with each other, and subsequent in situ hybridization analysis demonstrated that their mRNAs were ubiquitously and abundantly expressed in the intestinal epithelium, including in M cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20070607/DC1), we hypothesized that NKM 16–2-4 possesses specificity for the M cell–specific carbohydrate moiety containing α(1,2) fucose, as the precipitated antigens were commonly recognized by UEA-1 (Fig. 4 B).

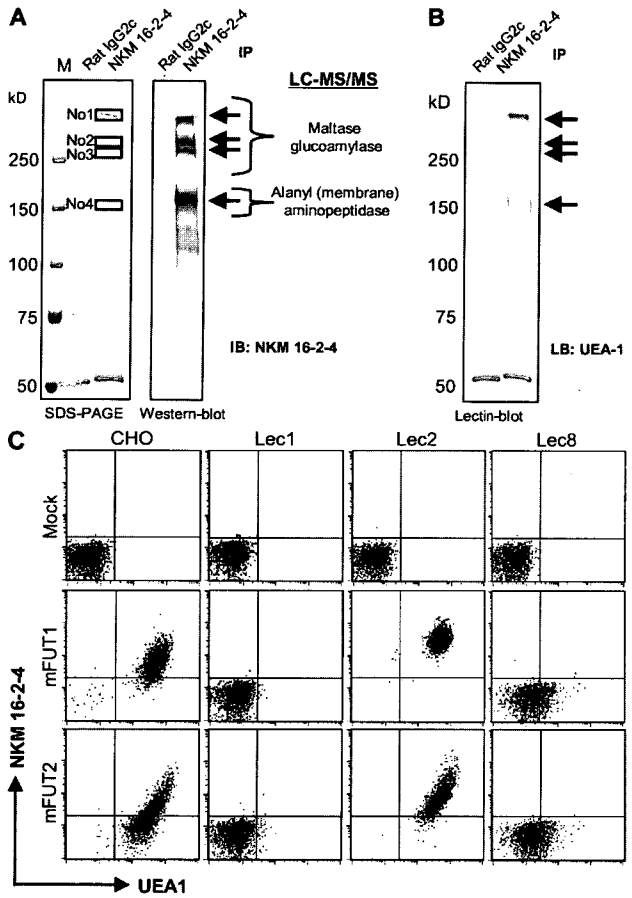

Figure 4. Identification of the antigen recognized by NKM 16–2-4. (A) Immunoprecipitation and Western blot analysis with NKM 16–2-4 were performed with an M cell lysate. 4 major bands (3 bands >250 kD and 1 band of ~150 kD) were precipitated. A subsequent LC-MS/MS analysis identified the three top bands as maltase glucoamylase and the bottom band as alanyl (membrane) aminopeptidase. (B) Lectin blot analysis performed after immunoprecipitation with NKM 16–2-4 showed that the precipitated antigens were all recognized by UEA-1. (C) mFUT1 and mFUT2 genes were transfected into original CHO cells and CHO-derived mutant lines (Lec1, Lec2, and Lec8 cells) with a pIRES2-EGFP expression system, and the specificity of NKM 16–2-4 and UEA-1 for EGFP-expressing transfectants was analyzed. NKM 16–2-4 and UEA-1 specifically reacted with mFUT1- or mFUT2-expressing original CHO cells. The reactivity of NKM 16–2-4 but not UEA-1 to mFUT1- or mFUT2-expressing Lec2 cells was enhanced compared with that to mFUT1- or mFUT2-expressing CHO cells. On the other hand, mFUT1- or mFUT2-expressing Lec1 or Lec8 cells were not recognized at all by NKM 16–2-4.

On the basis of our hypothesis, we transfected Chinese hamster ovary (CHO) cells with the genes encoding murine fucosyl transferase 1 (mFUT1) or mFUT2, which have been identified as α(1,2) fucose transfer enzymes (23). A flow cytometric analysis revealed that both NKM 16–2-4 and UEA-1 specifically reacted with CHO cells expressing mFUT1 or mFUT2, but neither NKM 16–2-4 nor UEA-1 showed specificity for original or empty vector–transfected CHO cells (Fig. 4 C). In addition, a blocking analysis showed that pretreatment of NKM 16–2-4 with α-L-fucose did not completely abolish reactivity to mFUT1- or mFUT2-expressing transfectants, although UEA-1 reactivity to these transfectants was dramatically decreased (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20070607/DC1), indicating that the epitope recognized by NKM 16–2-4 is an mFUT1- or mFUT2-mediated carbohydrate complex containing α(1,2) fucose that is different from the UEA-1–reactive portion of α(1,2) fucose.

Because immunohistochemical analysis demonstrated that UEA-1, but not NKM 16–2-4, recognized goblet cells in the intestinal villi (Fig. 1 A), we turned to examining the differences in recognition patterns between NKM 16–2-4 and UEA-1 by using a mutant line of CHO cells to elucidate the importance of the glycosylation of M cells and goblet cells in the mucosal immune system. When the mFUT1 or mFUT2 gene was introduced into a mutant line of CHO cells (Lec2) with an inactivated CMP-sialic acid transporter (24), the reactivity of NKM 16–2-4, but not of UEA-1, was higher in these transfectants than in the mFUT1- or mFUT2-expressing original CHO cells; however, mFUT1- or mFUT2-expressing Lec1 cells with inactivated GlcNAc transferase I (i.e., a lack of *N*-glycans) (25) or Lec8 cells with inactivated UDP-galactose transporter (26) were not recognized at all by NKM 16–2-4. On the other hand, we observed very low reactivity of UEA-1 to mFUT1- or mFUT2-expressing Lec8 cells, although mFUT1- or mFUT2-expressing Lec1 cells were not recognized by UEA-1. This is because UEA-1 might recognize α(1,2) fucose, which is linked to very low levels of galactose on *N* glycans in mFUT1- or mFUT2-expressing Lec8 cells because it has been reported that Lec8 cells retain 10–20% of their galactosylation (26), and no information is currently available on whether α(1,2) fucose links to anything other than galactose. These data suggest that sialic acid might be useful in distinguishing the reactivity of NKM 16–2-4, but not UEA-1 to galactose-binding α(1,2) fucose on *N*-glycans, although the reactivity to α(1,2) fucose regulated by *O*-glycans remains unclear. Thus, our initial immunohistochemical analyses demonstrated that the specificity of NKM 16–2-4 to UEA-1–positive M cells, but not UEA-1–positive goblet cells, is attributable to the existence of abundant sialic acids neighboring the α(1,2) fucose–containing carbohydrate moiety on goblet cells, but not on M cells. With the exception of their expression patterns at the tissue level, there is currently little reliable information available on the glycobiological and molecular biological differences between mFUT1 and mFUT2 as α(1,2) fucosyltransferases (23). Therefore, further studies, especially in terms of in situ expression patterns at a cellular level at inductive

sites, such as in PPs, are needed to elucidate the role of the carbohydrate moiety containing α(1,2) fucose in the mucosal immune system.

In summary, we established a novel M cell–specific mAb (NKM 16–2-4; rat IgG2c) that selectively recognizes M cells, but not goblet cells or epithelial cells, and we characterized the M cell–specific carbohydrate moiety containing α(1,2) fucose. Our strategy for M cell–targeted vaccination with NKM 16–2-4 is attractive for the development of mucosal vaccines.

MATERIALS AND METHODS

Animals. Female BALB/c mice, Crlj: CD1-*Foxn1*^{nu} mice, and SD rats between 6 and 8 wk old were obtained from CREA and Charles River Laboratories. All of them were maintained in the experimental animal facility at the Institute of Medical Science, the University of Tokyo, and experiments were performed according to the guidelines provided by the Animal Care and Use Committee of the University of Tokyo.

Establishment of an M cell–specific mAb. The M cell–enriched fraction was prepared from murine PPs as previously described, with some modification, by using UEA-1 (4). In brief, cells isolated from murine PPs were fixed in 4% paraformaldehyde (Wako) and stained with 500 ng/ml PE-conjugated UEA-1 (Biogenesis). UEA-1–positive cells were sorted by a FACSAria cell sorter (Becton Dickinson) and injected into the footpads of SD rats (10^6 cells/rat) 4 times at 2-wk intervals, with TiterMax Gold (TiterMax) as an adjuvant. 4 d after the final immunization, lymphocytes isolated from the spleen and inguinal lymph nodes of the immunized rats were fused with P3X63-AG8.653 myeloma cells (CRL-1580; American Type Culture Collection) in the presence of 50% (wt/vol) polyethylene glycol 1500 (Roche). Established hybridomas were injected into Crlj: CD1-*Foxn1*^{nu} mice, and mAbs were then purified from ascitic fluids by using protein G–Sepharose (GE Healthcare) and labeled with EZ-Link Sulfo-NHS-LC-biotin (Thermo Fisher Scientific), FITC (Sigma-Aldrich), or Alexa Fluor 647 (Invitrogen).

Immunohistochemical analysis. One monoclonal antibody (NKM 16–2-4; rat IgG2c) was selected on the basis of the initial screening and its specificity to M cells determined by immunohistochemical and whole-mount staining analyses, as described previously, with some modification (4). In brief, after a blocking step with 1% BSA, 7-μm fixed frozen sections or fixed tissues containing PPs were stained with 5 μg/ml FITC-conjugated NKM 16–2-4 or FITC-conjugated isotype control (FITC-conjugated rat IgG2c; MBL International) and 1 μg/ml tetrarhodamine isothiocyanate–conjugated UEA-1 (Vector Laboratories). The sections were then counterstained with 400 ng/ml DAPI (Sigma-Aldrich) for histochemical analysis and analyzed under a confocal laser-scanning microscope (TCS SP2; Leica). For electron-microscopic analysis, ultrathin sections (100 nm) were incubated with 1 μg/ml purified NKM 16–2-4 after blocking with 1% BSA, followed by 18-nm gold particle–conjugated goat anti–rat IgG (Jackson Immunoresearch Laboratories) diluted 1:10. Finally, the sections were stained with 4% uranyl acetate and analyzed under a transmission electron microscope (JEM100S; JEOL).

Uptake of NKM 16–2-4 by M cells. After the mice were anesthetized with 2 mg ketamine (Sigma-Aldrich), we injected 100 μg of FITC-conjugated NKM 16–2-4 or FITC-conjugated control rat IgG (Sigma-Aldrich) into intestinal loops containing PPs, in accordance with our previous study (4). The mice were killed 10 or 30 min, or 4 h, after the inoculation, and frozen sections (7 μm) of intestinal loop were prepared and analyzed under a confocal laser-scanning microscope after counterstaining with DAPI.

M cell–targeted vaccination. TT (provided by the Research Foundation for Microbial Diseases, Osaka University, Osaka, Japan) and type A BT (prepared according to a previous study; reference [27]]) were first treated with EZ-Link Sulfo-NHS-LC-biotin. Next, biotinylated TT or BT at 1 mg/ml was incubated with the same volume of avidin (1 mg/ml; Sigma-Aldrich). The complexes were then incubated with twice the volume of biotinylated NKM 16–2-4, biotinylated control rat IgG (Sigma-Aldrich), or biotinylated UEA-1 (Vector Laboratories; each 1 mg/ml). Mice were orally immunized with the complexes (in total, each 200 μg contained 50 μg TT or BT per mouse), noncoupled TT (50 or 500 μg per mouse), or PBS alone 3 times (once a week), together with 10 μg CT (List Biological Laboratories) as a mucosal adjuvant. 7 d after the final immunization, serum and fecal extracts were collected and analyzed for TT- or type A botulinum toxin–specific serum IgG and fecal IgA responses by ELISA, as previously described (5, 27). To examine the protective immunity, the mice were challenged via the i.p. route with 200 ng type A botulinum toxin (10,000× LD_{50} i.p.) diluted in 100 μl of 0.2% gelatin/PBS (27). To confirm the universality of M cell–targeted mucosal vaccine with NKM 16–2-4, OVA (Sigma-Aldrich) was conjugated with NKM 16–2-4 or control rat IgG and orally immunized together with 10 μg CT. In addition, intestinal loop assay was performed by using M cell–targeted OVA composed of Alexa Fluor 647–conjugated OVA (Invitrogen), FITC-conjugated avidin (Sigma-Aldrich), and NKM 16–2-4 or control rat IgG. Conjugation of NKM 16–2-4 or control rat IgG and the protein antigen was confirmed by sandwich ELISA (unpublished data).

Analysis of antigen recognized by NKM 16–2-4. To identify the antigen recognized by NKM 16–2-4, we performed an immunoprecipitation assay with NKM 16–2-4 followed by an LC-MS/MS analysis. In brief, a lysate of M cells was incubated with 10 μg/ml NKM 16–2-4 or an isotype control antibody (rat IgG2c; BD Biosciences) followed by protein G–Sepharose (GE Healthcare). Immune complexes were analyzed by SDS-PAGE and Western or lectin blot with 5 μg/ml biotinylated NKM 16–2-4, 5 μg/ml biotinylated isotype control antibody (biotin-conjugated rat IgG2c; BD Biosciences), or 5 μg/ml biotinylated UEA-1 (Vector Laboratories) and ABC–AP complex (Vector Laboratories). To identify the precipitated antigen, LC-MS/MS analysis was performed after digestion with 50 nM trypsin gold (Promega).

Transfection of cells. mFUT1 and mFUT2 genes were synthesized from mRNAs from intestinal tissue, including PPs, using specific primers (mFUT1: sense, 5′-TACTAAGCTAGCATGTGGACTCCCAGCCGGAGGCAG-3′, antisense, 5′-GCTAGCGGATCCTCAGACCAATCTAAAAAGACTGTC-3′; mFUT2: sense, 5′-ATCTAAGCTAGCATGGCGAGTGCCCAGGTAC-CTTTC-3′, antisense, 5′-TGCAGCGAATTCTTAGTGCTTAAGGAGT-GGGGACAG-3′; NheI and BamHI [mFUT1] and NheI and EcoRI [mFUT2] restriction enzyme sites are shown by underlining) by RT-PCR and inserted into pIRES2-EGFP vector (BD Biosciences). These plasmids were then transformed into CHO-K1 cells (CCL-61; American Type Culture Collection) and three CHO-cell–derived mutant lines (Lec1, CRL-1735 [reference 25]; Lec2, CRL-1736 [reference 24]; and Lec8, CRL-1737 [reference 26]). 2 d after transfection, the cells were stained with 500 ng/ml Alexa Fluor 647–conjugated NKM 16–2-4 and 500 ng/ml PE-conjugated UEA-1, followed by the application of 10 μl/test VIA-PROVE (BD Biosciences). They were then analyzed by flow cytometry with FACSCalibur (Becton Dickinson). For blocking analysis, 500 ng/ml Alexa Fluor 647–conjugated NKM 16–2-4 or 500 ng/ml PE-conjugated UEA-1 was first pretreated with 0.5 M α-L-fucose (Wako).

