisRS and other antisynthetase autoantibodies (1,9,10). These features include myositis, interstitial lung disease (ILD), arthritis, Raynaud's phenomenon, fever with exacerbations, and the skin lesion of the fingers referred to as mechanic's hands, and they appear to form a distinct syndrome referred to as the "antisynthetase syndrome" (8–11). Although the similarity of the clinical features associated with different antisynthetases is impressive (17,18), certain differences have been noted, which must be considered preliminary due to the small reported number of patients with non-HisRS antisynthetases (1,9,19). Patients with anti-AlaRS appear to be more likely than those with anti-HisRS to have ILD and/or arthritis either without myositis or with little evidence of muscle disease. Absence of significant myositis over the full disease course in patients with anti-HisRS is rare (<5%), although it may occur. Clinically significant myositis was seen in 60% of US patients with anti-AlaRS (13), whereas none of 6 Japanese patients with anti-AlaRS autoantibodies fulfilled criteria for myositis (20). Among patients with anti-IleRS, 2 of 10 had ILD without evidence of myositis, and 1 had ILD with subclinical myositis (14). In addition, antisynthetases may occur in either PM or DM, but PM is usually more common with anti-HisRS (10,16,21), and DM is usually more common with other antisynthetases, especially anti-GlyRS (15,22).

We recently described anti-KS autoantibodies and provided evidence that the KS antigen is asparaginyl–tRNA synthetase (AsnRS) (23). This sixth antisynthetase was found in sera from 3 patients with ILD and/or inflammatory arthritis without evidence of myositis. It immunoprecipitated a 65-kd protein and a unique tRNA that was distinct from that precipitated by any previously described antisynthetase or other reported tRNA-related antibody. Each of the 3 sera and their IgG fractions showed significant inhibition of AsnRS activity, but did not inhibit any of the other 19 aminoacyl–tRNA synthetase activities.

In this report, we describe the clinical and immunogenetic features of 5 additional patients with anti-AsnRS autoantibodies, most of whom had the syndrome of ILD with arthritis and/or myositis. Immunoprecipitation (IP) and aminoacylation inhibition studies with sera from these patients provide additional evidence that anti-KS (anti-AsnRS) reacts with asparaginyl–tRNA synthetase.

PATIENTS AND METHODS

Sera. Serum samples from a collection of sera from ~800 patients seen at the current or previous collaborating centers of the authors (Keio University, Tokyo, Japan; Kyoto University, Kyoto, Japan; Seoul National University, Seoul, Korea; Clinic and Research Institute for Rheumatic Diseases Aachen, Aachen, Germany; University of Oklahoma Health Sciences Center, Oklahoma City; National Institutes of Health, Bethesda, MD) or sera referred there for testing were stored at −20°C and were tested for the presence of anti-AsnRS autoantibodies. Sera from the following patients were included: 1) patients with PM or DM according to the criteria described by Bohan and Peter (24,25); 2) patients with a condition suggesting the clinical diagnosis of myositis; 3) patients with ILD who had no evidence of myositis and did not meet criteria for other connective tissue diseases; and 4) patients with serum anticytoplasmic antibodies, regardless of diagnosis. Approximately 1,700 other sera have also been tested, including sera from patients with other conditions including systemic lupus erythematosus, systemic sclerosis, and rheumatoid arthritis, as well as sera from normal subjects. Many of the sera were tested in studies of other autoantibodies. All samples were obtained after the patients gave their informed consent, as approved by the corresponding institutional review boards. Stored sera known to contain autoantibodies against synthetases for histidine, threonine, alanine, glycine, and isoleucine were used as controls.

ILD was considered to be present if an interstitial infiltrate was observed on chest radiography. DM was considered to be present if a heliotrope rash and/or Gottron's papules were observed.

IP. IP from HeLa cell extracts was performed as previously described (6,10). Ten microliters of patient sera was mixed with 2 mg of protein A–Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) in 500 μl of IP buffer (10 m*M* Tris HCl at pH 7.5, 500 m*M* NaCl, 0.1% Nonidet P40 [NP40]) and incubated with end-over-end rotation (Labquake shaker; Lab Industries, Berkeley, CA) for 2 hours at 4°C. The IgG-coated Sepharose was washed 4 times in 500 μl of IP buffer using 10-second spins in a microfuge tube, and resuspended in 400 μl of NET-2 buffer (50 m*M* Tris HCl at pH 7.5, 150 m*M* NaCl, 0.05% NP40).

