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 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).

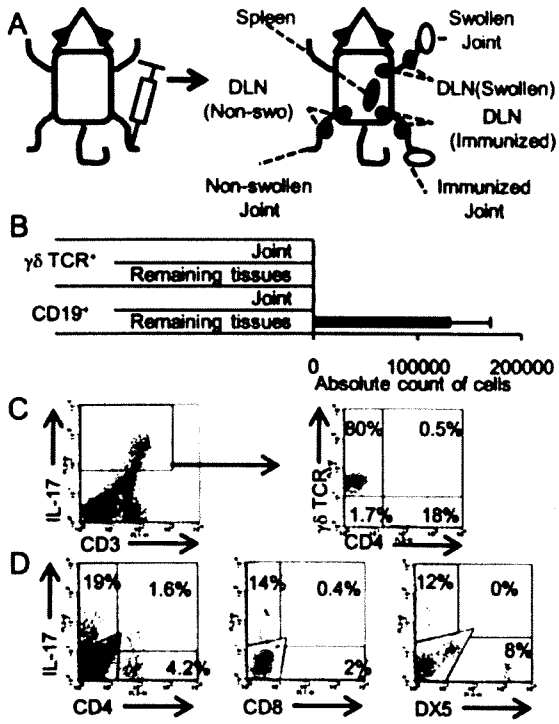

Figure 1. Predominance of interleukin-17 (IL-17)–producing γ/δ T cells in the swollen joints of mice with collagen-induced arthritis (CIA). **A**, Schematic of the analyzed joints and draining lymph nodes (DLNs) in mice with CIA. **B**, Comparison of the absolute counts of γ/δ T cells and CD19+ cells 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.

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

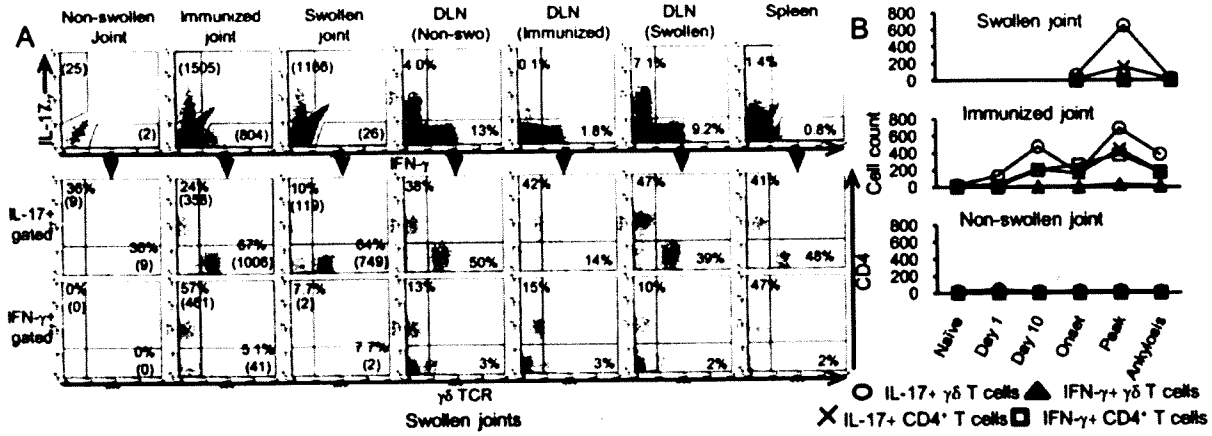

Figure 2. Distribution and kinetics of IL-17–producing γ/δ and CD4+ T cells in CIA. **A**, Cells were obtained from the joints, their DLNs, and the spleens of mice with CIA at the peak of arthritis. Viable lymphocytes were gated based on their forward and side scatter. Using intracellular cytokine staining, 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

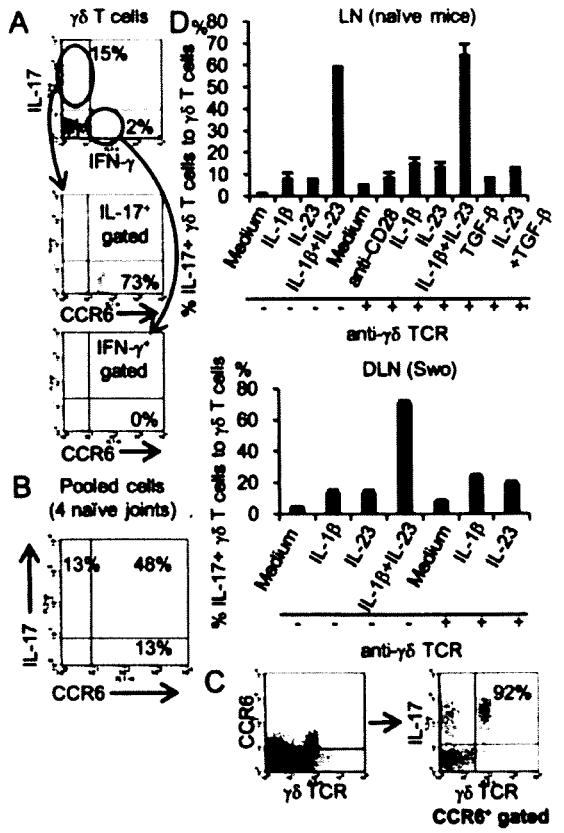

Figure 3. Efficient stimulation of IL-17 production from γ/δ T cells by IL-1β and IL-23. **A,** Thymocytes from naive mice were stimulated with phorbol myristate acetate and ionomycin for 4 hours. TCR+ cells were gated, and CCR6+ cells among IL-17–producing or interferon-γ (IFNγ)–producing γ/δ T cells 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).

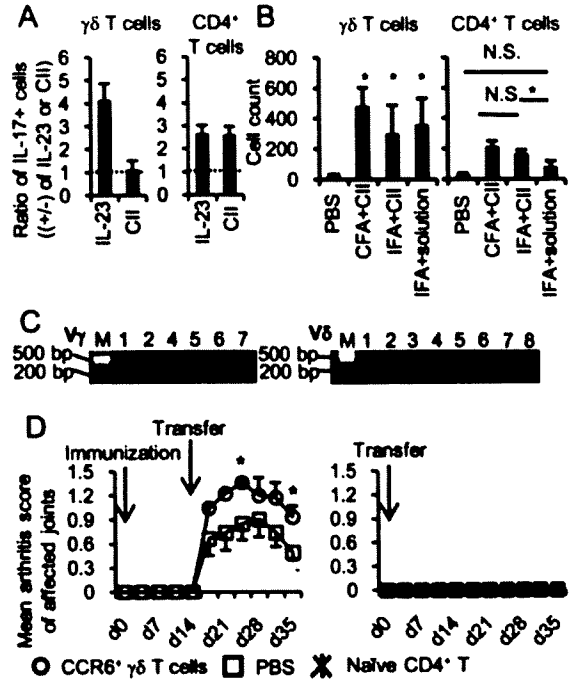

Figure 4. Exacerbation of arthritis by IL-17–producing γ/δ T cells. **A,** Cells were prepared from the DLNs of swollen joints and cultured for 7 days in the presence of IL-23, type II collagen (CII), or medium alone. IL-17–producing cells were detected by fluorescence-activated cell sorting (FACS) analysis. 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).