Data analysis. Data are expressed as the mean ± the SD. All analyses for statistically significant differences were performed by Tukey's *t* test, with $P < 0.01$ considered significant (denoted in the figures with an asterisk).

Online supplemental material. Fig. S1 shows the specificity of NKM 16–2-4 to isolated UEA-1–positive M cells. Fig. S2 shows that NKM 16–2-4 specifically reacts with M cells in NALT, similar to its reaction with PP-associated M cells. Fig. S3 shows the expression of maltase glucoamylase and alanyl aminopeptidase mRNAs in PPs. Fig. S4 shows that NKM 16–2-4 reacts with different form of UEA-1–reactive portion of α(1,2) fucose. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20070607/DC1.

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IL-17B and IL-17C Are Associated with TNF-α Production and Contribute to the Exacerbation of Inflammatory Arthritis[1]

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IL-17A is a T cell-derived proinflammatory cytokine that contributes to the pathogenesis of rheumatoid arthritis. Recently, six related molecules have been identified to form the IL-17 family, as follows: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. Whereas IL-17A and IL-17F up-regulate IL-6 in synovial fibroblasts, IL-17B and IL-17C are reported to stimulate the release of TNF-α and IL-1β from the monocytic cell line, THP-1 cell. However, their detailed function remains to be elucidated. We report in this study the effects of IL-17 family on the collagen-induced arthritis (CIA) progression by T cell gene transfer and bone marrow chimeric mice. The mRNA expressions of IL-17 family (IL-17A, IL-17B, IL-17C, and IL-17F) and their receptor (IL-17R and IL-17Rh1) genes in the arthritic paws of CIA mice were elevated compared with controls. Although IL-17A and IL-17F were expressed in CD4$^+$ T cells, IL-17B and IL-17C were expressed in the cartilage and in various cell populations in the CIA arthritic paws, respectively. In vitro, IL-17A, IL-17B, IL-17C, and IL-17F induced TNF-α production in mouse peritoneal exudate cells. In vivo, adoptive transfer of IL-17B- and IL-17C-transduced CD4$^+$ T cells evidently exacerbated arthritis. Bone marrow chimeric mice of IL-17B and IL-17C exhibited elevated serum TNF-α concentration and the high arthritis score upon CIA induction. Moreover, neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in CIA mice. Therefore, not only IL-17A, but also IL-17B and IL-17C play an important role in the pathogenesis of inflammatory arthritis. *The Journal of Immunology,* **2007, 179: 7128–7136.**

Interleukin-17A is a T cell-derived proinflammatory cytokine that is involved in the development of rheumatoid arthritis (RA).[3] IL-17A was originally named CTLA-8 after being cloned from activated T cells, and shares 57% homology to the protein encoded by the open reading frame 13 gene of the T lymphotropic herpesvirus saimiri (1). IL-17A is present at significant levels in the synovium and synovial fluid of patients with RA (2, 3). IL-17A is a potent inducer of various cytokines such as IL-1, TNF-α, and IL-6. T cell IL-17A stimulates the production of IL-1 and TNF-α from human PBMC-derived macrophages in vitro (4). IL-17A also enhances IL-1-mediated IL-6 production by RA synoviocytes in vitro as well as TNF-α-induced synthesis of IL-1, IL-6, and IL-8 (5, 6). These results indicate that IL-17A synergizes with IL-1 and TNF-α and contributes to inflammation of RA.

In in vivo studies, systemic as well as local overexpression of IL-17A in collagen-induced arthritis (CIA) has been shown to accelerate the onset of CIA and to aggravate the joint pathology (7). Moreover, treatment with anti-IL-17A Abs after the onset of CIA reduces the joint inflammation and histologic destruction of cartilage (8). IL-17A deficiency protects IL-1R antagonist-deficient mice from spontaneous development of destructive arthritis (9). Therefore, IL-17A plays a crucial role in the pathogenesis of arthritis through synergistic effects with IL-1 and TNF-α. However, IL-17A can directly induce joint destruction in an IL-1-independent manner and can bypass TNF-dependent arthritis (7, 10). This suggests that there is an IL-17A-dependent pathway to the destructive arthritis and anti-IL-17A cytokine therapy is an additional new antirheumatic strategy for RA besides anti-TNF/anti-IL-1 therapy.

Recently, the IL-17 family was determined to consist of six related molecules, as follows: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. These molecules have a molecular mass of 20–30 kDa and consist of 163–202 aa that bear 20–50% homology to IL-17A, especially within the C-terminal region. They share four conserved cysteine residues that may participate in the formation of intermolecular disulfide linkages (11, 12). The different IL-17 family members seem to have very distinct expression patterns, suggesting distinct biological roles.

Interestingly, IL-17F has the highest homology with IL-17A and is also expressed by activated T cells in response to IL-23 stimulation (13–15). However, the precise effect of IL-17F on arthritis has not been clarified. In contrast to the restricted expression of IL-17A and IL-17F, IL-17B mRNA can be detected in a wide range of tissues, including the spinal cord, testis, stomach, small intestine, pancreas, prostate, and ovary (16, 17). It has been recently reported that IL-17B is highly expressed in chondrocytes

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; BCII, bovine type II collagen; BM, bone marrow; CIA, collagen-induced arthritis; mIL, murine IL; MMP, matrix metalloproteinase; PEC, peritoneal exudate cell; pMIG, murine stem cell virus/internal ribosome entry site/GFP.

that are located at the mid and deep zones of normal bovine articular cartilage (11). In contrast, IL-17C expression has been confined only to rare expression sequence tags in adult prostate and fetal kidney libraries (17). However, the cell sources of IL-17B and IL-17C have not been identified in the development of inflammatory arthritis.

A common feature of IL-17 family members is the induction of neutrophil migration. IL-17A and IL-17F both mobilize neutrophils partly through granulopoiesis and CXC chemokine induction (12). Intranasal administration of adenovirus expressing IL-17A, IL-17C, or IL-17F resulted in neutrophilia in the bronchoalveolar lavage (18). Moreover, i.p. injection of human rIL-17B caused marked neutrophil migration in normal mice (17). In contrast, the members can be divided into two groups according to the induction of cytokine production. Although IL-17A and IL-17F up-regulate IL-6 and IL-8 in human fibroblasts (19, 20), IL-17B and IL-17C are reported to stimulate the release of TNF-α and IL-1β from the monocytic cell line THP-1 (17). Taken together, these results indicate that IL-17 family members induce inflammatory cytokines not only through activated T cells, but also through activated monocytes/macrophages.

Based on the structural and functional similarities among IL-17 family members, we speculated that not only IL-17A, but also other IL-17 family members are involved in the pathogenesis of many inflammatory and autoimmune disorders, especially in the development of RA. We focused on IL-17A, IL-17B, IL-17C, and IL-17F, which can affect inflammatory cytokine production of fibroblasts and macrophages. Recently, IL-17C expression in synovial fluid mononuclear cells and PBMCs of RA patients was reported (21). However, the biological effect of IL-17 family members in arthritis has not been analyzed.

In the present study, we investigated the expression and effect of IL-17 family members in arthritis. In vitro, not only IL-17A, but also IL-17B and IL-17C induced the mRNA expression of inflammatory cytokines such as IL-1β, IL-6, and IL-23 in the *3T3* cell line and peritoneal exudate cells (PECs). The supernatant of the PECs stimulated with each IL-17 family member all increased TNF-α production significantly compared with controls. In vivo, $CD4^+$ T cells transduced with each of IL-17B, IL-17C, or IL-17F exacerbated CIA in mice to the same degree as $CD4^+$ T cells transduced with IL-17A. Mice reconstituted with bone marrow (BM) cells transduced with each of IL-17B, IL-17C, or IL-17F suffered from severe CIA. Moreover, neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in CIA mice. Our results suggest that not only IL-17A, but also the other IL-17 family members (IL-17B, IL-17C, and IL-17F) are associated with inflammatory cytokines such as IL-1 and TNF-α and contribute to the exacerbation of autoimmune arthritis.

Materials and Methods

Animals

DBA/1J mice were purchased from Japan SLC. All mice were used at 6–8 wk of age. All animal experiments were conducted in accordance with the institutional and national guidelines.

Collagen-induced arthritis

CIA was induced, as described previously (22–24). In brief, bovine type II collagen (BCII) (Chondrex) was emulsified with an equal volume of CFA (Chondrex). DBA/1J mice were immunized intradermally at the base of the tail with 100 μg of BCII emulsified with CFA. On day 21, the mice were boosted by intradermal injection with 100 μg of BCII emulsified with IFA (Difco). The arthritis score was determined by erythema, swelling, or ankylosis per paw, as described previously (25, 26). The clinical arthritis score was defined as the sum of the scores of all four paws of each mouse.

Cytokines and cell lines

Recombinant murine IL (mIL)-17A, mIL-17B, mIL-17C, and mIL-17F were obtained from R&D Systems. The mouse fibroblast cell line *3T3* was obtained from American Type Culture Collection. This cell line was cultured with RPMI 1640 (Invitrogen Life Technologies) medium supplemented with 10% FCS, 2 mM γ-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-ME. Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM γ-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 ng/ml rmIL-3 (R&D Systems).

Murine PECs

Murine PECs were isolated after i.p. injection of 3 ml of 5% sterile fluid Brewer's thioglycolate broth (Sigma-Aldrich) into 8-wk-old DBA/1J mice (27). After culture of the PECs in a 6-well plate for 2 h, floating cells were removed by extensive washing, and attached cells were maintained in the medium described above for 3 days. More than 80% of the cultured cells were macrophages as determined by flow cytometric analysis of CD11b-positive cells. The following recombinant murine cytokines were added to the culture medium and incubated for 24 h: 50 ng/ml mIL-17A, mIL-17B, mIL-17C, or mIL-17F.

Preparation of retroviral constructs of mIL-17 family cDNAs

mIL-17A, mIL-17B, mIL-17C, and mIL-17F were isolated from the murine T lymphocyte cDNA library according to the reported nucleotide sequence from National Center for Biotechnology Information (mIL-17A NM_010552; mIL-17B NM_019508; mIL-17C NM_145834; mIL-17F NM_145856). The full-length fragments were subcloned into retrovirus vector murine stem cell virus/internal ribosome entry site/GFP (pMIG), as described previously (28).

Production of retroviral supernatants and retroviral transduction

Retroviral supernatants were obtained by transfection of pMIG carrying each of the IL-17 family genes into PLAT-E packaging cell lines using FuGENE 6 transfection reagent (Roche Diagnostic System), as described previously (29). For the detection of GFP-positive cells, we used an EPICS XL flow cytometer (Beckman Coulter).

Gene transduction to mouse splenocytes and adoptive transfer

Total splenocytes were cultured for 48 h in the presence of Con A (10 μg/ml) (Sigma-Aldrich) and mIL-2 (50 ng/ml) (R&D Systems). Retroviral gene transduction was performed, as described previously (30, 31). A $CD4^+$ T cell population was prepared by negative selection by MACS with anti-CD19 mAb, anti-CD11c mAb, and anti-CD8a mAb (BD Pharmingen). The gene-transduced $CD4^+$ T cells were suspended in PBS and injected i.v. (1×10^7) at 23 days after the first immunization of BCII.

BM precursor cell isolation, infection, and transfer

BM precursor cell isolation, retrovirus infection, and transfer were performed, as described previously (32). In brief, DBA/1J mice were treated with 5 mg/body 5-fluorouracil (Sigma-Aldrich) dissolved in PBS. After 5 days, BM cells were harvested and cultured with 50 ng/ml mIL-3, mIL-6, and murine stem cell factor (R&D Systems) for 48 h. Then the BM cells were spin infected with the retrovirus supernatants with 16 μg/ml polybrene (Sigma-Aldrich) for 90 min at 2400 rpm and 25°C. Recipient mice were treated by 700 rad of whole-body radiation and were injected with 1×10^6 of the BM cells i.v. Recipient mice were maintained for 6 wk until analysis or immunization.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

RNA of the cells was extracted using an RNeasy Micro Kit and RNeasy Mini Kit (Qiagen). RNA from the tissues was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using ISOGEN (Nippon Gene). RNA was reverse transcribed to cDNA with random primers (Invitrogen Life Technologies) and Superscript III, according to the manufacturer's protocol (Invitrogen Life Technologies). To determine the cellular expression of each protein, quantitative real-time PCR analysis was performed using an iCycler (Bio-Rad). The PCR mixture consisted of 25 μl of SYBR Green Master Mix (Qiagen), 15 pmol of forward and reverse primers, and the cDNA samples, in a total volume of 50 μl. We calculated the quantitative PCR data with δ cycle threshold method, and relative RNA abundance was determined based on control β-actin abundance. To measure the relative efficiency,

amplifications were performed on the serial diluted cDNA samples using primers for the target and the reference (β-actin) genes. We made plots of the log cDNA dilution vs δ cycle threshold, and confirmed that the efficiencies of the target and the reference genes were similar because the absolute value of the slope was close to zero (data not shown) (33, 34). The primer pairs used in the quantitative real-time PCR were as follows: mouse IL-17A, sense 5′-GCTCCAGAAGGCCCTCAGA-3′, antisense 5′-AGCTTTCCCTCCGCATTGA-3′; mouse IL-17B, sense 5′-CGGTGCCTATGTTTGGGTTGC-3′, antisense 5′-GGGTTG GTGGTTGGCTCAGAA-3′; mouse IL-17C, sense 5′-CACAGATGAG AACCGCTACCC-3′, antisense 5′-GCGGATGAACTCGGTGTGGA A-3′; mouse IL-17F, sense 5′-CAACGCTGCATACAAAAATCA-3′, antisense 5′-TTAAGTGAGGCATTGGGAACA-3′; mouse IL-17R, sense 5′-CCACTCTGTAGCACCCCAATG-3′, antisense 5′-CCTGGA GATGTAGCCCTGGTC-3′; mouse IL-17Rh1, sense 5′-GCAAGGAA GGAGCACGAAGAC-3′, antisense 5′-CTCGGCGATTTTCTTTTTCT G-3′; mouse TNF-α, sense 5′-CATCTTCTCAAAATTCGAGTGACA A-3′, antisense 5′-TGGGAGTAGACAAGGTACAACCC-3′; mouse IL-1β, sense 5′-CAACCAACAAGTGATATTCTCCATG-3′, antisense 5′-GATCCACACTCTCCAGCTGCA-3′; mouse IL-6, sense 5′-CACT TCACAAGTCGGAGGCTTA-3′, antisense 5′-GCAAGTGCATCATC GTTGTTC-3′; mouse IL-23, sense 5′-TGGCATCGAGAAACTGTGAG A-3′, antisense 5′-TCAGTTCGTATTGGTAGTCCTGTTA-3′; and mouse β-actin, sense AGAGGGAAATCGTGCGTGAC-3′, antisense 5′-CAATAGTGATGACCTGGCCGT-3′.