For analysis of RNAs, this suspension was incubated with 100 μl of extracts, derived from 6×10^6 cells, on the rotator for 2 hours at 4°C. The antigen-bound Sepharose was then collected with a 10-second centrifugation in the microfuge, washed 4 times with NET-2 buffer, and resuspended in 300 μl of NET-2 buffer. To extract bound RNAs, 30 μl of 3.0*M* sodium acetate, 30 μl of 10% sodium dodecyl sulfate (SDS), and 300 μl of phenol/chloroform/isoamyl alcohol (50:50:1; containing 0.1% 8-hydroxyquinoline) were added to the Sepharose beads. After agitation in a Vortex mixer and

spinning for 1 minute, RNAs were recovered in the aqueous phase after ethanol precipitation and dissolved in 20 μl of electrophoresis sample buffer, composed of 10*M* urea, 0.025% bromphenol blue, and 0.025% xylene cyanol FF (Bio-Rad, Hercules, CA) in Tris–borate–EDTA buffer (90 m*M* Tris HCl at pH 8.6, 90 m*M* boric acid, and 1 m*M* EDTA). The RNA samples were denatured at 65°C for 5 minutes and then resolved by 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE), with silver staining (Bio-Rad).

For protein studies, antibody-coated Sepharose was mixed with 400 μl of ^{35}S-methionine–labeled HeLa extract derived from 2×10^5 cells and rotated at 4°C for 2 hours. After 4 washes with IP buffer, the Sepharose was resuspended in SDS sample buffer (2% SDS, 10% glycerol, 62.5 m*M* Tris HCl at pH 6.8, 0.005% bromphenol blue). After heating at 90°C for 5 minutes, the proteins were fractionated by 10% SDS-PAGE, enhanced with 0.5*M* sodium salicylate, and dried. Labeled proteins were analyzed by autoradiography.

Aminoacylation. Aminoacylation inhibition reactions were performed as described previously, with minor modification (6,26). Six microliters of HeLa cell extract diluted 1:10 in Tris buffered saline was incubated with 3 μl of a 1:10 dilution of serum for 2 hours at 4°C. This was combined with 17 μl of reaction solution (50 m*M* Tris HCl at pH 7.5, 0.02*M* NaCl, 0.01*M* $MgSO_4$, 1 m*M* dithiothreitol) containing 8 units of yeast tRNA, 3 μl of ^{14}C-asparagine or other ^{3}H-labeled amino acid, and 1 μl of 200 m*M* cold amino acid. Ten-microliter aliquots were tested at 10 minutes and 20 minutes, spotted onto filter paper treated with 5% trichloroacetic acid (TCA), washed 5 times with 5% TCA, then with ethanol, then dried for counting. Results of inhibition testing were expressed as the percent inhibition of the average activity seen with the normal serum included in that experiment, as follows: % inhibition = [(average counts per minute with normal serum) − (cpm with test serum)] × 100/(average cpm with normal serum). Inhibition of >50% compared with the activity with normal serum was considered significant. In previous studies, although nonspecific effects on aminoacylation reactions by serum were common, nonspecific inhibition was usually <25%, and inhibition >50% reliably reflected specific antibody effects (6,7,12,13,26).

DNA typing of the HLA class II (DRB1, DQA1, DQB1, DPB1) alleles by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). Genomic DNA was isolated by phenol extraction of SDS-lysed and proteinase K–treated peripheral blood leukocytes, and then amplified by the PCR procedure using an automated PCR thermal cycler (PerkinElmer Cetus, Norwalk, CT). The primers used for specific amplification of the polymorphic exon 2 domains of the DRB1, DQA1, DQB1, and DPB1 genes were previously described (27). Amplified DNA was digested by all-specific restriction endonucleases and subjected to electrophoresis using a 12% polyacrylamide gel. Digested fragments were detected by staining with ethidium bromide, and HLA genotypes were determined on the basis of the RFLP patterns generated as previously described (27).