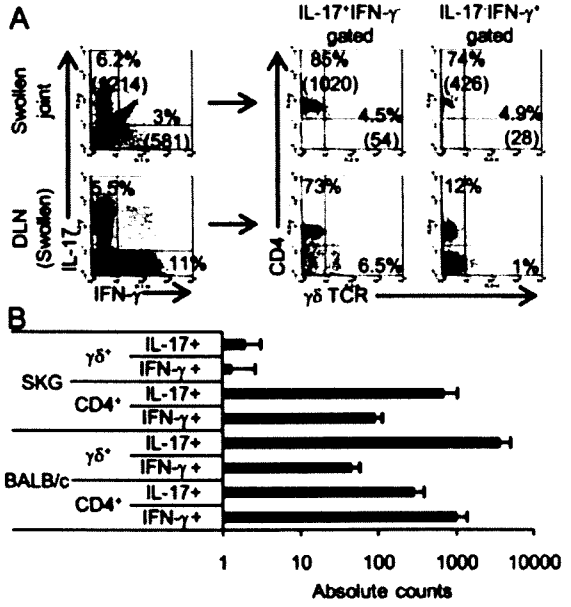

Figure 5. Absence of IL-17–producing γ/δ T cells in the swollen joints of SKG mice. **A**, Cells were collected from the ankles with maximum arthritis (and their DLNs) of SKG mice that had been treated with zymosan 7 weeks previously. Lymphocytes were gated based on their forward and side scatter. IL-17–producing 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.

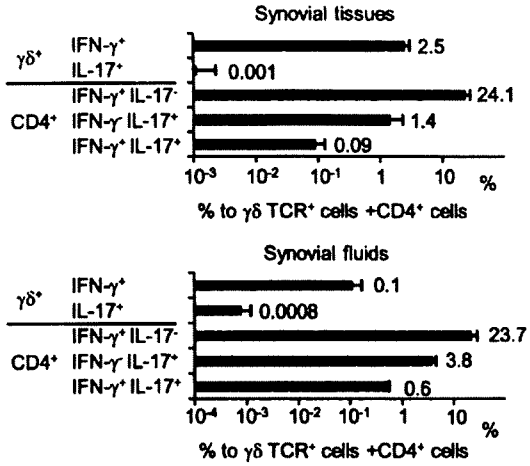

Figure 6. Absence of interleukin-17 (IL-17)–producing γ/δ T cells in the affected joints of patients with rheumatoid arthritis (RA). Cells in RA synovial tissue (n = 4) or synovial fluid (n = 7) were stained with antibodies against CD4 and γ/δ T cell receptor (TCR). IL-17–producing and interferon-γ (IFNγ)–producing 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.

Study conception and design. Y. Ito, Usui.

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Analysis and interpretation of data. Y. Ito, Usui.

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Hypoxia-induced Abrogation of Contact-dependent Inhibition of Rheumatoid Arthritis Synovial Fibroblast Proliferation

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ABSTRACT. ***Objective.*** Uncontrolled proliferation of synovial fibroblasts is characteristic of the pathology of rheumatoid arthritis (RA). Since synovial tissues in the rheumatoid joints are hypoxic, we investigated how hypoxia affects RA synovial fibroblast (RASF) proliferation.

Methods. RASF were cultured at 2000 cells (low density culture) or at 5000 cells (high density, growth-inhibitory confluent culture) per microtiter well under hypoxic (10%, 3%, or 1% O_2) or normoxic (21% O_2) conditions. Some RASF were treated with recombinant human interleukin 1 receptor antagonist (IL-1ra), anti-tumor necrosis factor-α (TNF-α)-neutralizing antibodies, anti-N-cadherin-blocking antibodies, or MG132. ^{3}H-labeled thymidine incorporation was quantified to assess their proliferation. Total RNA and cell lysates were prepared for real-time polymerase chain reaction and Western blot analyses.

Results. Hypoxia exerted no effect on proliferation of RASF cultured at low density. At high density, it abrogated contact-dependent growth inhibition of RASF, but not of human dermal fibroblasts. Addition of anti-TNF-α antibodies or IL-1ra did not affect the results. Upregulated expression of cyclin-dependent kinase inhibitor $p27^{Kip1}$ was observed in the cells cultured at high density under normoxic conditions, but not under hypoxic conditions. Hypoxia decreased N-cadherin expression on RASF. Addition of anti-N-cadherin-blocking antibodies mimicked the effects of hypoxic culture; it promoted proliferation of RASF cultured at high density under normoxic conditions. This antibody treatment also downmodulated $p27^{Kip1}$ expression.

Conclusion. Hypoxia downregulates N-cadherin expression on RASF, and thus prevents $p27^{Kip1}$ upregulation for their contact inhibition. It is likely that hypoxia in rheumatoid synovial tissues contributes to rheumatoid pathology by augmenting proliferation of synovial fibroblasts. (First Release Feb 15 2009; J Rheumatol 2009;36:698–705; doi:10.3899/jrheum.080188)

Key Indexing Terms:
HYPOXIA RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS
CYCLIN-DEPENDENT KINASE INHIBITOR CADHERIN

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by synovitis and subsequent destruction of cartilage and bone. In affected joints, inflammatory cells including lymphocytes and macrophages are recruited and activated to produce many cytokines at high concentrations. Among them, tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) are established targets in treatment of RA, and stimulate RA synovial fibroblasts (RASF) to proliferate and to produce inflammatory mediators[1]. In the normal joint, the synovial lining layer is at most a few cells thick. In the RA inflamed joint, RASF proliferate to form stratified hyperplastic synovial tissues called pannus. When isolated and transferred to *in vitro* culture, they still proliferate as if they were transformed cells, which show no contact-dependent proliferative inhibition (contact inhibition)[1].

Because of the uncontrolled synovial hyperplasia, capillary density becomes insufficient for oxygen demand by synovial cells in the rheumatoid joints[2]. Moreover, synovial fluid retention in affected joints increases the intraarticular pressure, leading to further reduction of blood perfusion[3].

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Thus, rheumatoid synovial tissues support vigorous proliferation of synovial fibroblasts, and are paradoxically hypoxic[3,4]. Lund-Olesen reported that oxygen levels of synovial fluid in patients with RA are reduced to less than half of those in healthy controls; mean pO_2 is 63 mm Hg in the normal joints and 27 mm Hg in the rheumatoid joints[3].

Hypoxia regulates gene expression of various inflammatory mediators and proteinases involved in bone and cartilage destruction[2,5,6], and which are suggested to contribute to rheumatoid inflammation. Effects of hypoxia on cellular proliferation depend on the cell types. Hypoxia promoted proliferation of endothelial cells[7] and fibroblasts[8,9]. It downregulated cyclin-dependent kinase inhibitor (CDKI) $p21^{Cip1}$ protein expression[9]. It also extended the lifespan of vascular smooth-muscle cells by activating telomerase[10]. In contrast, it halted cell-cycle progression by upregulation of CDKI, $p16^{INK4a}$ in the CV-1P monkey kidney cell line[11], and $p21^{Cip1}$ and $p27^{Kip1}$ in murine embryonic fibroblasts[12,13]. Thus, our objective was to determine how hypoxia affects the cell cycle of RASF.

Cell-cycle progression is largely regulated by kinase activity of cyclin/cyclin-dependent kinase complexes[14]. CDKI are intracellular molecules that halt cell-cycle progression via inhibition of cyclin/CDK kinase activities. We reported previously that gene transfer of CDKI inhibited RASF proliferation *in vitro*, and that intraarticular CDKI gene therapy ameliorated animal models of RA[15,16]. Thus, RASF proliferation is crucial in the pathology of RA, and could be a target of treatment for RA. We investigated the effect of hypoxia on RASF proliferation in the context of CDKI expression.