Immunoassays of cytokines and anti-type II collagen Ab

The concentrations of mIL-6, mTNF-α, and mIL-17A in mouse sera and culture supernatants were measured by sandwich ELISA, according to the manufacturer's protocol (BD Pharmingen). An automatic microplate reader (Bio-Rad 550) was used to measure the OD. Mouse serum IgG anti-type II collagen Ab titer was measured, as previously described (35).

Isolation of cartilage

Murine articular cartilage was isolated from patellae, as described previously (36). In brief, patellae were decalcified in 3.5% EDTA for 4 h at 4°C, when the whole cartilage layer was stripped off. Because old cartilage is more calcified, decalcification of the patellae of old mice (>3 mo) was performed overnight at 4°C.

Cell purification

Briefly, the arthritic paws of the CIA mice were cut into pieces, digested with collagenase type IV (Sigma-Aldrich), and stained with mAbs (Fc blocking with anti-mouse CD16/CD32 mAb, and staining with anti-mouse CD3-PE mAb, anti-mouse CD4-allophycocyanin mAb, anti-mouse CD11b-FITC mAb, anti-mouse CD11c-FITC mAb, anti-mouse CD19-FITC mAb, biotinylated anti-mouse I-A/I-E (MHC class II) mAb, and streptavidin PE Ab that were obtained from BD Pharmingen). Cell sorting of a specific cell population was performed with a FACSVantage flow cytometer (BD Biosciences).

Intracellular cytokine staining and flow cytometry

IL-17 family expressions of Ba/F3 cells transduced with each of IL-17 family members were examined using intracellular cytokine staining. Ba/F3 cells were infected with the retroviral supernatants in the presence of 10 μg/ml polybrene (Sigma-Aldrich) for 120 min. These cells were stained with anti-mouse IL-17A mAb conjugated to PE (BD Pharmingen), biotinylated anti-mouse IL-17B polyclonal Ab (R&D Systems), anti-mouse IL-17C polyclonal Ab (R&D Systems), and anti-mouse IL-17F mAb (R&D Systems), respectively. Bovine anti-goat IgG-PE (Santa Cruz Biotechnology) and F(ab′)$_2$ goat anti-rat IgG PE (Serotec) were used as secondary reagents for IL-17C and IL-17F staining, respectively. Cell fixation and permeabilization were performed using Cytofix/Cytoperm reagent (BD Pharmingen), according to the manufacturer's protocol (BD Pharmingen), and analyzed by flow cytometry. Splenocytes isolated from BM chimeric mice of IL-17A were also stained with anti-mouse IL-17A mAb in the same way.

Anti-IL-17B Ab treatment in CIA mice

CIA was induced in DBA/1J mice, as described above. Mice exhibited the first clinical signs of arthritis (arthritis score between 1 and 2) and were injected i.p. with 100 μg of polyclonal anti-mouse IL-17B Abs (R&D Systems). PBS was i.p. injected as a control. Arthritis was assessed using a scoring system, as described above. Mice were sacrificed at 10 days after the onset of arthritis, and the paws were removed. Joint pathology was evaluated on decalcified H&E-stained sections.

Histopathology

The tarsal joints of sacrificed CIA mice were embedded in paraffin wax after 10% formaldehyde fixation and decalcification. The sections were stained with H&E. Synovial tissues were graded by mononuclear cell infiltration and pannus invasion, as described previously (37).

Statistical analysis

Data are expressed as the means ± SD. All results were obtained by at least three independent experiments. Statistical significance was determined by the Mann-Whitney U test and unpaired Student's t tests. A value of $p < 0.05$ was considered statistically significant.

Results

IL-17 family genes (IL-17A, IL-17B, IL-17C, and IL-17F) were highly expressed in the arthritic paws of CIA mice

First, we examined the expressions of IL-17 family members and IL-17Rs in the arthritic paws of CIA mice by quantitative PCR. The mRNA expressions of all IL-17 family genes examined (IL-17A, IL-17B, IL-17C, and IL-17F) were highly elevated in the arthritic paws compared with the controls. In accordance with previous report of high in vivo expression of IL-17R in RA (38), mRNA expressions of IL-17Rs (IL-17R and IL-17Rh1) were also elevated (Fig. 1*A*). As expected, the mRNA expressions of inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-23) were also elevated in the arthritic paws compared with controls (Fig. 1*B*).

We next examined cell populations in the arthritic paws of CIA mice that express IL-17 family members. Subpopulations of the cells were sorted with various cell surface markers using a flow cytometer. As expected, CD4$^+$ T cells expressed IL-17A and IL-17F significantly. IL-17B was expressed exclusively in the inflammatory cartilage of CIA mice. In contrast, IL-17C was expressed in a broad range of cells, i.e., CD4$^+$ T cells, CD11b$^+$ MHC class II$^+$ macrophages, and CD11c$^+$ MHC class II$^+$ dendritic cells (Fig. 1*C*). These results suggested that CD4$^+$ T cells mainly express IL-17 family members, especially IL-17A, IL-17C, and IL-17F, at the inflammatory site.

IL-17 family induced several proinflammatory cytokines

We next investigated whether IL-17 family members have an influence on mouse fibroblast cell lines and mouse peritoneal macrophages. Cells of the mouse fibroblast line *3T3* were cultured with each of the IL-17 family members (50 ng/ml), and cytokine expression was examined after 24 h of incubation. IL-17A induced IL-1β and IL-6 expressions, as previously reported (2). Moreover, IL-17B, IL-17C, and IL-17F also induced IL-1β expression in *3T3* (Fig. 2*A*).

To examine the effects of IL-17 family members on mouse macrophages, thioglycolate-elicited PECs were isolated and cultured with each of the IL-17 family members (50 ng/ml). IL-17A induced IL-1β, IL-6, and IL-23 expressions in PECs. Interestingly, IL-17B also induced IL-1β, IL-6, and IL-23 expressions. Moreover, IL-17C induced IL-1β and IL-23 expressions in PECs (Fig. 2*B*). In addition, PECs stimulated with every IL-17 family member produced significantly increased amount of TNF-α protein compared with the control, and PECs stimulated with IL-17A and IL-17B produced significantly increased amount of IL-6 protein (Fig. 2*C*). These results suggested that IL-17A, IL-17B, IL-17C, and IL-17F stimulate fibroblasts and macrophages to produce inflammatory cytokines.

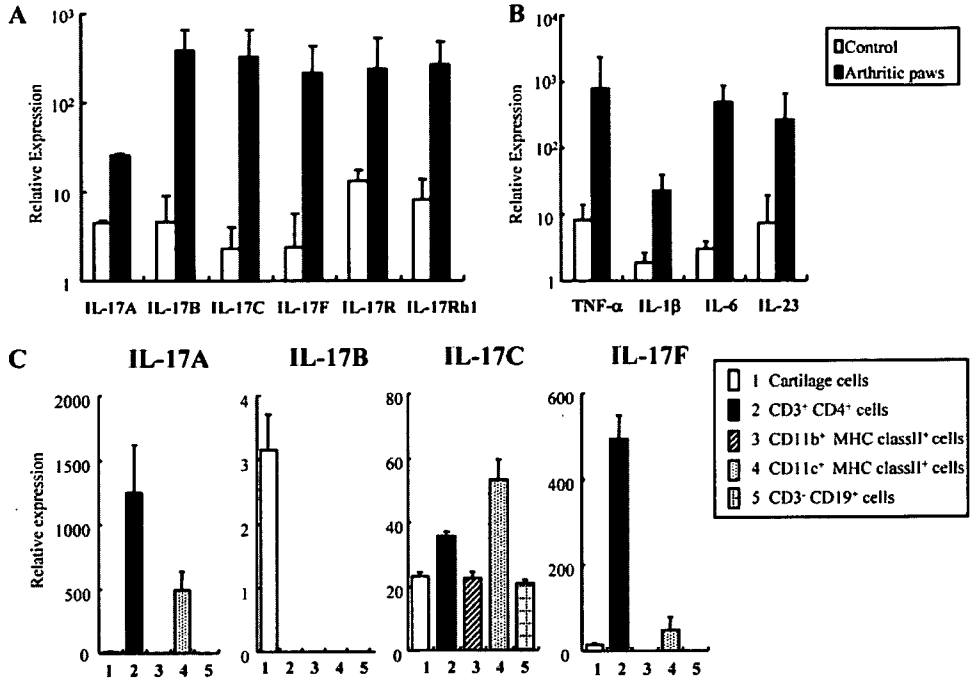

FIGURE 1. The expression of IL-17 family members and IL-17R genes in the arthritic paws of CIA mice. *A*, The expressions of IL-17 family genes and IL-17R genes were examined in the arthritic paws of CIA mice (■; $n = 3$) and in control mice (□; $n = 3$) by quantitative PCR. *B*, The expressions of inflammatory cytokines. *C*, The expressions of IL-17 family members in the sorted cell populations of the arthritic paws of CIA mice. The data are representative of three independent experiments.

Exacerbation of CIA by transfer of IL-17 family-transduced CD4$^+$ T cells

Because IL-17B and IL-17C induce the expression of inflammatory cytokines in fibroblasts and macrophages, we hypothesized that IL-17B and IL-17C have an effect on the process of arthritis. We subcloned cDNA fragment of mIL-17A, mIL-17B, mIL-17C, or mIL-17F to pMIG retrovirus vector. These vectors were retrovirally transduced to Ba/F3 cells, and protein expressions of IL-17 family members were confirmed with intracellular staining of each IL-17 family cytokine (Fig. 3*A*).

To examine the proinflammatory effects of the IL-17 family in vivo, we retrovirally transduced the IL-17 family genes to CD4$^+$ T cells. The transduction efficiencies were ~30% on average (Fig. 3*B*). These IL-17 family-transduced CD4$^+$ T cells were adoptively transferred to BCII-immunized DBA1 mice before the onset of arthritis. They exacerbated the progression of arthritis, as observed by the arthritis score (Fig. 3, *C* and *D*). The IL-17 family member-transduced CD4$^+$ T cells had no significant effect on the serum levels of anti-BCII IgG Abs at 14 days after the onset of CIA (data not shown). These results

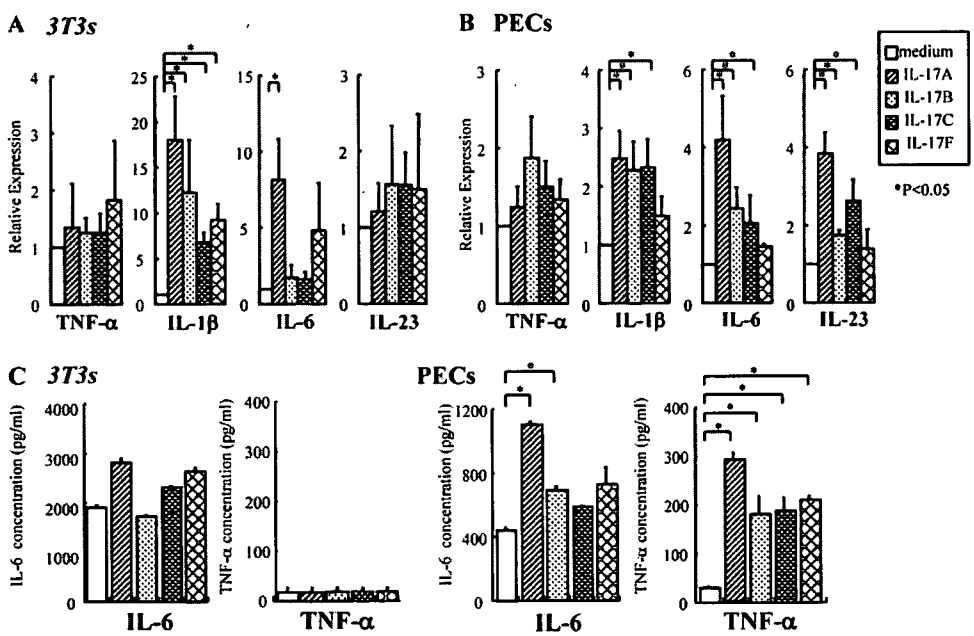

FIGURE 2. The proinflammatory effects of IL-17 family members on mouse fibroblasts and macrophages. *A*, Relative expression of the cytokine genes in *3T3* cell. The mouse fibroblast cell line *3T3* was cultured with each of mIL-17A, mIL-17B, mIL-17C, or mIL-17F for 24 h, and the expressions of inflammatory cytokines were measured by quantitative PCR. *B*, Relative expression of the cytokine genes in mouse thioglycolate-elicited PECs. PECs were cultured with each of mIL-17A, mIL-17B, mIL-17C, or mIL-17F for 24 h, and the expressions of inflammatory cytokines were measured by quantitative PCR. *C*, The secreted IL-6 and TNF-α levels in the supernatants of *3T3* and PECs were measured by ELISA. Error bars indicate ± SD. The data are representative of three independent experiments. Significance of differences between control (medium) and each IL-17 family was determined; *, $p < 0.05$.