Other. Ouchterlony double immunodiffusion was performed as described previously, using HeLa cell extract as antigen (10).

Cases. *Patient 1.* The patient, a 61-year-old Japanese woman, noticed chest pain, followed 3 months later by dyspnea on mild exertion. Chest radiography and computed tomography (CT) scanning showed bilateral basilar infiltrates. The patient had hypoxemia, with a restrictive pattern on pulmonary function tests. No muscle weakness was observed, and the creatine kinase (CK) level was normal (67 IU/liter). A lung biopsy specimen obtained by video-assisted thoracic surgery showed mild interstitial chronic inflammation and interstitial fibrosis lacking a temporal heterogeneity pattern, and a diagnosis of fibrotic nonspecific interstitial pneumonia was made.

Patient 2. The patient, a 51-year-old German woman, developed a nonproductive cough and dyspnea on exertion. Chest radiography showed bibasilar interstitial fibrosis, and pulmonary function tests showed a restrictive pattern with decreased diffusing capacity for carbon monoxide (DLco). A diagnosis of ILD was made, and the patient's pulmonary function remained stable throughout her disease course. She had polyarthralgia and developed erythema and keratosis of the palms and fingers consistent with mechanic's hands, but no cutaneous scleroderma, Raynaud's phenomenon, or DM rash (Gottron's papules or heliotrope rash) was observed. No muscle weakness was found, and the CK level was normal (56 IU/liter at the first visit) each time it was measured. When the patient was age 58 years, ovarian carcinoma was found, and surgery with subsequent irradiation was performed. She died of metastatic ovarian carcinoma at age 63 years.

Patient 3. The patient, a 72-year-old American woman, developed an itchy red eczematous rash that was thought to be due to a medication for hypertension. The rash was soon accompanied by progressive weakness, myalgias, mild dyspnea, and difficulty swallowing. She was started on prednisone and methotrexate, and 6 months after the rash had first appeared, she was referred to the Arthritis and Rheumatism Branch of the National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health. There was a widespread maculopapular rash of the trunk, extremities, and head, and Gottron's papules were observed. Proximal muscle weakness was present, and her CK level was 358 IU/liter. Magnetic resonance imaging of the thighs showed both atrophy and probable inflammation on the STIR images. A biopsy of the deltoid muscle showed changes of an active inflammatory myopathy. No malignancy was identified. She was treated with pulse methylprednisolone. However, her muscle weakness and rash were not significantly improved, and infectious complications limited the therapeutic options. Her disease course was subsequently complicated by herpes zoster and the Ramsay-Hunt syndrome as well as by skin infections and cellulitis, mastoiditis, heart failure, and a cerebrovascular accident.

Patient 4. The patient, a 53-year-old Korean woman with intermittent episodes of productive cough due to bronchiectasis, noticed easy fatigability and myalgia in 1994 and later developed muscle weakness and was admitted to Seoul National University Hospital in February 1995. Proximal muscle weakness in her extremities and a dark pigmentation over the extensor surface of both knees were observed. The CK level was elevated at 3,808 IU/liter. The findings on electromyogram and muscle biopsy were consistent with inflammatory myopathy. A diagnosis of DM associated with ILD was made, and she was treated with prednisolone (60 mg/day). Her muscle enzyme levels gradually normalized, and her muscle weakness improved. Her chest radiograph and high-resolution