MATERIALS AND METHODS

Cell culture. Synovial tissues were derived from 10 patients with RA who had responded poorly to antirheumatic drugs and underwent joint replacement or synovectomy at Tokyo Medical and Dental University Hospital, Tokyo Metropolitan Bokuto Hospital, National Shimoshizu Hospital, or Shinshu University Hospital. All patients fulfilled the American College of Rheumatology criteria for classification of RA[17]. All gave their consent to procedures in our studies, which were approved by the ethics committees of Tokyo Medical and Dental University and RIEN Research Center for Allergy and Immunology.

RASF were isolated and cultured as described[15]. Adult normal human dermal fibroblasts (HDF) were purchased from Cambrex, East Rutherford, NJ, USA. They were used at early passages (passages 5 to 9). These fibroblasts were cultured under normoxic conditions (21% O_2). Hypoxic conditions (10%, 3%, or 1% O_2) were generated in a hypoxic chamber filled with CO_2 (5%) and N_2 (85%, 92%, or 94%) gas mixture. Oxygen concentration was monitored with an oxygen electrode (Cosmo or Jiko, Tokyo, Japan). Dissolved oxygen levels in culture supernatants were measured with a dissolved oxygen monitor (Central Kagaku, Tokyo, Japan). RASF and HDF were cultured in a microtiter-plate at 5000 cells per well (high density culture) or 2000 cells per well (low density culture). RASF for RNA, total protein, and nuclear extraction were cultured at 3.5×10^5 cells per 60 mm dish (high density culture).

Cell proliferation assay. RASF were incubated for 24–72 h in a hypoxic chamber or under normoxic conditions. Some RASF were treated with 100 ng/ml recombinant human IL-1 receptor antagonist (IL-1ra; Prospec, Rehovot, Israel), 2 μg/ml anti-TNF-α-neutralizing monoclonal antibody (mAb; J2D10, Lab Vision, Fremont, CA, USA), 80 μg/ml anti-N-cadherin-blocking mAb (GC-4; Sigma, St. Louis, MO, USA), control mouse IgG1 mAb (MOPC-31; BD Biosciences, San Diego, CA, USA), and up to 200 μM cobalt chloride ($CoCl_2$; Sigma). During the last 24-h culture, 0.3 μCi of ^{3}H-labeled thymidine was present for quantification of the incorporated radioactivities.

Real-time polymerase chain reaction (PCR). Total RNA was isolated with an RNeasy kit (Qiagen, Valencia, CA, USA) and converted to cDNA with Superscript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase. Real-time PCR was carried out with iQ Syber Green supermix (Bio-Rad, Hercules, CA, USA) and a set of primers specific to N-cadherin[18] or $p27^{Kip1}$ cDNA (sense: GCT CTA GAT TTT TTG AGA GTG CGA GAG AG; antisense: GGG GTA GCC GCT TTT AGA GGC AGA TCA TT). Data were standardized with human 28S ribosomal RNA (sense: TTG AAA ATC CGG GGG AGA G; antisense: ACA TTG TTC CAA CAT GCC AG), and were analyzed with the cycle threshold method[19].

Western blot analyses. RASF were cultured for 24–72 h. Some RASF were treated with dimethyl sulfoxide (DMSO); Sigma), or 2.5 μM carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132; EMD Chemicals, Darmstadt, Germany) during the last 24-h culture. Total protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described[15]. Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Rabbit anti-human $p16^{INK4a}$, $p21^{Cip1}$, CDK4 (sc-468, sc-397, and sc-260; Santa Cruz Biotech, Santa Cruz, CA, USA), mouse anti-E-cadherin, N-cadherin, $p27^{Kip1}$ (HECD-1, clone 32, and clone 57; BD-Biosciences), cadherin-11 (clone 283416; R&D Systems, Minneapolis, MN, USA), and hypoxia inducible factor-1α 67; Gene Tex, San Antonio, TX, USA) antibodies were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit (NA-934; GE Healthcare Biosciences, Piscataway, NJ, USA) or anti-mouse IgG antibodies (6175-05; Southern Biotech, Birmingham, AL, USA) were used as secondary antibodies. Bound antibodies were visualized with ECL (GE Healthcare Biosciences). Signal intensities were quantified with ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

ELISA. ELISA kits for IL-1α, IL-1ß, IL-6, and TNF-α (Biosource International, Camarillo, CA, USA) were employed to quantify protein levels in the culture supernatants.

Statistics. Student's paired t test was used for statistical comparisons.

RESULTS

RASF proliferation accelerated by hypoxia in high density culture. The effect of hypoxia on RASF growth was studied with ^{3}H-thymidine incorporation. RASF were first cultured at low density in a microtiter plate for logarithmic cell growth. They grew equally under normoxic (21% O_2) and hypoxic (1% O_2) conditions. When cultured at high density under normoxic conditions, they stopped growing and incorporated less thymidine. Although more cells were present in the high density culture under normoxic conditions, these cells incorporated an amount of ^{3}H-thymidine comparable only to those in the low density culture. However, under hypoxic conditions, they incorporated more thymidine than those under normoxic conditions (Figure 1A). Thus, hypoxia attenuated growth suppression that was induced by high density culture.

Under normoxic (21% O_2) and hypoxic conditions (1% O_2), the dissolved oxygen concentrations in the culture supernatants of RASF cultured at low density were 8.01 ± 0.06 and 2.15 ± 0.14 mg/dl, and those of RASF cultured at high density were 8.00 ± 0.03 and 2.14 ± 0.07 mg/dl, respec-