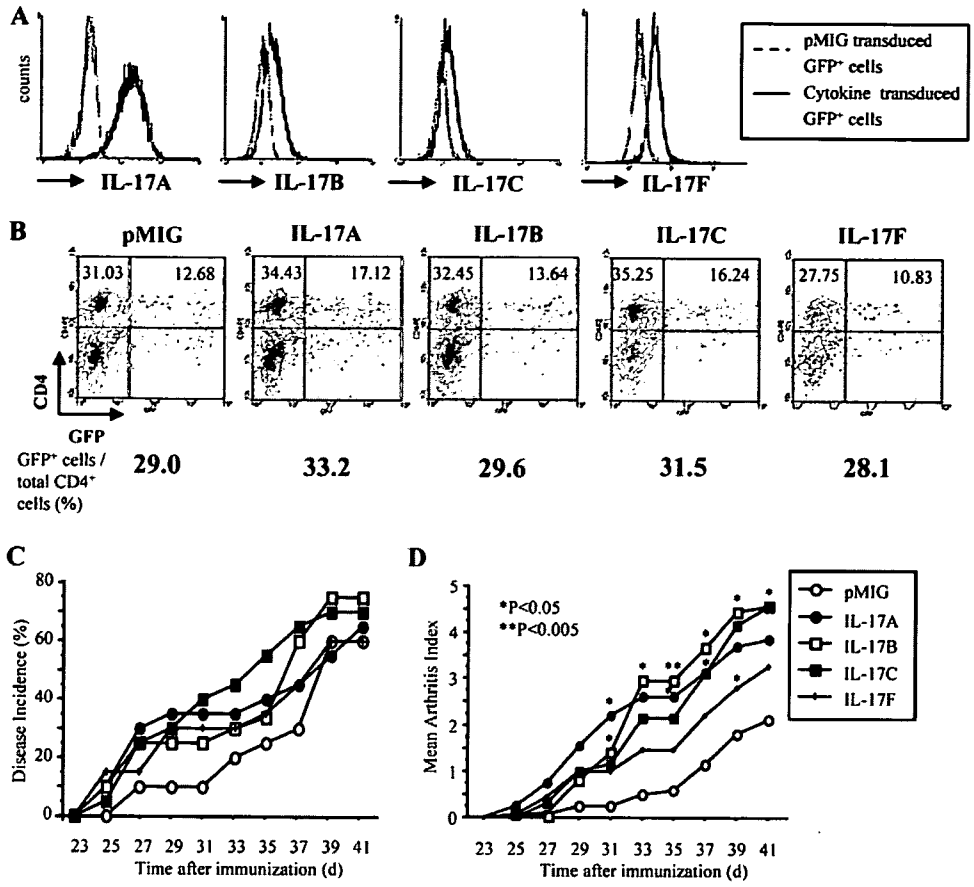

FIGURE 3. The effects of transfer of IL-17 family-transduced CD4$^+$ T cells on CIA. *A*, Intracellular IL-17 family expressions in Ba/F3 cells retrovirally transduced with each IL-17 family member. GFP-gated IL-17 family-transduced (mIL-17A, mIL-17B, mIL-17C, or mIL-17F) Ba/F3 cells were analyzed for IL-17A, IL-17B, IL-17C, or IL-17F expression compared with GFP-gated empty vector (pMIG)-transduced Ba/F3 cells. *B*, Representative FACS analysis of IL-17 family-transduced CD4$^+$ T cells was shown. Numbers in dot plots indicate the percentage of GFP$^+$ CD4$^+$ and GFP$^-$ CD4$^+$ cells, and the percentages of the GFP$^+$ cells within total CD4$^+$ cells were shown below. *C* and *D*, CD4$^+$ T cells transduced with each of IL-17 family genes were transferred to collagen-immunized mice before the onset of arthritis (day 23). The incidence of arthritis (*C*) and the progression of arthritis scores (*D*) are shown. Values are the mean of arthritis score (n = 20 mice per group). Significance of differences between control (pMIG) and each IL-17 family-transduced mice was determined; **, $p < 0.005$; *, $p < 0.05$.

indicated that the effect of IL-17 family members on the progression of arthritis was not associated with the elevations of anti-BCII Abs.

IL-17 family BM chimeric mice exhibited high arthritis scores upon CIA induction

To examine the proinflammatory effect of constitutively expressed IL-17 family members, we established IL-17 family BM chimeric mice by transfer of gene-transduced BM cells to lethally irradiated mice. In a previous study, the attempt to generate IL-17A-overexpressing mice with a conventional transgenic approach was unsuccessful because these mice were embryonic lethal (39). In accordance with the previous report, mice that expressed IL-17A with high efficiency (i.e., for which the percentage of GFP$^+$ cells in the spleen was >50%) became gaunt and died within 1 mo after BM transplantation (data not shown). When the percentage of GFP$^+$ cells in the spleen was 5–15%, the mice appeared to be healthy for several months. We therefore used BM chimeric mice that expressed IL-17 family genes in ~5–15% of spleen cells. Eight weeks after the BM transplantation, mIL-17A was readily detected by intracellular cytokine staining (Fig. 4*A*). Moreover, the serum concentration of mIL-17A was significantly elevated in these mice (Fig. 4*B*). Therefore, the BM chimeric mice were successfully allowed to express the transduced cytokines systemically. Then we immunized these mice with BCII 8 wk after BM transplantation. BM chimeric mice of IL-17A and IL-17F exhibited early onset and high arthritis scores upon CIA induction (Fig. 5, *A* and *B*). BM chimeric mice of IL-17B and IL-17C clearly exacerbated arthritis, as assessed by the arthritis score. In contrast, BM chimeric mice of IL-17B and IL-17C did not result in significant differences in the onset of disease (Fig. 5, *C* and *D*). BM expression of IL-17 family member did not affect the anti-BCII Ab responses at 14 days after the onset of CIA (data not shown). These results indicated that the effect of IL-17 family members on the exacerbation of arthritis was not associated with the responses of anti-BCII Abs.

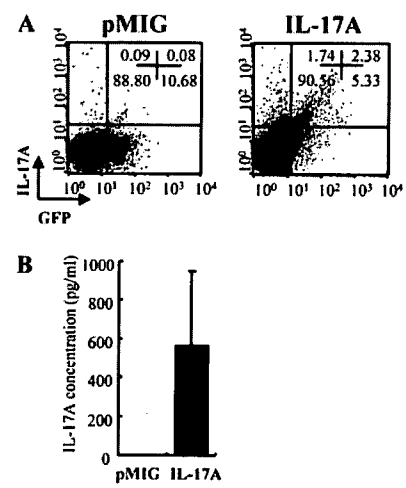

FIGURE 4. Generation of IL-17 family chimeric mice by BM transplantation of gene-transduced BM cells. Each of IL-17 family genes was transduced to BM cells with retrovirus vector and transferred to lethally irradiated mice. *A*, The intracellular expression of IL-17A protein in the spleen of IL-17A BM chimeric mice 8 wk after BM transplantation. The percentage of GFP$^+$ cells expressing IL-17A is indicated. The data are representative of three independent experiments. *B*, The concentration of IL-17A protein in the serum of IL-17A BM chimeric mice (n = 6) and control mice (pMIG BM chimeric mice) (n = 6). The levels of IL-17A were measured by ELISA.

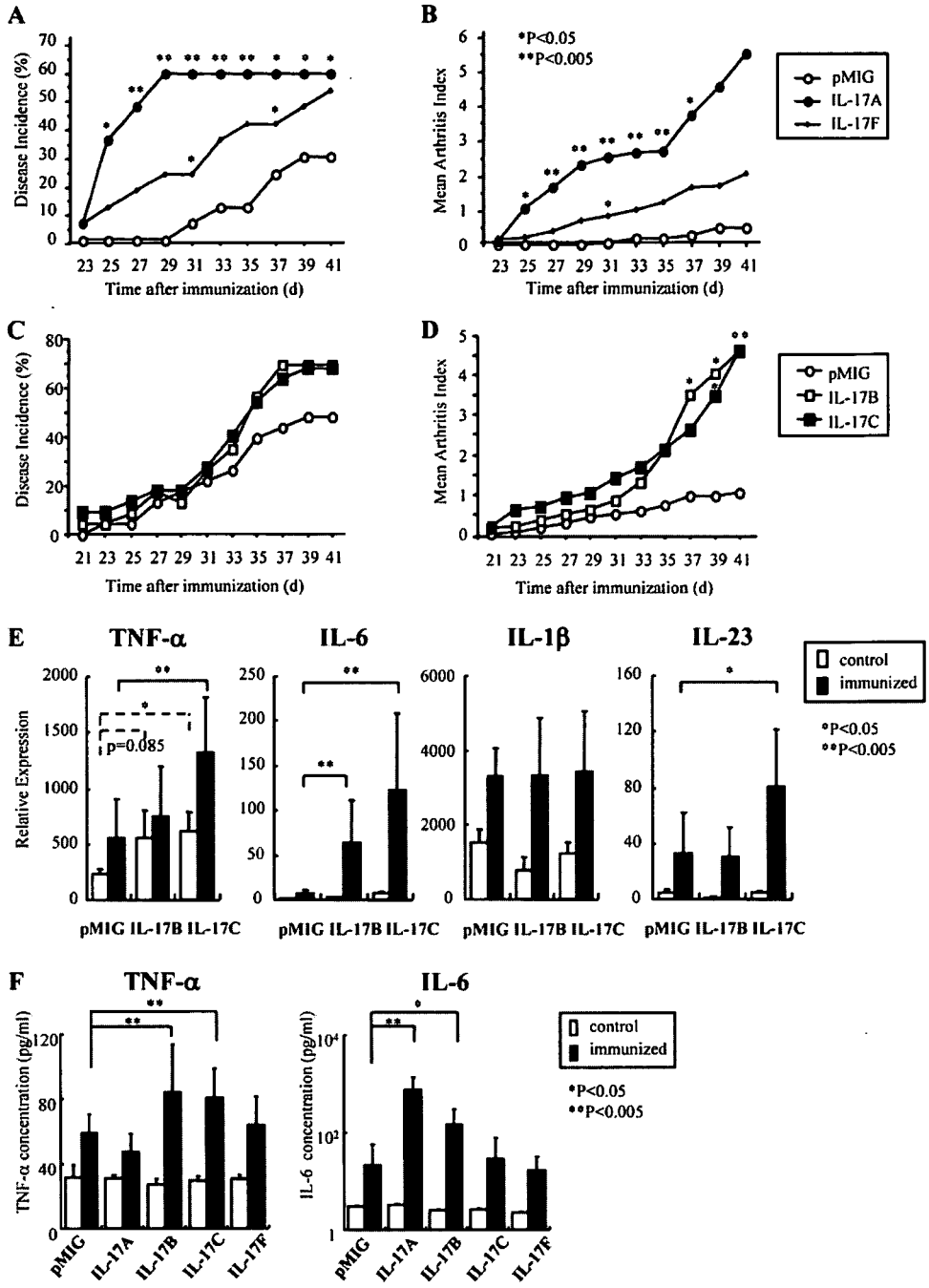

FIGURE 5. Incidence of CIA and arthritis scores in IL-17 family BM chimeric mice. Incidence of CIA and arthritis scores in IL-17A and IL-17F BM chimeric mice (*A* and *B*), and in IL-17B and IL-17C BM chimeric mice (*C* and *D*). Mice were immunized with BCII 8 wk after the BM transplantation. Values are the mean of experiments for IL-17A and IL-17F BM chimeric mice ($n = 20$ per group) and experiments for IL-17B and IL-17C BM chimeric mice ($n = 30$ per group). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$. *E*, The mRNA expression of inflammatory cytokines in the spleen of BM chimeric mice of IL-17B and IL-17C, which were immunized with BCII (■; $n = 15$ per group) or nonimmunized controls (□; $n = 6$ per group). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$. *F*, The secreted TNF-α and IL-6 levels in the serum of IL-17 family BM chimeric mice that were immunized with BCII (■; $n = 15$) or nonimmunized controls (□; $n = 6$). Significance of differences between control (pMIG) and each IL-17 family BM chimeric mice was determined; **, $p < 0.005$; *, $p < 0.05$.

We next examined the alterations of inflammatory cytokine production in these BM chimeric mice. Interestingly, nonimmunized IL-17C BM chimeric mice showed increased mRNA expression of TNF-α in the spleen compared with controls (Fig. 5*E*). Moreover, in the spleen of BCII-immunized IL-17C BM chimeric mice, the mRNA expressions of TNF-α, IL-6, and IL-23 were up-regulated. In contrast, BCII-immunized IL-17B BM chimeric mice showed increased mRNA expression of IL-6 in the spleen compared with controls (Fig. 5*E*). When we examined the concentrations of TNF-α and IL-6 protein in the sera of IL-17 family BM chimeric mice, the BCII-immunized IL-17B and IL-17C BM chimeric mice showed increased TNF-α concentration in the sera. And the BCII-immunized IL-17A and IL-17B BM chimeric mice showed increased IL-6 production in the sera (Fig. 5*F*). These results suggested that IL-17B and IL-17C enhanced inflammation in this mouse model of arthritis by increased inflammatory cytokine production.

Neutralization of IL-17B significantly suppressed the progression of arthritis

As shown in Fig. 5, we found that IL-17B exacerbated the progression of CIA as well as IL-17A with the method of retrovirus-mediated BM chimeric mice. Regarding IL-17A, neutralizing Abs against IL-17A have been previously shown to be effective in the treatment of CIA (8). We examined the effect of IL-17B blockade in CIA mice. CIA mice were systemically treated with polyclonal anti-mouse IL-17B Abs immediately after the first signs of arthritis. Neutralization of IL-17B significantly suppressed the progression of CIA compared with the controls (Fig. 6*A*). Moreover, histological analysis revealed significant reduction of cell infiltration

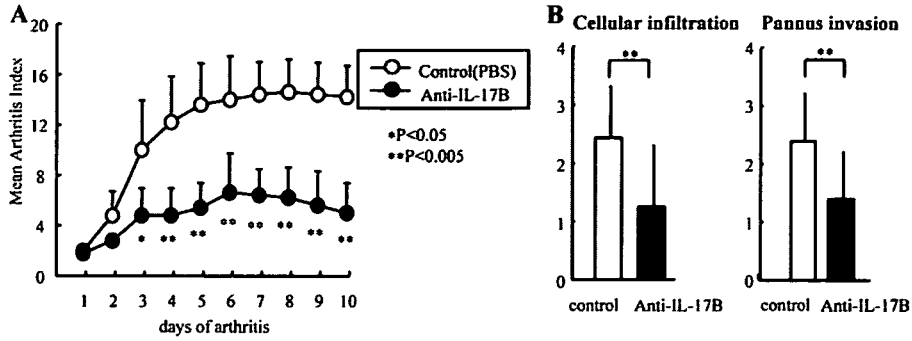

FIGURE 6. Effect of anti-IL-17B Ab treatment in CIA mice. *A*, CIA mice received i.p. injection of anti-mouse IL-17B Abs after the first clinical signs of arthritis (arthritis score between 1 and 2). As a control, PBS was injected. The arthritis score was shown. *B*, Histological score of the inflammatory joints of CIA mice treated with anti-IL-17B Abs was evaluated at 10 days after the onset of arthritis. Cellular infiltration and pannus invasion were graded in all four paws of the mice. Values are the mean of arthritis scores for anti-IL-17B Ab-treated mice and control mice (n = 5 per group). Significance of differences between control and anti-IL-17B Ab-treated mice was shown.

and pannus invasion in the anti-IL-17B Ab-treated mice (Fig. 6*B*). These results indicated that IL-17B was associated with the progression of arthritis in CIA mice.