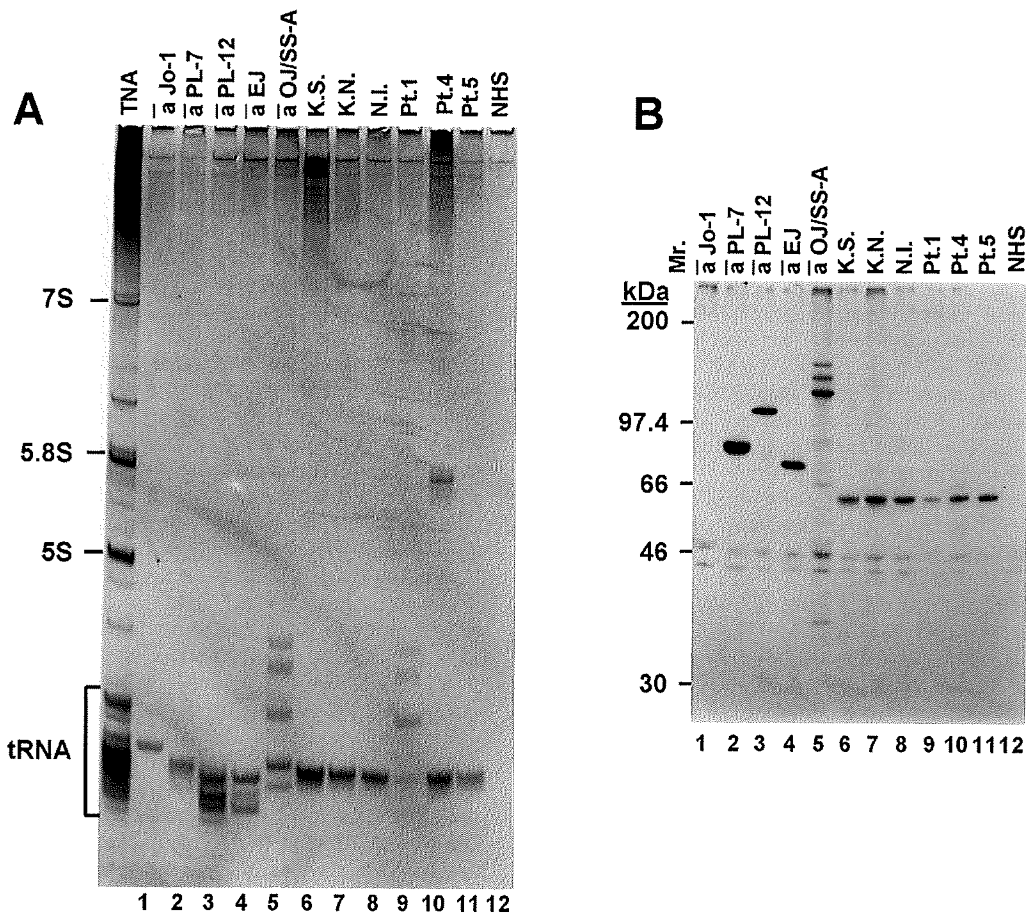

Figure 1. A, Immunoprecipitation (IP) for nucleic acids with anti-KS and control sera. Shown are patterns of transfer RNA (tRNA) resulting from 7*M* urea–10% polyacrylamide gel electrophoresis (PAGE) of phenol-extracted immunoprecipitates from HeLa cell extract, developed with silver stain. TNA = total nucleic acids, with the 5.8S and 5S small ribosomal RNAs and the tRNA region indicated. Antisynthetase sera used for IP are indicated. Lane 1, Anti–histidyl–tRNA synthetase (a Jo-1); lane 2, anti–threonyl–tRNA synthetase (a PL-7); lane 3, anti–alanyl–tRNA synthetase (a PL-12); lane 4, anti–glycyl–tRNA synthetase (a EJ); lane 5, anti–isoleucyl–tRNA synthetase (a OJ/SS-A); lanes 6–11, anti-KS sera from patients KS, KN, and NI in the previous study (23) and from patients 1, 4, and 5 in the present study; lane 12, normal human serum (NHS) control. The tRNA pattern with anti-KS sera is easily distinguishable from that of other antisynthetases. **B,** IP for proteins with anti-KS and control sera. Autoradiogram of 10% sodium dodecyl sulfate–PAGE of immunoprecipitates from ^{35}S-methionine–labeled HeLa cell extract. Mr. = molecular weight markers. Antisynthetase sera used for IP are indicated as in **A.** Anti-KS sera immunoprecipitated a very strong protein band from ^{35}S-methionine–labeled HeLa cell extracts (lanes 6–11), migrating at 65 kd, that was clearly different from the bands immunoprecipitated by sera with the described antisynthetases.

CT scan showed bilateral basilar interstitial fibrosis, and pulmonary function tests showed a restrictive pattern with decreased DLco. Her muscle weakness gradually improved, and the CK level normalized in January 1996. Prednisolone was tapered and discontinued in March 1996.