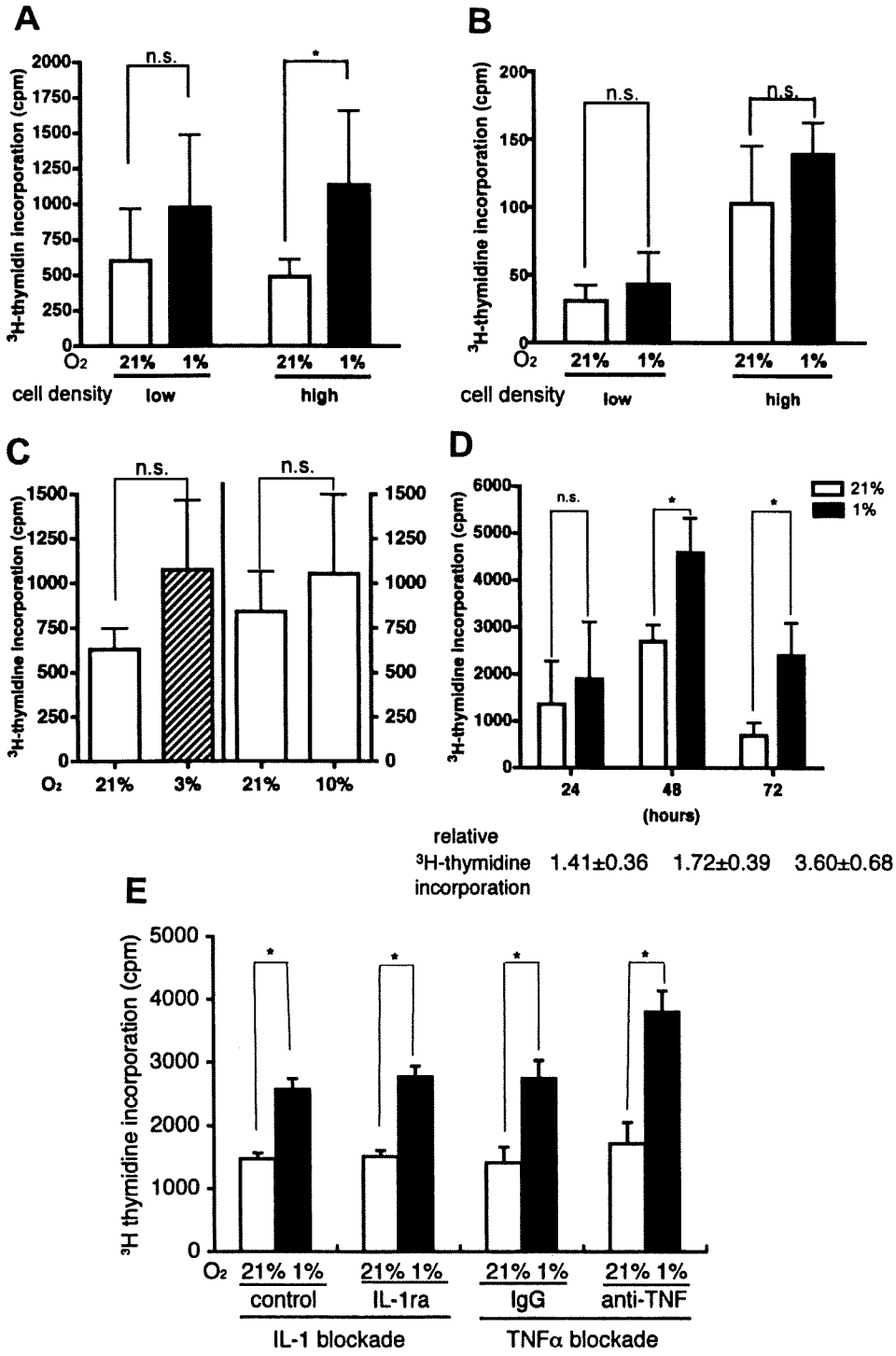

Figure 1. Hypoxia-induced augmentation of fibroblast proliferation. A. RA synovial fibroblasts (RASF) were cultured in microtiter plates at high or low density for 72 h under normoxic (21% O_2) or hypoxic (1% O_2) conditions. RASF proliferation was assessed using ^{3}H-thymidine incorporation in 5 wells per RASF sample, and the mean values were calculated. Columns and bars represent mean and SD values of 5 RASF samples. *$p < 0.05$. B. Effect of hypoxia on proliferation of human dermal fibroblasts was assessed in the same manner. Representative results of 1 of 2 experiments are shown. Columns and bars represent mean and standard deviations of 5 wells. C. The effects of hypoxia on the proliferation of RASF were assessed as in panel A. RASF were cultured in microtiter plates at high density for 72 hours under normoxic (21% O_2) or hypoxic (10% and 3% O_2) conditions. Columns and bars represent mean and SD of 3 RASF samples. D. RASF were cultured at high density for 24, 48, or 72 hours under normoxic (21% O_2) or hypoxic (1% O_2) conditions as described above. The effect of hypoxia on the proliferation of RASF was assessed in the same manner. Columns and bars represent mean and SD of 3 RASF samples. The relative ^{3}H-thymidine incorporations by RASF cultured in hypoxic conditions standardized with cultures in normoxic conditions at the same timepoints are shown at the bottom. *$p < 0.05$. E. The effect of hypoxia on the proliferation of RASF cultured at high density was assessed as in A. RASF were treated with culture medium only (control), 100 ng/ml human recombinant IL-1ra, 2 μg/ml isotype IgG1 control antibody, or 2 μg/ml anti-TNF-α-neutralizing mAb. Representative results of 1 of 2 experiments are shown. Columns and bars represent mean and SD of 5 wells. *$p < 0.05$. cpm: counts per minute; ns: not significant.

tively (mean ± SD of 3 samples). In these experiments, the cell density was found not to affect dissolved oxygen concentrations.

To determine if this was a general feature of fibroblasts, HDF were cultured in the same conditions. The results showed that hypoxia (1% O_2) did not promote proliferation of HDF in high density culture (Figure 1B).

The mean pO_2 is reported to be 63 mm Hg in normal joints and 27 mm Hg in rheumatoid joints[3]. To simulate oxygen supplies in normal and rheumatoid joints, we examined the effects of 10% O_2 (mean O_2 level in normal joints) or 3% O_2 (mean O_2 level in rheumatoid joints) on proliferation of RASF cultured at high density. RASF cultured under 3% O_2 incorporated slightly more ^{3}H-thymidine than fibroblasts under 10% O_2, but the differences were not statistically significant (Figure 1C).

To elucidate the time course of accelerated RASF proliferation by hypoxia, we examined the ^{3}H-thymidine incorporation by RASF cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions at 24 hours, 48 hours and 72 hours. The relative ^{3}H-thymidine incorporation by RASF under hypoxic conditions standardized with those under normoxic conditions at the same timepoints increased gradually with time, and it reached 2.64 ± 0.51 after 72 hours of culture (Figure 1D).

Effects of IL-1 and TNF-α on hypoxia-induced proliferation. When IL-1α, IL-1ß, and TNF-α in the supernatants of RASF culture were quantified with specific ELISA, they were all below detection limits (IL-1α and IL-1ß < 3.9 pg/ml, TNF-α < 1.7 pg/ml). This suggested that hypoxia does not stimulate RASF to produce these cytokines. Even when IL-1ra or anti-TNF-α-blocking mAb were included in the medium, RASF in the high density culture grew more under hypoxic conditions (Figure 1E). In separate experiments, the same concentrations of these blocking reagents suppressed the ^{3}H-thymidine incorporation and matrix metalloproteinase-3 production by RASF stimulated with 10 pg/ml IL-1ß and 10 pg/ml TNF-α (data not shown).

It has been reported that IL-6 suppresses RASF proliferation[20]. The supernatants of RASF cultured at high density under normoxic conditions and those cultured under hypoxic conditions contained comparable levels of IL-6: 4.6 ± 4.9 ng/ml and 6.4 ± 6.5 ng/ml (mean ± SD of 3 samples), respectively. Thus, IL-6 was not responsible for the overgrowth of RASF under the hypoxic conditions.

Downregulation of CDKI P27^{Kip1} expression by hypoxia. In general, logarithmic cell growth of nontransformed cells can be inhibited in high density culture. This contact inhibition involves upregulation of the CDKI p16^{INK4a}, p21^{Cip1}, and/or p27^{Kip1}, depending on the cell types[21-23]. Our previous studies revealed that RASF in confluent culture upregulated expression of these CDKI when they were incubated for more than 4 days[15]. We examined the effect of hypoxia in CDKI expression. When RASF were cultured at high density for 3 days, the protein level of p27^{Kip1} was upregulated. p16^{INK4a} and p21^{Cip1} protein were not upregulated at this timepoint. However, the high density culture did not induce p27^{Kip1} upregulation under hypoxic conditions (Figure 2A, 2B). The protein level of p27^{Kip1} was not upregulated when RASF were cultured at low density for 3 days (data not shown). Quantitative PCR of p27^{Kip1} mRNA transcripts showed that the difference did not depend on alteration of the p27^{Kip1} mRNA expression (Figure 2C). It has been reported that the p27^{Kip1} protein is degraded via the ubiquitin-proteasome pathway. To elucidate whether this pathway is involved in the attenuation of p27^{Kip1} protein expression by hypoxia, a proteasome inhibitor, MG132, was added to the culture medium. MG132 upregulates p27^{Kip1} protein expression through inhibition of protein degradation[24]. MG132 2.5 μM upregulated p27^{Kip1} protein concentrations in RASF cultured at high density under hypoxia (1% O_2; Figure 2D). This suggests that attenuation of p27^{Kip1} by hypoxia is regulated via the ubiquitin-proteasome pathway. These findings were in agreement with the fact that the protein level of p27^{Kip1} was primarily controlled at the post-transcriptional level, via the ubiquitin-proteasome pathway[25]. In contrast, when HDF were cultured at high density, the p27^{Kip1} protein was upregulated even in hypoxic conditions (Figure 2E).