Discussion

RA is considered to be an autoimmune disease, and is characterized by sustained inflammation of the joints and destruction of cartilage and bone. Several inflammatory cytokines are known to mediate the pathogenesis of arthritis, and TNF-α and IL-6 are the most important cytokines in the pathogenesis of RA. IL-17A, IL-17B, IL-17C, and IL-17F have the capacity to induce TNF-α production in PECs in vitro. In vivo, the mRNA expression of TNF-α was spontaneously increased in the spleen of IL-17C BM chimeric mice. Moreover, TNF-α productions in the sera of BCII-immunized IL-17B and IL-17C BM chimeric mice were up-regulated. Although IL-17A induced TNF-α production in PECs, IL-17A BM chimeric mice did not show up-regulated production of TNF-α. This result is consistent with previous observation in THP-1 cell line that IL-17B and IL-17C stimulated the release of TNF-α, whereas IL-17A has only a weak effect on TNF-α (17). In contrast to IL-17B and IL-17C, IL-17A may not be directly associated with TNF-α production in vivo. Moreover, the mRNA expression in the spleen and serum concentration of IL-6 were significantly up-regulated in IL-17B BM chimeric mice that were immunized with BCII. These results showed the close association of IL-17B and IL-17C with TNF-α and IL-6 in vivo and clearly suggested the importance of IL-17B and IL-17C in the pathogenesis of RA.

To date, the cell sources of IL-17B and IL-17C have not been identified. In this study, we showed that IL-17B was expressed in the inflammatory cartilage of CIA mice, whereas IL-17C was expressed in a broad range of cells, i.e., $CD4^+$ T cells, $CD11b^+$ MHC class II^+ macrophages, and $CD11c^+$ MHC class II^+ dendritic cells. IL-17A and IL-17F were expressed in $CD4^+$ T cells, as expected. These results suggested that $CD4^+$ T cells are involved in the expression of IL-17 family members, especially IL-17A, IL-17C, and IL-17F, at the inflammatory site. Although we did not detect a unique cell source of IL-17C, the arthritis-promoting effect of IL-17C-transduced $CD4^+$ T cells suggests the importance of IL-17C expressed in $CD4^+$ T cells.

In our in vivo analysis, we observed arthritis-promoting effects of the IL-17 family members. As shown in Fig. 3, the transfer of mIL-17A-, mIL-17B-, mIL-17C-, and mIL-17F-transduced $CD4^+$ T cells evidently exacerbated arthritis as assessed by the arthritis score. This effect was also confirmed in the CIA of the mIL-17A, mIL-17B, mIL-17C, and mIL-17F BM chimeric mice. The arthritis-promoting effect of IL-17A was previously reported in a study using adenovirus vector (5, 40). In contrast to IL-17A, which hastened the onset of arthritis, IL-17B and IL-17C did not affect the onset of arthritis evidently. This fact suggests that IL-17B and IL-17C affect arthritis rather in the effector phase. To our knowledge, this is the first observation of an in vivo arthritis-promoting effect of IL-17B and IL-17C.

Blockade of IL-17A has recently been shown to be effective in the treatment of CIA (8). In the present study, we have demonstrated the therapeutic potential of IL-17B blockade after the onset of CIA. Because blockade of TNF-α or IL-1β is not always effective in RA patients, blockade of additional cytokine might be a useful therapeutic option. Therefore, our data strongly suggest that IL-17B as well as IL-17A could be an important target for the treatment of inflammatory arthritis.

In a recent study, the combination of IL-6 and TGF-β was reported to strongly induce IL-17A production in Th17 cells (41). Moreover, it was recently recognized that IL-23 contributes to the expansion of autoreactive IL-17A-producing T cells and promotes chronic inflammation dominated by IL-17A, IL-6, IL-8, and TNF-α (14, 42). Thus, IL-17B and IL-17C may exacerbate arthritis via IL-6- and IL-23-mediated promotion of IL-17A production. However, the possibility that IL-17B and IL-17C exert a cooperative proinflammatory response together with IL-17A and IL-17F in arthritis by regulating the release of cytokines such as IL-6, IL-1β, and IL-23 still remains to be examined.

IL-17F has the highest homology with IL-17A and, like IL-17A, is produced by activated T cells. IL-17F appears to have an effect similar to that of IL-17A on cartilage proteoglycan release and inhibition of new cartilage matrix synthesis (11). Although IL-17F is thought to contribute to the pathology of inflammatory disorders such as RA, the in vivo effect of IL-17F on arthritis was not elucidated. In this study, we found that transduction of BM-expressed IL-17F resulted in both an earlier onset and a subsequent aggravation of arthritis.

We also found that the mRNA expression of all IL-17 family and IL-17R genes examined (mIL-17A, mIL-17B, mIL-17C, mIL-17F, mIL-17R, and mIL-17Rh1) was elevated in the arthritic paws of CIA mice compared with the paws of the control mice. The receptor for IL-17A is IL-17R (also named IL-17AR), which is extensively expressed in various tissues or cells tested, in contrast to the exclusive expression of IL-17A in activated T cells. Recently, IL-17R signaling has been suggested to play a crucial role in driving the synovial expression of proinflammatory and catabolic mediators, such as IL-1, IL-6, matrix metalloproteinase

(MMP)-3, MMP-9, and MMP-13, in streptococcal cell wall-induced arthritis (43). IL-17R-deficient (IL-17R$^{-/-}$) mice that were locally injected five times with streptococcal cell wall fragments into the knee joints showed a significant reduction of joint thickness and cartilage damage that was accompanied by reduced synovial expression of IL-1, IL-6, and the MMPs 3, 9, and 13 compared with arthritic wild-type mice. Therefore, these results indicate the critical role of IL-17R signaling during progression from an acute, macrophage-driven joint inflammation to a chronic, cartilage-destructive, T cell-mediated synovitis. There are four additional receptor-like molecules that share homology to IL-17R, i.e., IL-17Rh1 (also named IL-17RB or IL-17BR), IL-17RL (also named IL-17RC), IL-17RD, and IL-17RE. IL-17Rh1 was shown to bind to IL-17B, but with higher affinity to IL-17E (11, 12).

Although IL-17A transgenic mice have been reported to be embryonic lethal (39), we established BM-overexpressing mice that constitutively expressed IL-17A. The adequate control of the expression level was critically important. In our experiment, the serum concentration of IL-17A was elevated to ~600 pg/ml in IL-17A BM chimeric mice. This serum concentration of IL-17A was similar to those in patients with inflammatory diseases such as RA, inflammatory bowel diseases, familial Mediterranean fever, and the acute stage of Kawasaki disease (3, 44–46). Therefore, our BM chimeric mice approach may be useful to elucidate the physiological role of inflammatory cytokines that show lethal phenotypes in the conventional gene-transgenic technique.

In conclusion, we found that IL-17 family genes were up-regulated in association with their receptors in CIA. Each of the IL-17 family members clearly exacerbated the progression of CIA with the method of retrovirus-mediated BM chimeric mice. IL-17B and IL-17C have the capacity to exacerbate inflammatory arthritis in association with increased TNF-α and IL-6 productions from macrophages. Moreover, neutralization of IL-17B significantly suppressed the progression of arthritis and bone destruction in CIA mice. Therefore, our results suggest that not only IL-17A, but also the IL-17 family members IL-17B, IL-17C, and IL-17F play an important role in the pathogenesis of inflammatory arthritis and should be a new therapeutic target of arthritis.

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Disclosures

The authors have no financial conflict of interest.

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Gene Therapy of Arthritis with TCR Isolated from the Inflamed Paw[1]

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In recent years, the treatment of autoimmune diseases has been significantly advanced by the use of biological agents. However, some biologics are accompanied with severe side effects, including tuberculosis and other types of infection. There is thus a critical need for nonsystemic and lesion-specific methods of delivering these therapeutic agents. We attempted to treat a mouse model of arthritis by using T cells that expressed a regulatory molecule and were specifically directed to the inflamed paw. To this end, we first identified the TCR $\alpha\beta$ genes accumulating in the inflamed paw of mice with collagen-induced arthritis (CIA) by a combination of single-strand chain polymorphism analysis of TCR and single-cell sorting. We identified an expanded clone B47 which is autoreactive but is not specific to type II collagen. In vivo, TCR genes from B47-transduced T cells accumulated in the inflamed paw. Injection of cells cotransduced with the B47 and soluble TNFRIg genes resulted in a significant suppression of CIA. The suppression was correlated with the amount of TNFRIg transcripts in the hind paw, not with the serum concentrations of TNFRIg. Moreover, T cells cotransduced with the *B47* and intracellular *Foxp3* genes significantly suppressed CIA with reductions in TNF-α, IL-17A, and IL-1β expression and bone destruction. T cells cotransduced with *B47* and *Foxp3* genes also suppressed the progression of established CIA. Therefore, immunosuppressive therapy with autoreactive TCR is a promising therapeutic strategy for arthritis whether the TCRs are used to deliver either soluble or intracellular suppressive molecules. *The Journal of Immunology,* **2006, 177: 8140–8147.**

Progress in molecular biology reveals many molecular bases for the autoimmune diseases. In recent years, the treatment of autoimmune diseases has been significantly advanced by the use of biological agents. Treatment of rheumatoid arthritis (RA)[3] has long been insufficient to prevent joint destruction. However, anti-TNF therapy has been a breakthrough in the treatment of RA. Anti-TNF therapy significantly ameliorates arthritis symptoms, acute phase reactants, and bone destruction (1–3). In contrast, anti-TNF therapy is accompanied by increased risk of serious infection, including tuberculosis (4). Therefore, it is important to develop an optimal molecular delivery system for anti-TNF drugs and other biological agents.

In addition to the interference of cytokines, there are several other candidate molecules that may suppress autoimmune diseases. For example, Foxp3, a master transcription factor for regulatory T cells (5), is an important candidate for autoimmune suppression. Consequently, specific delivery of intracellular molecules is also important for future molecular therapy.

Because T cells systemically survey specific Ags and migrate to specific organs upon Ag recognition, they are an appropriate candidate vehicle for molecular delivery. T cell therapy has been used for the treatment of several kinds of autoimmune diseases (6–8). However, it is difficult to isolate and culture lesion-specific T cells to realize an amount sufficient for treatment. To date, type II collagen (CII)-specific T cell hybridoma and TCR-transgenic cells have been used for in vivo therapy of arthritis (9, 10), and OVA-specific TCR-transgenic cells have been used to treat OVA-induced arthritis (11). However, tumor cells and transgenic cells are evidently not applicable in human treatment. Moreover, T cells specific for the disease-priming autoantigen have the possibility to exacerbate arthritis inflammation.

We previously established a technique for analyzing T cell clonality by the reverse transcription (RT)-PCR/single-strand conformational polymorphism (SSCP) method (12). This method detects nucleotide changes of the CDR3 regions of clonally expanded T cells in vivo. Using this method, we have demonstrated oligoclonal expansion of T cells in patients with RA and solid tumors (12, 13). These findings indicate that the knowledge of the specific TCR accumulated at the inflammatory site may make it possible to reconstitute functional and organ-specific T cells. Indeed, we have previously identified the TCR α and β genes of expanded T cell clones infiltrated into p815 tumors (14).

In this study, we isolated a pair of TCR α and β genes, B47 from the paw of a mouse with collagen-induced arthritis (CIA). This TCR was not specific to immunized CII. We reconstituted this clonotype on peripheral $CD4^+$ T cells as a therapeutic vehicle. Cells cotransduced with B47 and TNFRIg suppressed CIA. The suppression was correlated with the amount of TNFRIg transcripts in the hind paw, not with the serum concentrations of TNFRIg. Moreover, T cells cotransduced with B47 and intracellular Foxp3 significantly suppressed CIA with reductions in TNF-α, IL-17A,

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; SSCP, single-strand conformational polymorphism; CIA, collagen-induced arthritis; RT, reverse transcription; CII, type II collagen; bCII, bovine CII; mCII, murine CII; IRES, internal ribosomal entry site; ILN, inguinal lymph node.

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and IL-1β expression and bone destruction. Therefore, an in vivo cloned TCR can be considered an efficient tool for molecular therapy.

Materials and Methods

Induction of CIA and scoring of joint swelling and histology

DBA1 mice were purchased from SLC and maintained in our specific pathogen-free facility. Mice were immunized intradermally at the base of the tail with 100 μg of bovine CII (bCII; Chondrex) emulsified with CFA (Chondrex). On day 21, mice were boosted by intradermal injection with 100 μg of bCII emulsified with IFA (Difco). Inflammation of the four paws was graded from 0 to 4 as follows: grade 0, no swelling; grade 1, swelling of the finger joints or focal redness; grade 2, mild swelling of the wrist or ankle joints; grade 3, severe swelling of the entire paw; and grade 4, deformity or ankylosis. Each paw was graded and the four scores were totaled so that the possible maximal score per mouse was 16. All animal experiments were conducted in accordance with the institutional and national guidelines.

Vector construction

We constructed the vectors pMX-CIIT TCR (pMX-CIITα-IRES-CIITβ) and pMX-B47 (pMX-B47α-IRES-B47β) to transduce the desired TCR clonotype to activated CD4$^+$ T cells. bCII-specific TCR, CIIT, α- (Vα11) and β- (Vβ8.2) chains were constructed based on the published sequences of clone 173 α- and β-chains (15) as previously described (16). A TNFRIg fragment was constructed by fusing murine TNFR (p75) to the hinge and Fc region of a murine IgG2a H chain. The resulting TNFRIg fragment was subcloned into a pMX retrovirus vector. We also constructed the vector pMX-Foxp3-IRES-GFP. Retroviral gene transfer was performed as previously described (16).