Patient 5. The patient, a 64-year-old Japanese man with a previous history of prostatic carcinoma, was admitted to the hospital due to bilateral infiltrates on chest radiography. He did not notice cough or dyspnea at that time, but a chest CT scan revealed bibasilar interstitial fibrosis. A transbronchial lung biopsy was performed, with histology showing usual interstitial pneumonia. He was started on prednisolone (40 mg/day), resulting in slight improvement seen on his chest radiograph. Prednisolone was tapered and discontinued in April 1998. He then developed polyarthritis and was treated with a nonsteroidal antiinflammatory drug. No muscle weakness was found, and the CK level was normal (50 IU/liter at the first visit) throughout his disease course.

RESULTS

Identification of anti-KS (anti-AsnRS) antibodies. Sera from all 8 patients (the 3 patients with ILD and/or inflammatory arthritis without evidence of myositis in our previous study [patients KS, KN, and NI; see

Table 1. Clinical features of 8 patients with anti-KS antibodies*

	Patient							
	KS	KN	NI	1	2	3	4	5
Age at onset, years/sex	36/F	44/F	61/F	60/F	51/F	72/F	53/F	65/M
Ethnic background	Japanese	Japanese	Japanese	Japanese	German	US	Korean	Japanese
ILD	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Myositis	No	No	No	No	No	Yes	Yes	No
DM rash	No	No	No	No	No	Yes	Yes	No
Arthritis	Yes	No	No	No	Yes	Yes	No	Yes
Malignancy	No	No	No	No	Ovarian cancer	No	No	Prostate cancer
Raynaud's phenomenon	No	Yes	No	No	No	No	No	No
Other autoantibodies	No	No	No	Anti-SSA/Ro	No	No	No	No
Diagnosis	ILD with arthritis	Idiopathic ILD	Idiopathic ILD	Idiopathic ILD	Idiopathic ILD	DM	DM	ILD with arthritis

* ILD = interstitial lung disease; DM = dermatomyositis.

ref. 23] and the 5 additional patients described above) were shown to immunoprecipitate a characteristic, identical pattern of tRNA, with a strong predominant nucleic acid band of tRNA size, accompanied by a faster faint band (Figure 1A). This gel pattern of tRNA was clearly distinguishable from the pattern of tRNA precipitated by the 5 other described antisynthetases (Figure 1A) and was identical in mobility and appearance to that of serum KS, the originally reported anti-KS serum (23) (Figure 1A).

A very strong band from ^{35}S-methionine–labeled HeLa cell extracts (Figure 1B), migrating at 65 kd, that was also identical in mobility to that of serum KS, was found by IP for all 8 sera, with 5 representative sera shown in Figure 1B. This was clearly different from the characteristic bands immunoprecipitated by sera with the other described antisynthetases (Figure 1B).

Five of the newly recognized anti-KS antibody–positive sera were tested for their ability to inhibit the in vitro enzymatic function of AsnRS (aminoacylation of $tRNA^{Asn}$). Four of the 5 new anti-KS sera significantly inhibited (by >50% at 10 minutes) AsnRS activity compared with normal serum or other controls (serum from patient KS by 87%, serum from patient KN by 99%, serum from patient NI by 91%, serum from patient 1 by 82%, serum from patient 2 by 100%, serum from patient 3 by 18%, serum from patient 4 by 87%, and serum from patient 5 by 91%). This inhibition was strong and comparable with that seen with serum KS, for 4 of the 5 new anti-KS sera. Purified IgG from the third new serum (from patient 3) showed significant, but not strong, inhibition (52%) that increased at 20 minutes (to 84%).

There was no significant inhibition of other synthetases. Normal control serum and anti-KS–negative myositis serum did not show significant inhibition of AsnRS, although sera with other antisynthetases inhibited the expected enzymes. These results indicated that sera with anti-KS by IP showed specific inhibition of AsnRS, further supporting previous data indicating that anti-KS reacted with AsnRS.