Hypoxia-induced N-cadherin downregulation responsible for accelerated RASF proliferation. Cadherins are a group of cell-surface molecules that recognize direct cell-cell contact and mediate signal pathways into the cells. Homophilic interaction of surface N-cadherin and E-cadherin promotes p27^{Kip1} protein accumulation for contact inhibition[26,27]. Recently, it was reported that cadherin-11 is expressed by RASF and contributes to organization of the lining-like structure of RA synovial tissues[28,29]. RASF grown under normoxic conditions expressed N-cadherin and cadherin-11, but not E-cadherin. When the same cells were cultured under hypoxic conditions, their expression of N-cadherin, but not cadherin-11, was reduced (Figure 3A, 3B). Quantitation of mRNA with real-time PCR revealed that this reduction was regulated at the mRNA level (Figure 3B). These data suggested that hypoxia suppressed N-cadherin expression, resulting in augmentation of RASF proliferation at high density. In support of this, hypoxia did not alter the N-cadherin protein expression in HDF (Figure 3C).

Next, anti-N-cadherin blocking mAb was used to interfere with homophilic interaction of N-cadherins. When RASF were cultured with this mAb at high density under normoxic conditions, they proliferated more than those treated with control IgG (Figure 3D). In analogy to hypoxia, treatment with the blocking mAb downmodulated expression of p27^{Kip1} protein in the cultured RASF (Figure 3E).

DISCUSSION

Hypoxia promoted proliferation of RASF by attenuating

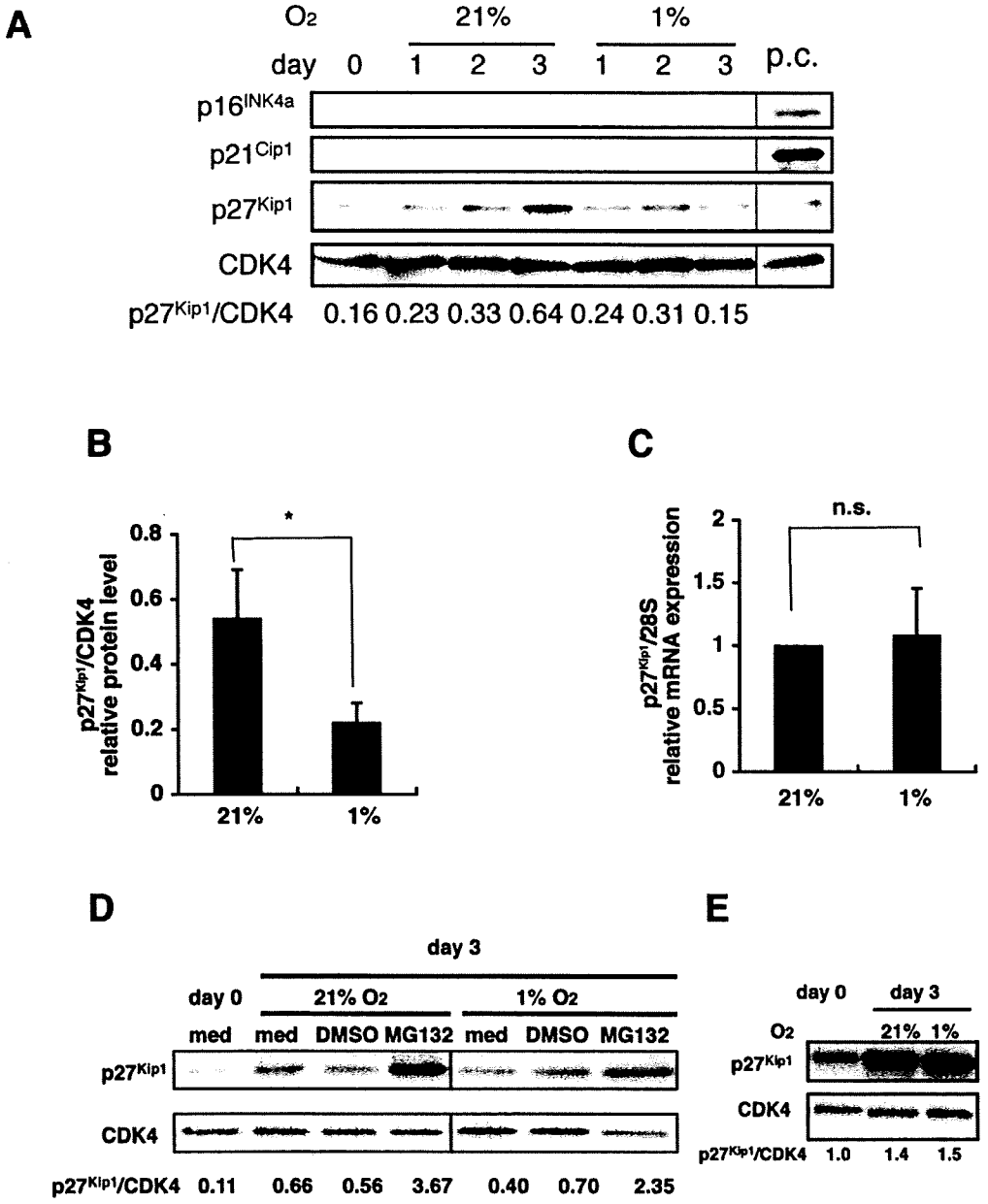

Figure 2. Downregulation of $p27^{Kip1}$ expression by hypoxia. A. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions, and whole-cell lysates were collected after indicated number of days. Expression of CDKI $p16^{INK4a}$, $p21^{Cip1}$, and $p27^{Kip1}$ by RASF was immunodetected by Western blot. CDK4 was stained as loading control. Expression levels of $p27^{Kip1}$ protein standardized with those of the CDK4 protein in the same blot are shown at the bottom. pc: positive control. B. $p27^{Kip1}$ levels cultured under normoxic (21% O_2) and hypoxic (1% O_2) conditions for 3 days were standardized with levels of CDK4. Columns and bars represent mean and SD of 3 samples. *$p < 0.05$. C. RASF were cultured for 3 days at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions before extraction of total RNA. $p27^{Kip1}$ mRNA expression was quantified by real-time PCR using 28S ribosomal RNA as an endogenous control. Columns and bars represent mean and SD of 3 samples. ns: not significant. D. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions, and whole-cell lysates were collected after 3 days. RASF were treated with culture medium alone (med), 0.1% DMSO, or 2.5 μM MG132 during the last 24 hours of culture. $p27^{Kip1}$ expression by RASF was immunodetected by Western blot. Representative results of 2 samples are shown. Expression levels of $p27^{Kip1}$ protein standardized with those of the CDK4 protein in the same blot are shown at the bottom. E. Human dermal fibroblasts were cultured for 3 days at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions before extraction of whole-cell lysates. Expression of $p27^{Kip1}$ and CDK4 was assessed as in A. Representative blots of 2 independent experiments are shown.