Single-cell sorting and RT-PCR

The CD4$^+$ T cells at the inflammation site were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-Vβ8.1/8.2 (BD Pharmingen). The CD4$^+$/Vβ8.1/8.2$^+$ cells were sorted at a ratio of one cell per well using an automatic cell dispensing unit driven by the FACSVantage and Clone-Cyt software (BD Biosciences). Each cell was sorted into a well of 96-well plate containing 20 μl of RT reaction mixture (10 nM CαRT primer, 10 nM CβRT primer, 1× RT reaction buffer, 100 μM each dNTP (Takara), 0.5% Nonidet P-40 (Boehringer Mannheim), 0.5 U/μl RNasin (Promega) in a 96-well microtiter plate. Immediately, 20 U/μl Superscript II (Invitrogen Life Technologies) reagent was added to each well and the plate was held at 37°C for 90 min. After the reaction mixture received heat inactivation for 10 min at 65°C, an equal volume of TdT solution (2× TdTase reaction buffer, 2.5 mM dATP (Amersham Biosciences), 0.5 U/μl TdT (Invitrogen Life Technologies)) was added to each well and the plate was incubated for 15 min at 37°C (17). From the single-cell RT reaction mixtures, 2 μl of cDNA was added to 23 μl of the first PCR premix (1.6 pM/μl each 1st primer, 200 mM each dNTP, and 0.25 U/μl KOD-plus- *Taq* polymerase (Toyobo)) and amplified by a 25-cycle program (95°C for 1 min, 52°C for 1 min, and 72°C for 2 min). Two microliters of first PCR products was used for the second PCR (30 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min), using the second PCR premix (1.6 pM/μl of each second primer, 200 mM of each dNTP, and 0.25 U/μl *Taq* polymerase (Promega)). Then, 2 μl of the second PCR products was used for further amplification reaction (35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min), using the third PCR premix (1.6 pM/μl each third primer, 200 mM each dNTP, 0.25 U/μl *Taq* polymerase).

Single-strand conformational polymorphism

The SSCP study was performed as described previously (14, 18). In brief, the synthesized cDNA was amplified by PCR with a pair of Vβ1 to Vβ19 primers and a Cβ common primer. The amplified DNA was electrophoresed on a nondenaturing 4% polyacrylamide gel. After transfer onto a nylon membrane, the cDNA was hybridized with a biotinylated internal common Cβ oligonucleotide probe and visualized by subsequent incubations with streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate system (Phototope-Star Chemiluminescent Detection kit; New England Biolabs).

Cell purification

A CD4$^+$ T cell population was prepared by negative selection with MACS (Miltenyi Biotec) using anti-CD19 mAb, anti-CD11c mAb, and anti-CD8a mAb. CD11c$^+$ DCs were prepared as previously described (19, 20).

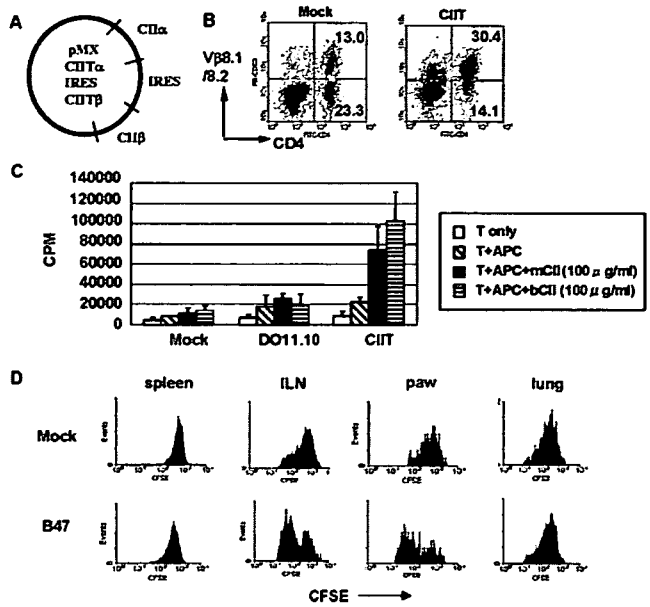

FIGURE 1. Reconstitution of CII-specific TCR, CIIT, on splenic CD4$^+$ T cells of DBA1 mice. *A*, Schematic representation of the bicistronic retrovirus construct of CIIT. *B*, Representative result of retroviral transduction of CIIT in DBA1 splenocytes. The cells were stained for Vβ8.1/8.2 and CD4. *C*, Ag specificity of CIIT-transduced CD4$^+$ T cells. Mock-, DO11.10-, and CIIT-transduced CD4$^+$ T cells were cultured with no APC, 1×10^5 APC (irradiated splenocytes), APC plus 100 μg/ml mCII, or APC plus 100 μg/ml bCII. *D*, CFSE-labeled mock- or CIIT-transduced T cells were transferred into CIA-induced DBA1 mice. Ninety-six hours later, CD4$^+$ T cells in the spleen, ILN, paws, and lungs were examined for Vβ8.1/8.2$^+$CD4$^+$ gated CFSE-positive cells.

Briefly, spleen cells or lymph node cells were digested with collagenase type IV (Sigma-Aldrich) and DNase I, and the CD11c$^+$ cells were selected twice by positive selection using MACS CD11c microbeads and magnetic separation columns. The purity (85% in average) was determined by visualization with anti-CD11c-biotin followed by streptavidin-PE. A CD19$^+$ B cell population was prepared by positive selection with MACS using anti-CD19 mAb. For CFSE-labeling (Molecular Probes), cells were resuspended in PBS at 1×10^7/ml and incubated with CFSE at a final concentration of 5 mM for 30 min at 37°C, followed by two washes in PBS. An Anti-FITC MultiSort kit (Miltenyi Biotec) was used in the negative selection experiment in the CD11c$^+$ population.

Paw tissues were prepared by removing the skin and separating the limb below the ankle joint. Finely minced tissues were incubated in complete RPMI 1640 medium with 1 mg/ml type IV collagenase (Sigma-Aldrich) for 60 min. The cell suspension was strained through nylon mesh and washed with PBS. In the single-cell sorting experiment, anesthetized mice were sacrificed by cardiac perfusion with PBS before the paw preparation.

Proliferation assay

At 24 h postinfection, purified CD4$^+$ T cells were cultured at $0.5–1 \times 10^4$ cells/well, with 1×10^5 cells/well of irradiated splenocytes or 1×10^4 cells/well of irradiated CD11c$^+$ DCs in 96-well, flat-bottom microtiter plates in volumes of 100 μl of complete medium with or without 100 μg/ml heat denatured bCII or murine CII (mCII) (Chondrex). After 24 h of culture, the cells were pulse-labeled with 1μCi of [^{3}H]thymidine/well (NEN Life Science Products) for 15 h and the [^{3}H]thymidine incorporation was determined.

Flow cytometry

The percentage of TCR gene transduced cells in each organ was determined by FACS analysis. Cell suspensions were first incubated with anti-CD16/CD32 (BD Pharmingen) to block FcRs. The cells were then stained with anti-CD4-allophycocyanin-Cy7, anti-Vβ8.1/8.2-PE, anti-Vα2-biotin followed by streptavidin-allophycocyanin (BD Pharmingen). Flow cytometry was performed using FACSVantage.

Table I. *Major clones in Vβ8.1/8.2$^+$CD4$^+$ T cells from seven arthritic mice*

	Vβ8.2	CDR3	Jβ
Mouse 1 major	Y F C A	S G **D** R **G** N S D Y	T F G S G
Mouse 2 major	Y F C A	S G D V F N E R L	F F G H G
Mouse 3 major	Y F C A	S **D** R L **G** G L Y E Q	V F G P G
Mouse 4 major	Y F C A.	S G **D** S **G G** E R L	F F G H G
Mouse 5 major	Y F C A	S G **D** A **G** D T Q	Y F G P G
Mouse 6 major	Y F C A	S G V P G Q G A N E R	F F G H G
Mouse 7 major	Y F C A	S G **D** P **G G** Q D T Q	Y F G P G

Real-time PCR

The skin was stripped from the mouse paws and the paws were frozen in Isogen (Nippon Gene). mRNA extraction and cDNA preparation were performed according to the manufacturer's (Nippon Gene) instructions. Real-time quantitative PCR was performed using CyberGreen master-mix (Qiagen) and an iCycler (Bio-Rad). Primer pairs were selected as previously described for β-actin, GAPDH, TNF-α, IFN-γ, IL-1β, and IL-10 (21). IL-17 primer pairs were as follows: IL-17 forward 5′-GCTCCAGAAGG CCCTCAGA-3′ and IL-17 reverse 5′-AGCTTTCCCTCCGCATTGA-3′. The PCR parameters were 95°C for 15 min, followed by 50 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 60 s.

Results

Reconstitution of paw specificity by gene transfer of the CII-specific TCR

Our aim was to generate an inflamed paw-directed T cell that expresses a regulatory molecule using TCR cloned from an arthritic paw. To this end, we first examined whether TCR reconstituted CD4$^+$ T cells could accumulate in the arthritic paw. We selected TCR αβ sequences of a known CII-specific TCR (15) for the reconstitution and subcloned them into a bicistronic retrovirus vector. This TCR was designated as CIIT (Fig. 1*A*).

In the gene transduction experiment, control cells that were transduced with an empty vector (pMX) were designated as mock-transduced cells. In a representative experiment, the percentage of Vβ8.1/8.2$^+$ T cells in the CD4$^+$ population was increased from 36% (100 × 13.0/(13.0 + 23.3)) to 68% (100 × 30.4/(30.4 + 14.1)) after infection of CIIT α- (Vα11) and β- (Vβ8.2) chains into DBA1 splenocytes (Fig. 1*B*). The calculated efficiency of β-chain transduction into initially Vβ8.1/8.2-negative cells was ~50% (100 × (68 − 36)/(100 − 36). We speculated that the transduction efficiency of the α-chain was equal to that of the β-chain. Therefore, the clonotypic transduction efficiency was estimated to be ~25%.

We next examined the specific reactivity of CIIT-transduced cells. Though CIIT-transduced cells showed only marginal proliferation in the presence of autologous irradiated splenocytes alone, these cells proliferated strongly in the presence of mCII and bCII (Fig. 1*C*). Moreover, this proliferation was blocked by anti-I-A^q Ab (data not shown). The reactivity of CIIT-transduced cells to mCII is consistent with a previous report that a T cell hybridoma expressing this TCR was accumulated in the inflamed joints of mice (9). There was no significant difference in proliferation between mock- and DO11.10 TCR (I-A^d restricted, OVA$_{323-339}$-specific TCR) (22) transduced cells in the presence of DCs with or without CII. Thus, CIIT gene transfer can reconstitute Ag specificity on CD4$^+$ T cells of DBA1 mice in vitro.

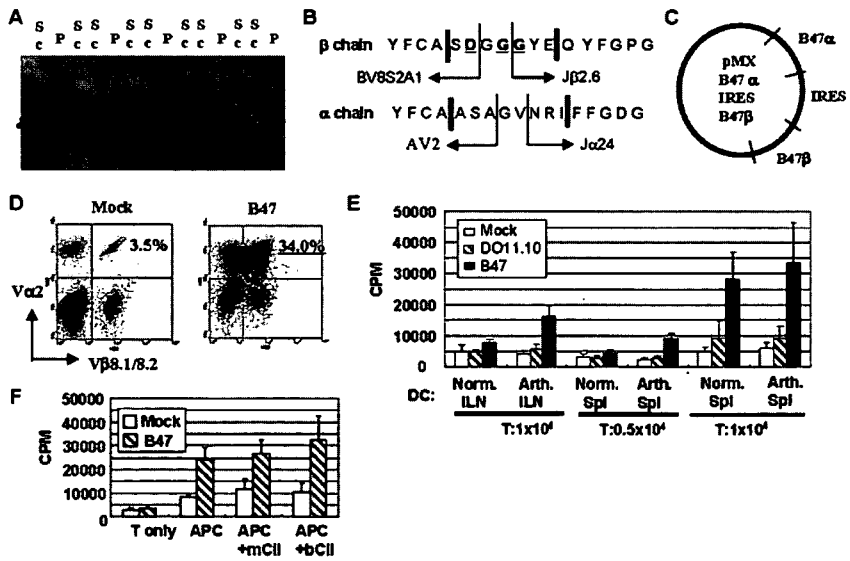

FIGURE 2. Identification and reconstitution of B47 TCR, which was autoreactive and not specific to CII. *A*, An example of identification of an expanded TCR in CIA by the TCR-SSCP method. RT-PCR was performed with Vβ8.1/8.2-specific and Cβ primers for cDNA of sorted single cells and the total paw. PCR products were subjected to electrophoresis. *Lane Sc*, Single-cell-sorted T cells. *Lane P*, Total paw T cells. A few TCR β-chains from sorted single cells exhibited the same mobility as that from the total paw (arrow). *B*, Amino acid sequences of identified B47 TCR α- and β-chains expanded in CIA. *C*, Schematic representation of the bicistronic retrovirus construct of B47 TCR. *D*, Representative result of retroviral transduction of B47 TCR in DBA1 splenocytes. The cells were triple stained for Vα2, Vβ8.1/8.2, and CD4. CD4 gated dot plots are shown. *E*, Representative result of autoreactivity of B47-transduced CD4$^+$ T cells. Indicated numbers of mock-, DO11.10-, or B47-transduced CD4$^+$ T cells and 1 × 10^4 of CD11c$^+$ dendritic cells were cultured in 96-well plates. *F*, A total of 1 × 10^4 mock- or B47-transduced cells were cultured with no APC, 1 × 10^4 splenic CD11c$^+$ cells, splenic CD11c$^+$ cells plus 100 μg/ml mCII, or splenic CD11c$^+$ cells plus 100 μg/ml bCII.