Clinical findings. The clinical features of the 5 newly identified patients (patients 1–5) and the 3 patients with anti-AsnRS reported previously (patients KS, KN, and NI) (23) are summarized in Table 1. All patients with anti-AsnRS antibodies were middle-aged or elderly, and 7 of them were women. Five patients were Japanese, 1 was from the US, 1 was German, and 1 was Korean. Seven of these 8 patients (88%) had ILD, documented in each case by both chest radiography and pulmonary function tests. In addition, 2 patients had myositis and a diagnosis of DM. Their clinical courses of ILD were classified as the chronic type. Four patients (50%) had nonerosive arthritis or arthralgia. Raynaud's phenomenon was seen in only 1 patient. None of the patients had sclerodactyly or overlap syndromes with other connective tissue diseases. Malignant diseases (ovarian carcinoma and prostatic carcinoma) were observed in 2 patients. Regarding other autoantibodies, anti-SSA/Ro antibodies were detected in only 1 patient.

Anti-AsnRS was found in 0% of Japanese patients with myositis, but was found in 3% of Japanese patients with "idiopathic" ILD. Thus, most patients with anti-AsnRS antibodies had chronic ILD with or without features of PM/DM or other connective tissue disease.

Immunogenetic features. The HLA class II gene was determined in 4 Japanese patients (Table 2). All 4 patients had DR2 (DRB1*1501 or DRB1*1502) compared with 33% of healthy local controls. It should be noted that all patients with anti-AsnRS antibodies had DR2, but the frequency of DR2 did not reach statistical significance ($P > 0.05$).

Table 2. HLA class II genes in Japanese patients with anti-KS autoantibodies

	Patient			
	KS	KN	NI	1
DR	2/5	2/1	2/2	2/4
DRB1*	1502/1101	1501/0101	1502/1502	1501/0405
DQA1*	0103/0501	0102/0101	0103/0103	0102/0303
DQB1*	0601/0301	0602/0501	0601/0601	0602/0401
DPB1*	0901/1401	0201/0501	0901/0901	0201/0402

DISCUSSION

We have identified anti-KS (anti-AsnRS) autoantibodies in 8 patients with ILD and DM, by IP of the same distinctive set of tRNA and protein that differed from those precipitated by the other 5 antisynthetases. Most of the anti-KS sera showed specific inhibition of the enzyme target, AsnRS, without inhibiting other synthetases.

Several interesting characteristics of the previously studied antisynthetases have been described: 1) they are associated with a distinctive clinical syndrome referred to as the antisynthetase syndrome, 2) they are directed at functionally related enzymes (performing the same function for different amino acids), 3) they do not cross-react with other synthetases, and 4) they tend to be mutually exclusive. Anti-AsnRS antibodies seem to have the same features. No serum with any other antisynthetase has had antibodies to AsnRS, and none of the 8 anti-AsnRS sera reported here showed signs of reaction with other synthetases. The mechanism of this phenomenon remains unknown.

Multiple tRNA bands immunoprecipitated by anti-AsnRS were found on urea-PAGE. The patterns of tRNA for each of the 8 patients were very similar, highly restricted compared with total tRNA, and distinctive compared with the pattern of other anti–aminoacyl tRNA synthetase autoantibodies. These bands are likely to represent different forms of tRNA for asparagine, which can include tRNA with different asparagine anticodons (uracil-uracil-adenine, uracil-uracil-guanine) or tRNA with the same anticodon but differences in other parts of the sequence. Most sera with anti-HisRS, anti-ThrRS, anti-GlyRS, and anti-IleRS had not been described to react directly with tRNA, suggesting indirect precipitation of tRNA. However, approximately one-third of anti-HisRS–positive sera were reported to contain autoantibodies recognizing $tRNA^{His}$ (28). Most anti-AlaRS sera react directly with the sets of $tRNA^{Ala}$ with the inosine-guanine-cytosine anticodon (29). We previously found that the 3 original anti-KS (anti-AsnRS) sera did not immunoprecipitate any RNA from deproteinized HeLa extracts (23). This suggests that anti-AsnRS antibodies can precipitate $tRNA^{Asn}$ indirectly, through its affinity for AsnRS, although the possibility of conformational epitopes on the tRNA has not been excluded (28). Further analysis will be necessary to determine the sequence and specificity of tRNA immunoprecipitated by anti-AsnRS.