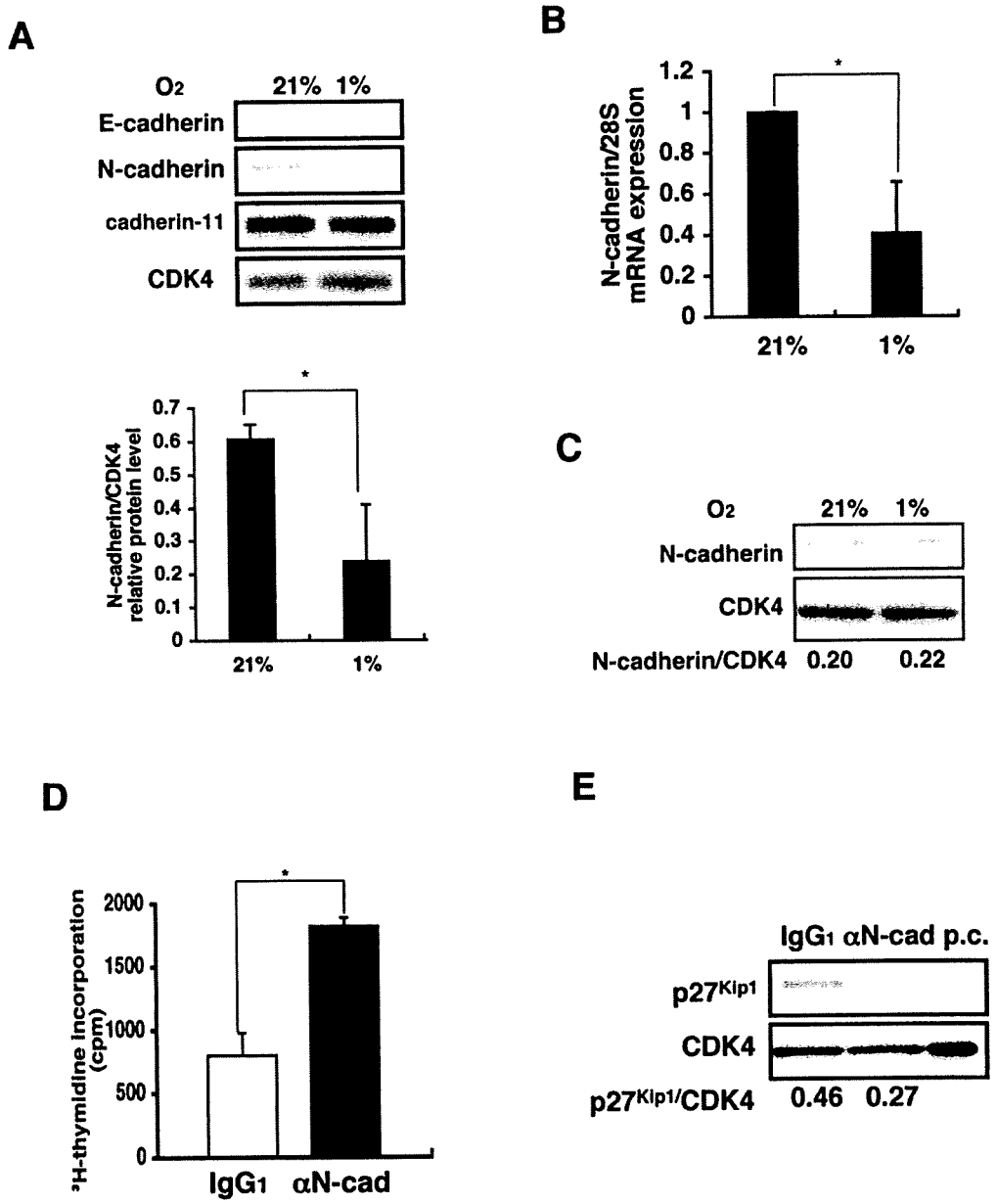

Figure 3. Downregulation of N-cadherin expression by hypoxia. A. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 3 days before collection of whole-cell lysates. N-cadherin protein expression was immunodetected by Western blot. Representative blots of 3 samples are shown in the upper panel. Expression levels of N-cadherin protein were standardized with those of CDK4 protein (lower panel). Columns and bars represent mean and SD of the 3 samples. *$p < 0.05$. B. RASF were cultured as in A before extraction of total RNA. N-cadherin mRNA expression was quantified by real-time PCR using 28S as an endogenous control. Columns and bars represent mean and SD of the 3 samples. *$p < 0.05$. C. Expression of N-cadherin and CDK4 in human dermal fibroblasts cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions was assessed as in A. Representative blots of 2 independent experiments are shown. D. RASF were cultured at high density, and treated with anti-N-cadherin-blocking mAb (αN-cad) or isotype IgG1 control mAb. After 3 days, effect of N-cadherin blockade on RASF proliferation was examined by ^{3}H-thymidine incorporation. Representative results of 3 samples are shown. Columns and bars represent mean and SD of 5 wells. *$p < 0.05$. E. Expression of $p27^{Kip1}$ and CDK4 by RASF treated with αN-cad or IgG1 for 3 days were immunodetected by Western blot. Representative results of 2 independent experiments are shown. pc: positive control

their contact inhibition. The accelerated proliferation was mediated by suppressed expression of N-cadherin, which should otherwise upregulate $p27^{Kip1}$ expression on their homophilic interaction. It has been reported that hypoxia stimulates RASF to produce several inflammatory mediators and proteinases that are involved in the pathology of RA[2,5]. We observed that hypoxia may contribute to RA pathology by promoting proliferation of RASF as well (Figure 4).

Hypoxia did not affect logarithmic-phase proliferation of RASF, but abrogated their contact inhibition by modulating N-cadherin and subsequent $p27^{Kip1}$ expression. The same