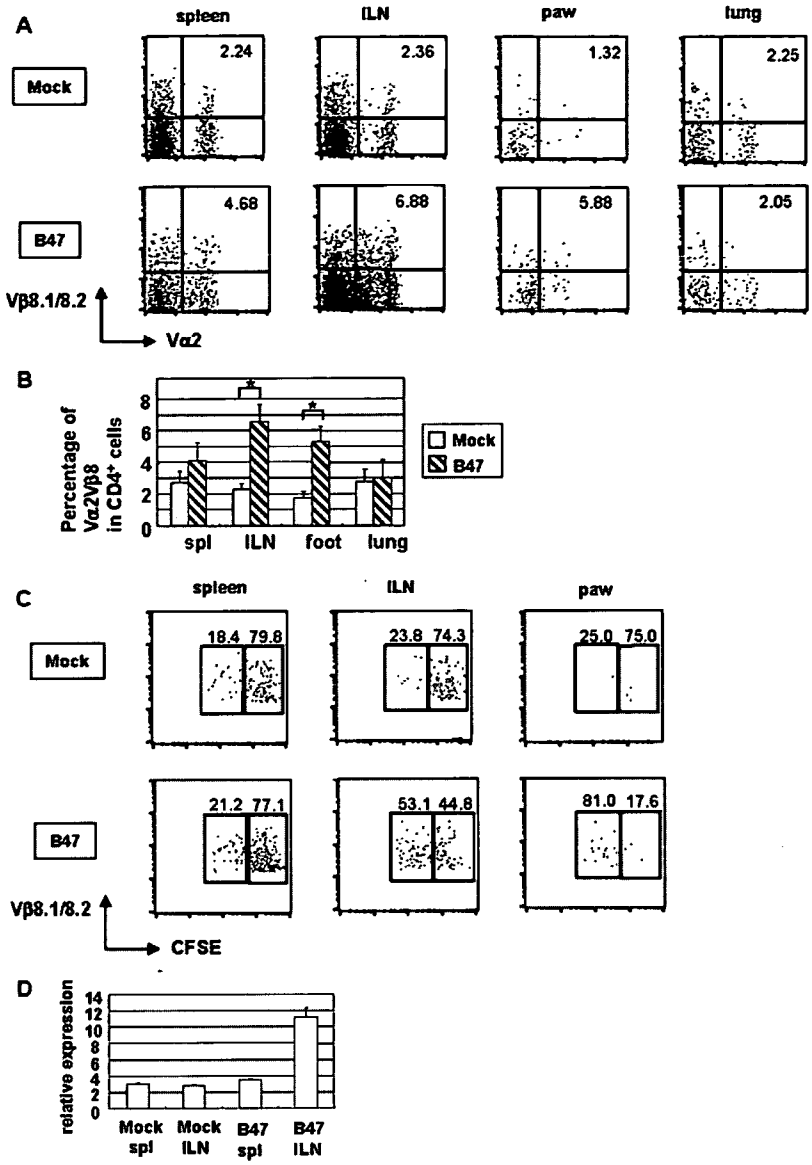

FIGURE 3. Kinetics of B47-transduced T cells in arthritic mice. *A*, Mock- or B47-transduced cells were labeled with CFSE and i.v. transferred to arthritic mice. Five days later, the spleen, ILN, paws, and lungs were analyzed for $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells by FACS. *B*, The average percentages of $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells in the indicated organs from three independent experiments. *, A significant difference ($p < 0.05$) compared with mock group. *C*, CFSE analysis of $V\alpha2^+V\beta8.1/8.2^+CD4^+$ T cells in the indicated organs. $V\alpha2^+CD4^+$ gated profiles are shown. *D*, IFN-γ expressions were quantified with real-time PCR in $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells from the indicated organs.

To investigate in vivo migration capacity of CIIT-transduced cells, CFSE-labeled CIIT-transduced cells were transferred to arthritic mice via the tail vein. The spleen, inguinal lymph nodes (ILN), paws, and lungs were analyzed 4 days after the transfer. Mock-transduced cells showed a relatively convergent peak of high CFSE fluorescence in all organs examined. CIIT-transduced cells also showed a relatively convergent peak of high CFSE fluorescence in the spleen and lungs. In contrast, CIIT-transduced cells showed a significant increase of cells with weak fluorescence in the ILN and paws (Fig. 1*D*). This result indicated that T cells reconstituted by paw Ag-specific TCR are able to accumulate at the site of arthritis.

Identification of a $V\beta8.1/8.2^+CD4^+$ T cell clone expanded in the arthritic paw using the TCR-SSCP method

To identify the TCR clone expanded in the arthritic paw, we focused on the TCR Vβ8.1/8.2 subfamily, which is one of the largest TCR Vβ subfamilies. We first examined sequences of the CDR3 motif of $V\beta8.1/8.2^+$ T cells in the inflamed paw of CIA mice. When we analyzed major clones in $V\beta8.1/8.2^+CD4^+$ T cells from seven arthritic mice, five of the seven mice had major clones with a similar motif containing aspartic acid and glycine in their CDR3, DXGG, DXXG, and DXGX (Table I).

To obtain a pair of TCR α- and β-chains from a cell expanded in the arthritic paws, we performed single-cell sorting of $V\beta8.1/8.2^+CD4^+$ T cells. cDNA was synthesized and the sequence of the TCRβ chain was determined by three-step nested PCR. The sequence of the TCRα chain was determined by three-step seminested PCR using a series of Vα1–22 primers. The β-chain sequences of ~50% of the sorted cells were determined. We compared TCR-SSCP of total paw $V\beta8.1/8.2^+CD4^+$ T cells and those of single cells (Fig. 2*A*). Some sorted single-cell clones had TCRβ chains that were identical with the major clone in the arthritic paw. Among identified clones, B47 was found to be expanded in the arthritic paw. The TCRβ chain of B47 made up 9.1% of total Vβ8.1/8.2 sequences in SSCP. The DXGG motif in CDR3 of the B47 β-chain suggests that B47 recognizes a common Ag in arthritis (Fig. 2*B*). The TCRα chain of B47 belonged to the Vα2 subfamily.

B47-transduced cells showed strong autoreactive response to $CD11c^+$ DCs from arthritic mice

We subcloned cDNA of the B47 α- and β-chain into a bicistronic retrovirus vector (Fig. 2*C*). The transduction efficiency of the B47 clonotype was determined by anti-Vα2 and Vβ8.1/8.2 Abs, and the clonotypic transduction efficiency was 30–40% on average

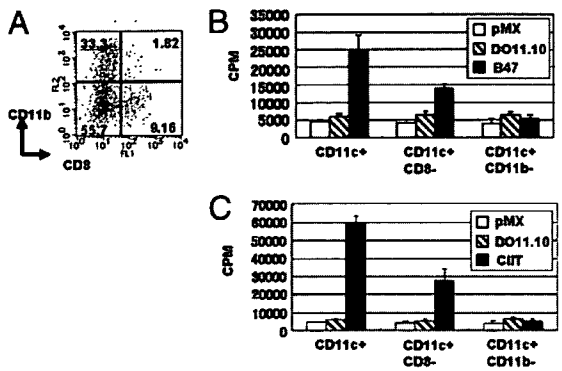

FIGURE 4. A DC subpopulation that stimulated B47- and CII-transduced $CD4^+$ T cells. *A*, ILN cells were stained with CD8-FITC, CD11b-PE, and CD11c-Tricolor and analyzed by FACS. A representative CD11c gated dot plot is shown. *B*, Stimulation of B47 TCR-transduced cells with ILN whole $CD11c^+$ cells, CD8-depleted $CD11c^+$ cells, and CD11b-depleted $CD11c^+$ cells. *C*, Stimulation of CII TCR-transduced cells with ILNl whole $CD11c^+$ cells, CD8-depleted $CD11c^+$ cells, and CD11b-depleted $CD11c^+$ cells.

(Fig. 2*D*). B47-transduced $CD4^+$ cells proliferated in the presence of autologous $CD11c^+$ DCs of the spleen and draining lymph nodes from naive mice (Fig. 2*E*). Though mock- and DO11.10-transduced $CD4^+$ cells were stimulated weakly by $CD11c^+$ DCs from naive and arthritic mice, B47-transduced $CD4^+$ cells proliferated more strongly in the presence of $CD11c^+$ DCs from arthritic mice (Fig. 2*E*). In addition, B47-transduced $CD4^+$ cells did not show increased proliferation in response to mCII and bCII (Fig. 2*F*). Therefore, B47 TCR was found to recognize an autoantigen that is presented more efficiently in arthritic mice.

We next examined the kinetics of B47-transduced $CD4^+$ cells in the arthritic mice. Mock- or B47-transduced $CD4^+$ cells were labeled for CFSE and transferred to arthritic mice via the tail vein. These mice groups were designated as the mock group and the B47 group, respectively. Five days after transfer, the accumulation of $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells was similar in the spleen and lung of the mock group and the B47 group (Fig. 3, *A* and *B*). In contrast, the accumulation of $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells in the ILN and paws of B47 group was significantly greater than that in mock group (Fig. 3, *A* and *B*). Moreover, $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells in the ILN and paws showed lower CFSE fluorescence than those in the spleen of the B47 group and in the ILN and paws of the mock group (Fig. 3*C*). $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells in the ILN of the B47 group showed higher expression of IFN-γ than those in the spleen of the B47 group or in the spleen and ILN of the mock group (Fig. 3*D*). This result indicated that transfer of B47 allowed $CD4^+$ T cells to accumulate in the draining lymph nodes and arthritic paws.

In Fig. 3*A*, the $V\alpha2^+V\beta8.1/8.2^-$ population also increased in these mice. In in vitro experiments using GFP-reported TCR α and β expression vectors (pMIG-TCRα and pMIG-TCRβ), the expression of the transduced TCR β-chain was rather unstable compared with that of the transduced TCR α-chain (K. Fujio, unpublished data). We suppose that this phenomenon was related to phenotypic allelic exclusion of the TCR β protein, because internal ribosomal entry site (IRES)-driven GFP expression was sustained despite a decrease of TCR β-chain expression. We think that at least a part of the $V\alpha2^+V\beta8.1/8.2^-$ population may have come from B47-transduced cells that lost TCR β expression.

We next explored the subpopulation of $CD11c^+$ DCs that can present arthritis-associated autoantigens. $CD11c^+$ DCs in ILN can classified into three groups, $CD11c^+CD11b^-CD8^-$ cells, $CD11c^+CD11b^+CD8^-$ cells, and $CD11c^+CD11b^-CD8^+$ cells (Fig. 4*A*). We compared the Ag presentation of total ILN $CD11c^+$ DCs, MACS-depleted $CD8^-CD11c^+$ DCs, and $CD11b^-CD11c^+$ DCs to

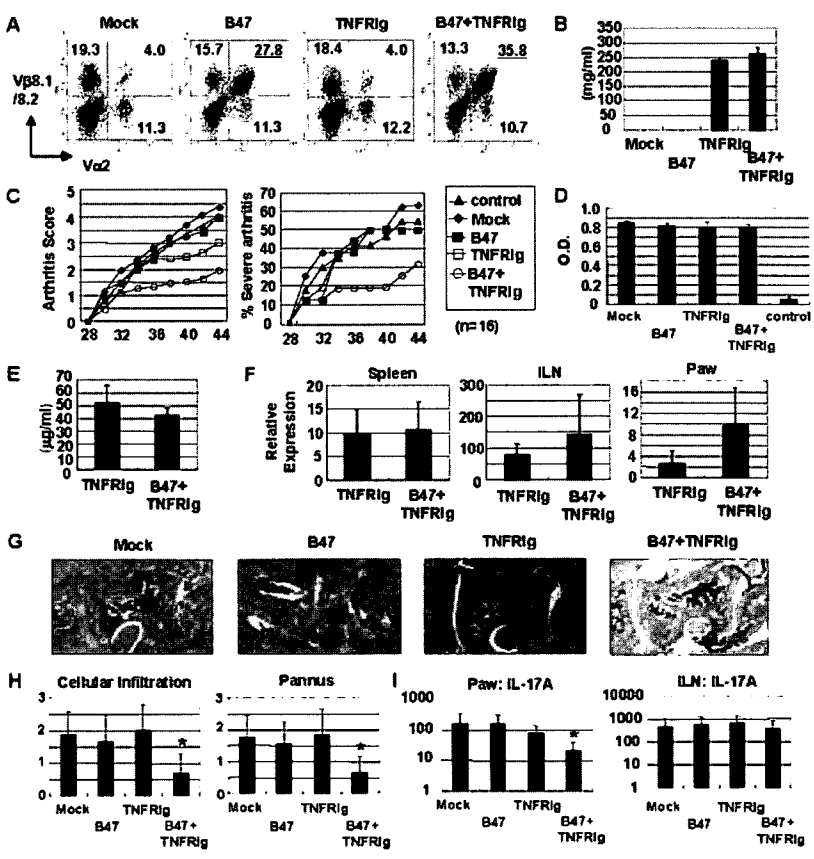

FIGURE 5. B47 and TNFRIg cotransduced $CD4^+$ T cells suppressed CIA progression. *A*, Retroviral gene transfer of B47 and TNFRIg in DBA1 splenocytes. The results shown are representative of three independent experiments. The cells were triple stained for Vα2, Vβ8.1/8.2, and CD4. CD4 gated dot plots are shown. *B*, TNFRIg concentrations in the culture supernatant of each experimental group. *C*, B47+TNFRIg-transduced $CD4^+$ cells containing 0.5–1 $\times$ 10^6 B47 and TNFRIg cotransduced cells were transferred to bCII-immunized mice just before the onset of arthritis (day 28). Data are shown as the mean of the clinical scores (*left panel*) and the incidence of severe arthritis (arthritis score $\geq$4) (*right panel*) at the indicated time points after the bCII priming (n = 16 per each group). ℄, PBS; ◆, Mock; f, B47; □, TNFRIg; E, B47+TNFRIg. *D*, Serum concentrations of anti-CII Ab in each experimental group. *E*, Serum concentrations of TNFRIg protein in the TNFRIg and B47+TNFRIg groups. *F*, Accumulation of the TNFRIg gene in arthritic paws. The relative expressions were determined by quantitative PCR. *G*, Histologic examination of each experimental group. *H*, The cellular infiltration and pannus invasion were scored for each experimental group. Data represent the mean values and SEs. * indicates a significant difference (p < 0.05) compared with mock group. *I*, The expressions of cytokine mRNAs were determined by quantitative PCR analysis. cDNAs were synthesized from the paws of each experimental group. Cytokine expressions in the paws (*left panel*) and ILN (*right panel*) are shown.