The specific inhibition of AsnRS function by most of the sera found to have anti-KS is consistent with findings observed for other antisynthetases. It should be noted that our anti-KS sera also demonstrated inhibition of enzymatically active recombinant AsnRS (30). Most sera with any of the 5 reported antisynthetases specifically inhibit the aminoacylation of the respective tRNA, indicating inhibition of the enzymatic function of the synthetase (3,5–7,12). This functional inhibition may indicate that the autoantibodies are recognizing the active sites of the synthetases. In contrast, it has been reported that animal antisera raised against synthetases do not consistently show such inhibition, suggesting that active sites tend not to be immunogenic for animals (31). Hypothetically, this could relate to relative conservation of the active site. However, there might be an alternative mechanism for inhibition. For example, binding of antibodies outside the active site may alter the structure of the enzyme or interfere with enzyme activity sterically. Further studies of the precise epitope on the aminoacyl–tRNA synthetase might help to explain the development of these autoantibodies.

Each of the 5 previous antisynthetases was first identified in patients with myositis and then found to be associated with ILD. In previous studies, these autoantibodies were associated with myositis with a high frequency of ILD (50–80%) and arthritis (50–90%) (1,2,17,18), as well as an increase in Raynaud's phenomenon (60%), fever with exacerbations (80%), and the skin lesion of the fingers referred to as mechanic's hands (70%) when compared with the overall population of patients with myositis (9–11). The similarities between patients with different antisynthetases have been noted, whereas certain differences have been found, which must be considered preliminary due to the small reported number of patients with non-HisRS antisynthetases. Absence of significant myositis over the full disease course in patients with anti-HisRS is rare (<5%) (32), whereas patients with anti-AlaRS are more likely than patients with anti-HisRS to have ILD and/or arthritis without clinical evidence of myositis (19). Anti-ThrRS

resembles anti-HisRS more than anti-AlaRS in Japanese patients (33).

In the present study, 7 of 8 patients (88%) with anti-AsnRS autoantibodies had ILD, some with other associated features of connective tissue disease including arthritis and Raynaud's phenomenon. In this respect, anti-AsnRS appears to resemble anti-AlaRS more than anti-HisRS. It is noteworthy that the 2 patients with both anti-AsnRS and myositis were among the 3 patients from outside Japan, while none of 5 patients from Japan had myositis. Thus, as with patients with anti-AlaRS, for patients with anti-AsnRS, the frequency of ILD without myositis may be higher in Japanese patients. However, most of the group of patients with ILD without myositis who were tested in this study were from Japan.

The features of these 8 patients with anti-KS appeared to reside within the spectrum of the antisynthetase syndrome that has been associated with other antisynthetases. ILD is one of the most important features of the antisynthetase syndrome, and Raynaud's phenomenon and arthritis, as seen in some patients with anti-AsnRS, are also likely to be part of the syndrome. The syndrome associated with anti-AsnRS may be one end of the spectrum of patients with antisynthetase. This highlights the clinical importance of looking for such antibodies in patients with ILD even if there are no signs of myositis or connective tissue diseases.

The typical cutaneous features of DM were observed in 2 patients with anti-AsnRS antibodies. PM has been reported to be much more common (60–80% or more) than DM in patients with anti-HisRS in most studies, whereas DM was most frequent with anti-GlyRS (15) and was also found to be common among patients with anti-AlaRS (13). Like anti-GlyRS and anti-AlaRS antibodies, anti-AsnRS antibodies were more associated with DM in the small number of patients available.

Malignancy has been reported to be unusual in patients with antisynthetases. In our studies, 2 patients were found to have malignancy during their disease course. However, malignancy in these patients may not be related to the DM or ILD, since these malignancies occurred separated in time from each other.

Immunogenetic studies of connective tissue disease have been performed, but HLA associations produced conflicting results. However, a strong correlation of HLA class II antigens with some autoantibodies has been reported (34). With regard to antisynthetase antibodies, HLA–DR3 (DRB1*0301), DQA1*0501, or DQA1*0401 was found to be significantly increased in myositis patients with antisynthetases (9,21). In Japanese patients, we have reported that 7 of 9 patients (78%) with anti-HisRS tested had the HLA class II DRB1*0405;DQA1*0302;DQB1*0401 haplotype, compared with 22% of healthy controls (odds ratio [OR] 13, $P = 0.002$), while 4 of 7 patients (57%) with anti-AlaRS had the DRB1*1501;DQA1*0102;DQB1*0602 haplotype, compared with 9% of healthy controls (OR 14, $P = 0.006$) (35). Interestingly, all 4 Japanese patients tested had DR2 (DRB1*1501/1502), compared with 33% of healthy controls, although a definite statistical association could not be established. These results suggest that the stronger association of anti-AlaRS and anti-KS with ILD may be related to the DR2 phenotype. However, it has been noted that different ethnic groups exhibit different immunogenetic profiles that link with specific autoantibodies (36). Therefore, further studies including analysis of more patients with anti-KS antibodies in different ethnic groups and major histocompatibility complex–restricted T cell responses could provide important clues for understanding the possible mechanisms for the development of antisynthetase antibodies.