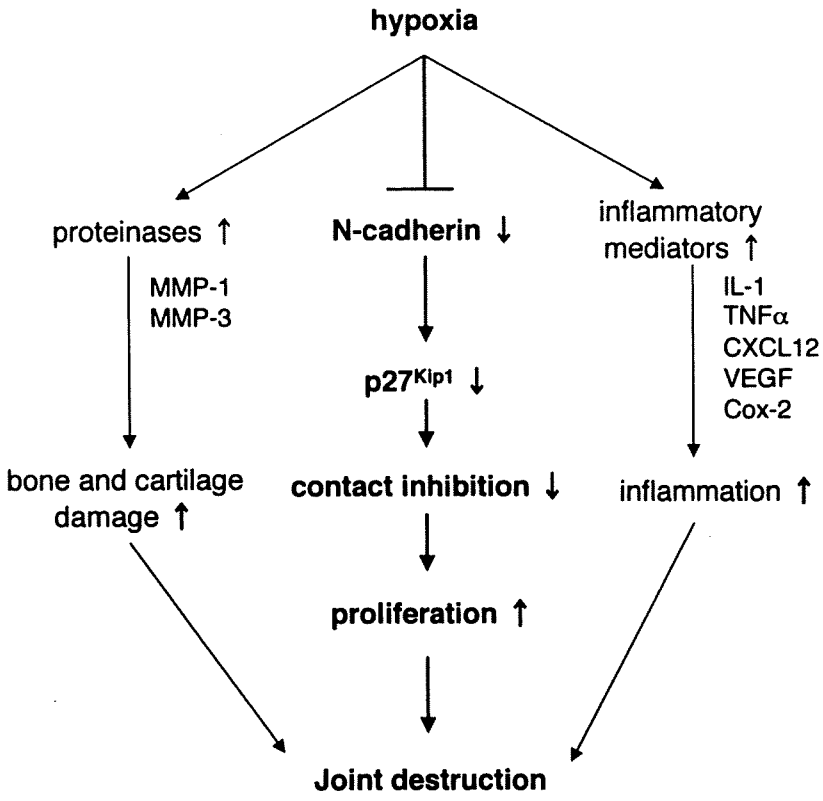

Figure 4. Abrogation of contact-dependent proliferative inhibition of RASF by hypoxia. Hypoxia downregulates N-cadherin and subsequently $p27^{Kip1}$ expression to augment RASF proliferation. It also upregulates inflammatory mediators and proteinases involved in RA. These results suggest that hypoxia in rheumatoid synovial tissues should contribute to joint destruction through multiple pathways. MMP: matrix metalloproteinase; VEGF: vascular endothelial growth factor.

changes were not observed in HDF. Thus, hypoxia-induced augmentation of proliferation via N-cadherin and $p27^{Kip1}$ might be one specific feature of RASF. RASF were found to be prone to express a few types of CDKI in longterm *in vitro* cultures[14]. Although the short-term hypoxic culture in our experiments disclosed only its effect on $p27^{Kip1}$, we assume that expression of other CDKI might be affected by hypoxia.

Our results showed that RASF proliferation was augmented in the presence of 1% O_2, but not in the presence of 3% O_2. Treuhaft, *et al* reported that the knees of 30% of patients with RA exhibited profound hypoxia, with pO_2 less than 10 mm Hg, and the lowest pO_2 in a rheumatoid joint was 8 mm Hg[4]. Thus, 1% O_2 corresponds to the lowest levels in rheumatoid joints. Further, it has been reported that movement of inflamed joints can reduce the blood flow in synovial capillaries, and thus further aggravate synovial hypoxia[30]. Hypoxia may augment RASF proliferation in such severely hypoxic rheumatoid joints, especially during exercise.

The time-course experiments revealed that the relative ^{3}H-thymidine incorporation by RASF was increased gradually with time, and peaked after 72 hours of culture. Further, hypoxia downregulated the expression of $p27^{Kip1}$ and N-cadherin to a statistically significant level. However, the absolute level of ^{3}H-thymidine incorporation by RASF peaked after 48 hours, and decreased thereafter, i.e., at 72 hours, even under hypoxic conditions. These data suggest that hypoxia-induced augmentation of RASF proliferation does not completely overcome contact inhibition. After culture for 48 hours, hypoxia augmented RASF proliferation at a statistically significant level, and downregulated the protein expression of $p27^{Kip1}$, but the latter effect was not significant. Thus, some mechanism other than the attenuation of $p27^{Kip1}$ protein expression may be involved in the hypoxia-induced augmentation of RASF proliferation.

It is known that quite a few cellular responses to hypoxia are mediated by hypoxia inducible factor-1 (HIF-1)[31]. Its expression was upregulated in the rheumatoid synovial tissues, reflecting the hypoxic environment of the affected joints[2]. In a separate experiment, HIF-1 in cultured RASF was upregulated artificially with $CoCl_2$ [2]. However, this treatment did not affect the RASF proliferation or the protein level of $p27^{Kip1}$ (data not shown), suggesting that an HIF-1-independent pathway should operate in the hypoxia-triggered augmentation of RASF proliferation.

Cadherins maintain the integrity of multicellular structures. One of the intracellular outcomes of cadherin activation is induction of CDKI, including $p21^{Cip1}$ and $p27^{Kip1}$, that inhibit cell-cycle progression. E-cadherin interaction mediates contact-dependent proliferative inhibition by

increasing the level of $p27^{Kip1}$ expression in thyroid cancer cells[32], while N-cadherin interaction induces contact inhibition through upregulation of $p27^{Kip1}$ in CHO cells[26]. These data point to a link between cadherin signaling and CDKI protein expression. In addition, hypoxia suppresses E-cadherin expression on cancer cell lines[33] and N-cadherin expression in cortex proximal renal tubules[34]. Thus, there is another link between hypoxia and cadherins.

RASF expressed N-cadherins, but not E-cadherins. Recently, it was reported that cadherin-11 is expressed by RASF and contributes to organization of the lining-like structure of synovial tissues[28,29]. A cadherin-11–Fc fusion protein and an anti-cadherin–11-blocking mAb prevented and reduced arthritis in mouse models[35]. Thus, cadherins, which should control proliferative reactions to hypoxia, appear to be crucial molecules for formation and maintenance of the rheumatoid synovial structure.

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Association study of *TRAF1-C5* polymorphisms with susceptibility to rheumatoid arthritis and systemic lupus erythematosus in Japanese

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Association study of *TRAF1-C5* polymorphisms with susceptibility to rheumatoid arthritis and systemic lupus erythematosus in Japanese

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▶ Additional data (supplementary methods and supplementary table 1) are published online only at http://ard.bmj.com/content/vol69/issue2

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ABSTRACT

Objective: The primary aim of this study was to investigate the association of polymorphisms of *TRAF1-C5*, a newly identified rheumatoid arthritis (RA) risk locus in Caucasians, with susceptibility to RA and systemic lupus erythematosus (SLE) in Japanese populations. Gene expression levels of *TRAF1* and *C5* to assess the functional significance of genotypes were also analysed.

Methods: A multicentre association study consisting of 4 RA case-control series (4397 cases and 2857 controls) and 3 SLE case-control series (591 cases and 2199 shared controls) was conducted. Genotyping was performed using TaqMan genotyping assay for two single nucleotide polymorphisms (SNPs) that showed the best evidence of association in the previous Caucasian studies. Quantifications of *TRAF1* and *C5* expression were performed with TaqMan expression assay.

Results: Significant differences in allele frequency for both SNPs were observed between RA and control subjects (combined odds ratio = 1.09), while no significant difference was detected between patients with SLE and controls. Interestingly, alleles rs3761847 A and rs10818488 G had increased the risk for RA in the present study, while they decreased the risk in the original studies. A significant difference was found between risk allele carriers and non-carriers of rs10818488 for the expression level of *TRAF1* in phorbol myristate acetate-stimulated lymphoblastoid cell lines ($p = 0.04$).

Conclusion: Association of *TRAF1-C5* locus with RA susceptibility was detected in the Japanese populations with modest magnitude, while no significant association was observed for SLE. Significant positive effect of genotype on the expression of *TRAF1* might support the genetic association between *TRAF1* and RA.

Recent advances in the technology of dense genotyping chips and increasing coverage of polymorphisms across the whole human genome has made it possible to perform genome-wide association studies (GWASs). The GWAS is now a popular and powerful tool to detect novel genetic variations related to common complex diseases, and numerous disease-associated loci have been documented.