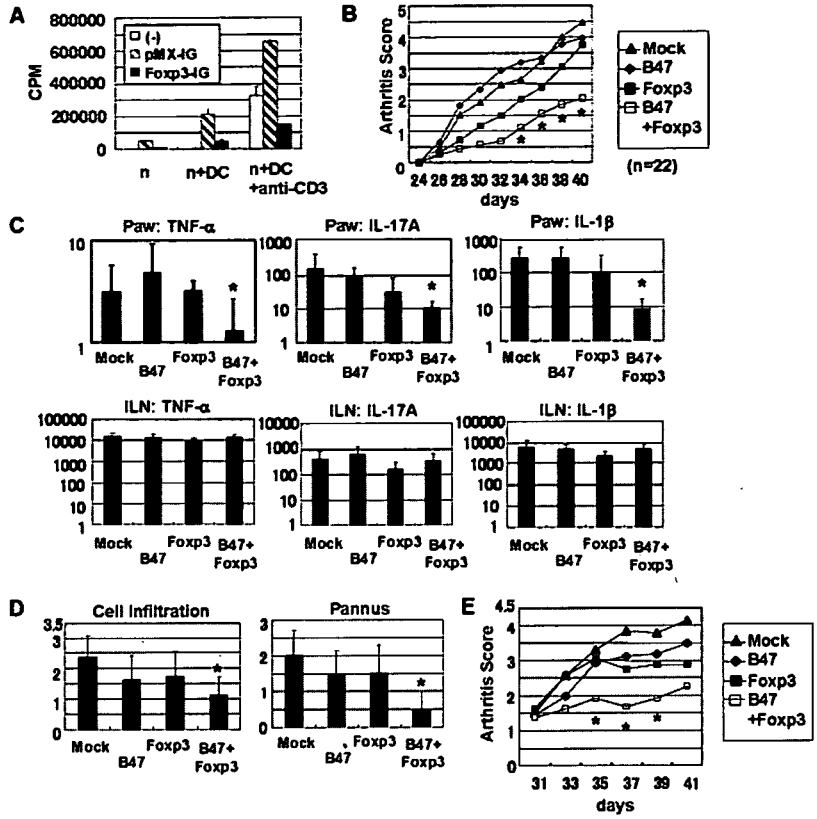

FIGURE 6. B47 and Foxp3 cotransduced T cells suppressed CIA and expression of pathogenic cytokines. *A*, Foxp3-reconstituted cells showed suppressive activity. A total of 1×10^5 naive $CD4^+$ cells (*n*) and 1×10^4 $CD11c^+$ cells (DC) in the presence or absence of anti-CD3 Ab (10 μg/ml) were cultured with no cells, 1×10^5 mock (pMX-IRES-GFP)-transduced $CD4^+$ cells, or Foxp3-transduced $CD4^+$ cells. Representative results of three experiments are shown. *B*, B47 and Foxp3 cotransduced T cells suppressed CIA progression. B47+Foxp3-transduced cells containing $0.5–1 \times 10^6$ of $V\alpha2^+V\beta8^+GFP^+CD4^+$ cells were transferred to bCII-immunized mice before the onset of arthritis (day 23). Equivalent numbers of mock, B47, and Foxp3-transduced cells were transferred as controls. Data are shown as the mean of clinical scores at the indicated time points after the bCII priming (n = 22 per each group). ▲, Mock; ◆, B47; ■, Foxp3; □, B47+Foxp3. *, A significant difference ($p < 0.05$) compared with the mock-transduced cells. *C*, The expressions of cytokine mRNAs were determined by quantitative PCR analysis. cDNAs were synthesized from the paws of each experimental group. Cytokine expressions in the paws (*upper panel*) and ILN (*lower panel*) are shown. *D*, The cellular infiltration and pannus invasion were scored for each experimental group. Data represent the mean values and SEs. *, A significant difference ($p < 0.05$) compared with the mock group. *E*, Reconstituted regulatory T cells were transferred to bCII-immunized mice just after the arthritis score reached approximately two points. Data are shown as the mean of clinical scores at the indicated time points after the bCII priming (n = 12 per each group). ▲, Mock; ◆, B47; ■, Foxp3; □, B47+Foxp3. *, A significant difference ($p < 0.05$) compared with the mock group.

B47-transduced $CD4^+$ cells. As shown in Fig. 4*B*, CD11b-depleted $CD11c^+$ DCs lost their autoantigen presentation to B47-transduced $CD4^+$ cells. We next examined the autoantigen presentation to CIIT-transduced $CD4^+$ cells. CD11b-depleted $CD11c^+$ DCs from ILN cells of CIA mice also lost their autoantigen presentation to CII-specific T cells (Fig. 4*C*). These results indicated that $CD11b^+CD11c^+$ DCs are important APCs in arthritis.

B47 plus TNFRIg-transduced cells suppressed CIA

We next attempted to use paw-directed B47-transduced $CD4^+$ cells as a vehicle for therapeutic molecules. We constructed a TNFRIg-expressing vector by fusing the murine p75 TNFR and Fc domain of IgG2a. TNFRIg-producing paw-directed cells were generated by triple gene transfer of B47 TCR and TNFRIg. We prepared three groups receiving controlled gene transfer of either mock vector, B47 alone, or TNFRIg alone. The clonotypic transduction efficiency was ~30% on average (Fig. 5*A*). Though we could not directly detect the transduction efficiency of TNFRIg, the TNFRIg protein concentrations in the culture supernatant of B47 plus TNFRIg-transduced $CD4^+$ cells were equivalent to those of TNFRIg (Fig. 5*B*). Therefore, the transduction efficiency of the *TNFRIg* gene was considered to be almost equal in these two groups.

These mock, B47, TNFRIg, or B47 plus TNFRIg transduced cells were i.v. transferred to CII-immunized mice via the tail vein just before the onset of arthritis at day 28. These mice groups were designated as mock group, B47 group, TNFRIg group, and B47 plus TNFRIg group, respectively. The arthritic score of B47 plus TNFRIg group was evidently suppressed compared with those of the mock and B47 groups (Fig. 5*C*). The arthritis score of the TNFRIg group was slightly suppressed. In terms of the incidence of severe arthritis, the B47 plus TNFRIg group clearly showed the lowest rate.

Accumulation of TNFRIg transcript in the paws was important for arthritis suppression

We next examined the kinetics of the transduced *TNFRIg* gene. Because the titers of anti-CII IgG at day 38 were equivalent in all experimental groups, TNFRIg did not directly affect the humoral immune response (Fig. 5*D*). The serum concentrations of TNFRIg protein in the B47 plus TNFRIg group were equivalent to those in the TNFRIg group at day 38 (Fig. 5*E*). This result indicated that the serum concentration of TNFRIg was not the main determinant of arthritis suppression in the B47 plus TNFRIg group.

We then checked the accumulation of TNFRIg transcript in the lymphoid organs and paws. The amount of TNFRIg transcript was

determined by real-time PCR of cDNAs from tissues of day 46 (Fig. 5*F*). The amount of TNFRIg transcript was equivalent between these two groups in the spleen and ILN. This result was consistent with the equality of the serum concentration of TNFRIg. In contrast, the amount of TNFRIg in the paws of the B47 plus TNFRIg group was significantly higher than that in the paws of the TNFRIg group. Therefore, local accumulation of the TNFRIg transcript suppressed arthritis in the B47 plus TNFRIg group.

On histologic examination, although the control groups showed severe inflammation, the B47 plus TNFRIg group showed only marginal inflammation (Fig. 5*G*). We graded mononuclear cell infiltration and cartilage/bone destruction by histopathological determination of the pannus invasion. Mononuclear cell infiltration and pannus formation were significantly suppressed in the B47 plus TNFRIg group (Fig. 5*H*).

We next evaluated the gene expression profiles of the paws and ILN by quantitative PCR. Among the cytokines important for arthritis progression, the expression of IL-17A was significantly suppressed in the paws, but not in ILN (Fig. 5*I*). In contrast, the expressions of TNF-α and IL-1β were not significantly suppressed in either the paws or ILN.

B47 plus Foxp3-transduced T cells suppressed CIA

We next tried to generate paw-directed regulatory T cells by cotransfer of B47 and Foxp3. It has previously been shown that retroviral transduction of Foxp3 confers a regulatory function onto $CD4^+$ T cells (5) (Fig. 6*A*). We generated B47 plus Foxp3-transduced cells (B47 plus pMX-Foxp3-IRES-GFP); three groups received controlled gene transfer of either the mock vector (pMX plus pMX-IRES-GFP), B47 alone (B47 plus pMX-IRES-GFP) or Foxp3 alone (pMX plus pMX-Foxp3-IRES-GFP). These gene-transduced cells were i.v. transferred to bCII-immunized mice before the onset of arthritis (day 23). These mice groups were designated as mock group, B47 group, Foxp3 group, and B47 plus Foxp3 group, respectively. B47 plus Foxp3 group showed a significant suppression in the development of arthritis (Fig. 6*B*). Foxp3 group showed only a marginal suppression of arthritis.

The titers of anti-CII Abs did not differ among these experimental groups (data not shown). When we evaluated the gene expression profiles of the paws and ILN by quantitative PCR, TNF-α, IL-17A, and IL-1β were found to be significantly suppressed (Fig. 6*C*). A suppressive cytokine, IL-10, was not up-regulated in the B47 plus Foxp3 group (data not shown). In ILN, the expression of TNF-α, IL-17A, and IL-1β was not suppressed in the B47 plus Foxp3 group (data not shown).

On histologic examination, although the control groups showed severe inflammation, the B47 plus Foxp3 group showed only marginal inflammation (data not shown). Mononuclear cell infiltration and pannus formation were suppressed in the B47 plus Foxp3 group (Fig. 6*D*). These results suggest that regulatory T cells at arthritic sites suppress bone destruction as well as inflammation. In contrast, Foxp3-transduced T cells without Ag specificity were not sufficient for arthritis suppression. Reconstituted regulatory cells also showed effective suppression when transferred after the onset of arthritis, at which time the average arthritic score reached around two points (Fig. 6*E*).

Discussion

We demonstrated the therapeutic efficacy of T cells transduced with an arthritis-associated TCR and a soluble and intracellular molecule. To obtain these paw-homing T cells, we cloned TCR from T cells expanded in the arthritic paws using a combination of single-cell sorting and TCR-SSCP. This method enabled us to identify the TCRs expanded in the inflamed tissues.

In response to the treatment with TNFRIg, T cells coexpressing B47 and TNFRIg exhibited suppressive activity associated with local accumulation. This result suggested that the main determinant of therapeutic efficacy in anti-TNF therapy is local accumulation, not serum concentration. Therefore, the conventional systemic administration of an anti-TNF drug that depends on serum concentration may not be a reasonable therapy. An elevated serum concentration is associated with systemic immunosuppression and high cost of treatment. Local injection of an anti-TNF drug is another approach to avoid a systemic suppressive effect (23, 24). However, this approach is not ideal due to the polyarthritic nature of RA. In contrast, T cells that produce TNFRIg and accumulate in the paws at the arthritic sites can reach multiple paws with reduced systemic effect.

The TCR transfer was also effective in the treatment with intracellular Foxp3 expression. Though suppression of murine arthritis with polyclonal regulatory T cells have been reported (25), the importance of T cell specificity has not been addressed. In the Foxp3 transfer experiment, Foxp3-expressing T cells with arthritis-associated TCR were effective. Once activated, regulatory T cells exhibit suppression in an Ag nonspecific manner (26). However, Ag specificity is important in the migration and expansion of regulatory T cells (27, 28). Indeed, Ag-specific regulatory T cells are efficient in suppressing various autoimmune diseases. The problem is how to obtain a sufficient amount of organ-Ag-specific regulatory T cells for therapeutic transfer. TCR and Foxp3 gene transfer is one possible approach to overcome this problem. Many mice spleens may be required to obtain $0.5–1.0 \times 10^6$ of $CD4^+CD25^+$ regulatory T cells, which is required to treat one mouse in the prior CIA treatment (25). In contrast, in vitro-expanded cells derived from a quarter of a spleen were sufficient to treat one mouse in our experiment.

Several groups have reported that regulatory T cells are accumulated in the joints of arthritis patients (29, 30). These joint-accumulating $CD4^+CD25^+$ T cells display a greater ability to suppress arthritis than blood $CD4^+CD25^+$ T cells. However, the precise role that these accumulating regulatory T cells play in the pathology of arthritis has not been clarified. Our experiments suggest that regulatory T cells in arthritic joints have the capacity to suppress pathogenic cytokine expression and bone destruction. Moreover, it is noteworthy that reconstituted regulatory T cells suppressed ongoing arthritis (Fig. 6*E*). There are several evidences that blocking of a specific inflammatory cascade ameliorates CIA after the onset. IL-10 and anti-IL-17A have been reported to inhibit ongoing CIA (31, 32). Our results suggested that regulatory T cells suppress arthritis by blocking the continuous inflammatory process. Therefore, regulatory T cells or Foxp3 therapy may be a feasible approach for established RA patients.

In therapeutic experiments for autoimmune diseases, use of a TCR without specificity to the disease-priming Ag can be an advantage. In our experiment, transfer of B47-transduced cells did not exacerbate arthritis. If CII-specific TCR is used for treatment, there is a possibility that the arthritis will be exacerbated due to enhancement of anti-CII immunity. This potential risk is important for the priming Ag-specific T cell-based treatment of other autoimmune diseases or human diseases that last for a significantly longer period than the diseases in mouse models. Indeed, it is necessary to clarify the specificity of these TCRs associated with arthritis or other autoimmune disorders before clinical application. Despite epitope screening with synthetic combinatorial peptide libraries in a positional scanning format (PS-SCL) (33), the precise autoantigen for B47 has not been determined.

We confirmed the clonal expansion of autoreactive $CD4^+$ T cells that were not specific to the priming Ag in the arthritic paws

of this mouse model. This result may have important implications for the treatment of autoimmune inflammation. Because $CD11c^+CD11b^+$DCs present both CII and an Ag recognized by B47, this DC population may be associated with copriming of B47 upon CII immunization.

In summary, we identified a TCR that is expanded in arthritic paws by a combination of TCR-SSCP and single-cell sorting. This arthritis-associated TCR that was not specific to the disease-priming Ag was used as a highly effective therapeutic vehicle for both soluble and intracellular molecules.

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Disclosures

The authors have no financial conflict of interest.

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