The mechanism for the association of antisynthetases with ILD is unknown, but it seems to be related to etiologic factors (37). Recently, a new association of anti-HisRS–positive PM and ILD was reported in a patient with hepatitis C virus infection (38). It was hypothesized that viruses might interact with the synthetases and induce autoantibodies by molecular mimicry or antiidiotype mechanisms in the anti-HisRS–positive patient with myositis associated with ILD (3,39). Another mechanism for generating autoantigenic epitopes of synthetase by granzyme B cleavage in apoptosis was also described recently (40,41). However, these proposed mechanisms remain speculative, and further studies could provide important clues for understanding the possible mechanisms for the development of these antibodies. Studies of these antibodies may provide insight into the etiologic and pathogenetic mechanisms of ILD and myositis.

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AUTHOR CONTRIBUTIONS

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

Study design. Hirakata.
Acquisition of data. Hirakata, Nagai, Genth, Song, Targoff.
Analysis and interpretation of data. Hirakata, Suwa, Takada, Sato, Mimori.
Manuscript preparation. Hirakata, Takada, Targoff.
Statistical analysis. Hirakata, Suwa, Targoff.

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ORIGINAL ARTICLE

Combining effects of polymorphism of tumor necrosis factor α 5′-flanking region and HLA-DRB1 on radiological progression in patients with rheumatoid arthritis

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Abstract We examined whether polymorphisms upstream of the TNF-α gene (*TNFA*) were associated with the radiological progression of rheumatoid arthritis (RA). One hundred and twenty-three patients with early RA (disease duration <1 year) were enrolled in a prospective follow-up study. The laboratory findings (ESR, CRP, and RF) were evaluated every 2 months for 2 years. Radiological progression in hands/wrists and feet was evaluated every 6 months for 2 years using Larsen's score. *HLA-DRB1* genotype was determined by PCR-RFLP method. The genotypes for -1031, -863, and -857 single-nucleotide polymorphisms in the upstream 5′-flanking region of *TNFA* were determined by a PCR-preferential homoduplex formation assay in patients with RA and 265 healthy controls. Four *TNFA* alleles (U01, U02, U03, and U04) were identified. The frequency of individuals with U02 was significantly higher in patients than in controls ($P = 0.0025$). Radiographs of hands/wrists/feet were available for 72 patients after 1 year and for 73 patients after 2 years. When the *HLA-DRB1* genotype was analyzed simultaneously, patients possessing U02 without an *HLA-DRB1* shared epitope (SE) (U02+SE−) showed the lowest progression of Larsen's score (12 months). There was no difference in the level of ESR, CRP, or RF at the first visit among U02+SE+, U02+SE−, U02−SE+, and U02−SE− groups. The combination of the polymorphism of the *TNFA* upstream promoter region and *HLA-DRB1* allele was associated with radiological progression in the early stage of RA.

Keywords Polymorphism · Rheumatoid arthritis · Tumor necrosis factor

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease, and its etiology is unknown. *HLA-DRB1* alleles encoding the shared epitope (SE) are associated with RA. On the other hand, a variety of cytokines have been detected in the synovial fluid and synovial tissue of patients with RA. TNF-α, a proinflammatory cytokine, plays a pivotal role in RA [1]. TNF-α is produced mainly by monocytes and macrophages, but also by B cells, T cells, and fibroblasts. Transgenic mice that overexpress the human TNF gene develop polyarthritis similar to RA [2]. Moreover, significant clinical benefit has been observed in RA patients who received TNF blocking agents.