One such disease is rheumatoid arthritis (RA), in which Plenge *et al* recently identified the *TRAF1-C5* locus on 9q33–34 as a novel genetic risk for anti-cyclic citrullinated peptide (anti-CCP) antibody-positive RA in European descent populations.[1] Simultaneously, Kurreeman *et al* reported the association by a candidate gene approach in Caucasian RA case-control populations.[2] In addition to these two studies, other studies validated the association of genetic polymorphisms on the *TRAF1-C5* locus with RA in Caucasian populations.[3–6] These results strongly support the theory that *TRAF1-C5* is one of the RA risk loci in populations of European ancestry.

The *TRAF1* gene encodes tumour necrosis factor (TNF) receptor associated factor 1, which is considered to be associated with multiple TNF receptor family members and regulates cytokine signalling networks including TNFα by binding several protein kinases and adaptor proteins.[7] C5 is a pivotal member of the complement system, and unregulated complement activation is likely to play a crucial role in the pathogenesis of several autoimmune diseases including RA.[8 9] Thus, *TRAF1* and *C5*, which lie adjacent to one another on 9q33–34, are considered to together play an important role in the pathogenesis of autoimmune diseases.

Replicating previously reported genetic associations in other populations is essential to establish the association as well as to reveal the magnitude of the genetic risk in each population, because genetic risks may differ among different populations and especially among various ethnicities. One of the successful examples of replication is the association between *HLA-DRB1* and susceptibility to RA that has been replicated repeatedly among Caucasian, Asian and other ethnic populations.[10–12] *HLA-DRB1* is now considered as the strongest genetic factor for RA in most ethnic populations. *STAT4*, which was recently identified as an RA risk gene in European ancestry,[13] was also associated by successful replication studies in Korean and Japanese populations.[14 15] However, many other replication studies across different ethnic populations targeted at genetic factors have failed to replicate the previous associations found in the original reported population. For example, a missense single nucleotide polymorphism (SNP) on *PTPN22*, R620W, which is a strong susceptibility polymorphism for RA in populations with European ancestry, is not polymorphic in Asian populations.[16] By contrast, *PADI4*, a frequently reported RA-associated gene in Asian populations, is seldom replicated in Caucasian populations.[17]

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One of the aims of the present study was to define the role of *TRAF1-C5* locus in Japanese patients with RA. For this, we conducted a multicentre case-control study involving over 7000 samples to increase the statistical power. We also tested the association between *TRAF1-C5* locus and systemic lupus erythematosus (SLE), because some RA susceptible genes have previously been reported to be associated with other autoimmune diseases including SLE.[15] The sample sizes of RA and SLE used in the present association study are the largest among those that have been conducted in Japan thus far.

Although *TRAF1* and *C5* are both considered to be reasonable candidate genes that might play some role in the pathogenesis of autoimmune diseases, functional evidence of the polymorphisms' effect on disease susceptibility remains to be found. Investigating the genotypic effect on gene expression levels of *TRAF1* and *C5* is another aim of this study.

MATERIALS AND METHODS

Subjects for association study and disease criteria

All patients with RA fulfilled the American College of Rheumatology (ACR) 1987 revised criteria for RA,[18] and the diagnosis of SLE was established using the ACR 1982 revised criteria for SLE.[19] Written informed consent was obtained from all subjects and the study was approved by the local ethics committee of each institute.

To evaluate the association of the *TRAF1-C5* locus with RA, we utilised subjects from four unrelated case-control studies from three institutions in Japan: the Institute of Rheumatology Rheumatoid Arthritis cohort (IORRA) series,[20] the RIKEN series,[21] the RIKEN/BioBank series,[14] and the Tokushima series.[22] The details of each case-control series are summarised in table 1. In total, 4397 patients with RA and 2857 controls were enrolled in this study. IORRA DNA collection was also used to analyse the effect of the polymorphism on radiographic severity in Japanese patients with RA. Anteroposterior radiographs of the hands at 5-year disease duration were available for 625 patients from the IORRA cohort. Radiographic severity was assessed using the Sharp/van der Heijde score (SHS) by a single experienced reader.[23]

We used three SLE case-control studies to evaluate the association of the *TRAF1-C5* locus with SLE: the Tokyo Women's Medical University (TWMU) series, the RIKEN series and the Tokushima/Fukuoka series.[14] The details of each case-control series are summarised in table 2. In total, 591 case subjects and 2199 shared control subjects were enrolled in this analysis.

All case-control studies were matched for ethnicity.

Selection and genotyping of SNPs

Two intergenic polymorphisms on *TRAF1-C5* locus (rs3761847 and rs10818488) from the studies by Plenge *et al* and Kurreeman *et al* were selected because they showed the most significant association in each study, respectively.[1 2] Genotyping of the selected SNPs were performed by 5′ nuclease allelic discrimination assay according to the manufacturer's instructions (Applied Biosystems, Tokyo, Japan) as described elsewhere.[14] Duplicate samples and negative controls were included to ensure accuracy of genotyping.

Quantification of gene expression

Reagents and detailed methods for quantifying gene expression in lymphoblastoid cell lines (n = 40) are presented in the Supplementary material.

Statistical analysis

The χ^2 test was performed to compare allelic frequencies of each SNP between cases and controls and for stratified analyses on autoantibody status in patients with RA. The χ^2 test was also used to test the Hardy–Weinberg equilibrium. To assess the feasibility of combining the results of studies, genotype heterogeneity among studies was assessed using Breslow–Day test. The Mantel–Haenszel procedure was performed to provide a common odds ratio when the heterogeneity was denied. Differences in medians of SHS (hands) or the gene expression level ($\log_{10}$-transformed expression level) between risk allele carriers of rs10818488 and risk allele non-carriers were tested by the Mann–Whitney test. The expectation maximisation algorithm implemented in the PENHAPLO program was used for the linkage disequilibrium (LD) analysis.[24] These analyses were performed using the R software package (http://www.r-project.org/).

RESULTS

The overall genotyping success rate was 99% and the genotype concordance rate was 100%, as assessed by duplicate samples. The rs3761847 and rs10818488 SNPs were both in Hardy–Weinberg equilibrium within each case-control series ($p>0.05$), and the non-heterogeneity of allelic distributions was confirmed among all case-control series of RA or SLE ($p>0.1$ by Breslow–Day test). Both SNPs were in tight LD in IORRA controls ($r^2 = 0.96$).

Association of *TRAF1-C5* polymorphisms with RA

Significant association of both SNPs with RA susceptibility in allele frequency (odds ratio (OR) 1.09, 95% CI 1.02 to 1.17) were revealed by combining all 4 case-control studies, comprising 4397 RA subjects and 2857 control subjects (table 3). Interestingly, the alleles reported to be risk alleles for RA in the original studies (alleles rs3761847 G and rs10818488 A) had decreased risk in the Japanese populations.

Table 1 Case-control series for rheumatoid arthritis

	Patients					Controls		
Series	Number of samples	Mean age, years	Female (%)	RF positive (%)	Anti-CCP positive (%)	Number of samples	Mean age, years	Female (%)
IORRA[20]	1504	59.3	83.8	88	87	752	36.4	50.0
RIKEN[21]	830	64.3	83.7	75	NA	658	49.5	52.0
RIKEN/BioBank[14]	1113	60.4	82.0	70	84	940	52.6	25.0
Tokushima[22]	950	61.8	79.1	NA	NA	507	39.8	74.6
Total	4397					2857		

CCP, cyclic citrullinated peptide; IORRA, Institute of Rheumatology Rheumatoid Arthritis; NA, not available; RF, rheumatoid factor.