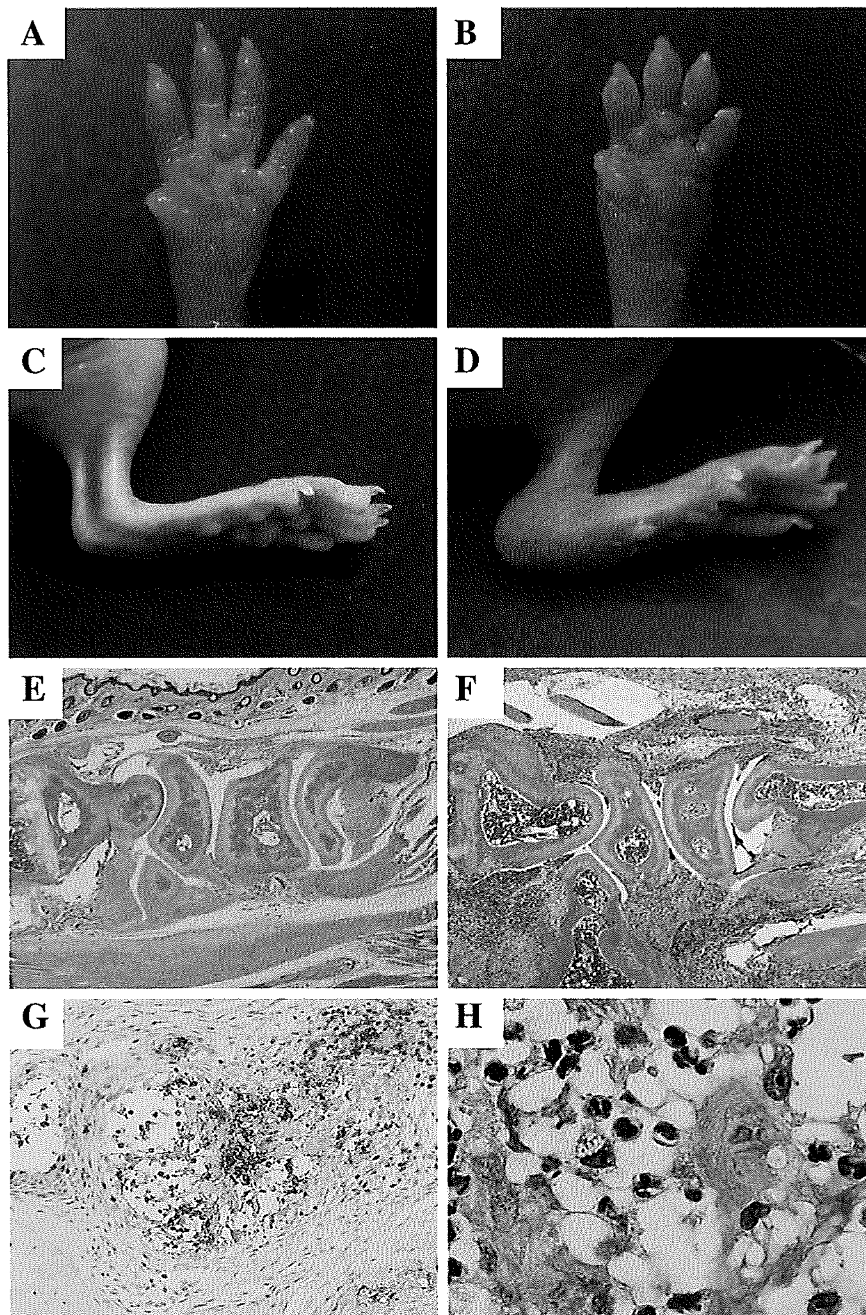

Fig. 5. Arthritis in athymic nude mice transferred with SKG T cell clones. (A–D) Macroscopic views of a forepaw (A) and a hind paw (C) of a recipient of control dengue 2F7 and a forepaw (B) and a hindpaw (D) of a recipient of 35S. (E–H) Histology of the joints of recipients of control dengue 2F7 (E)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

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

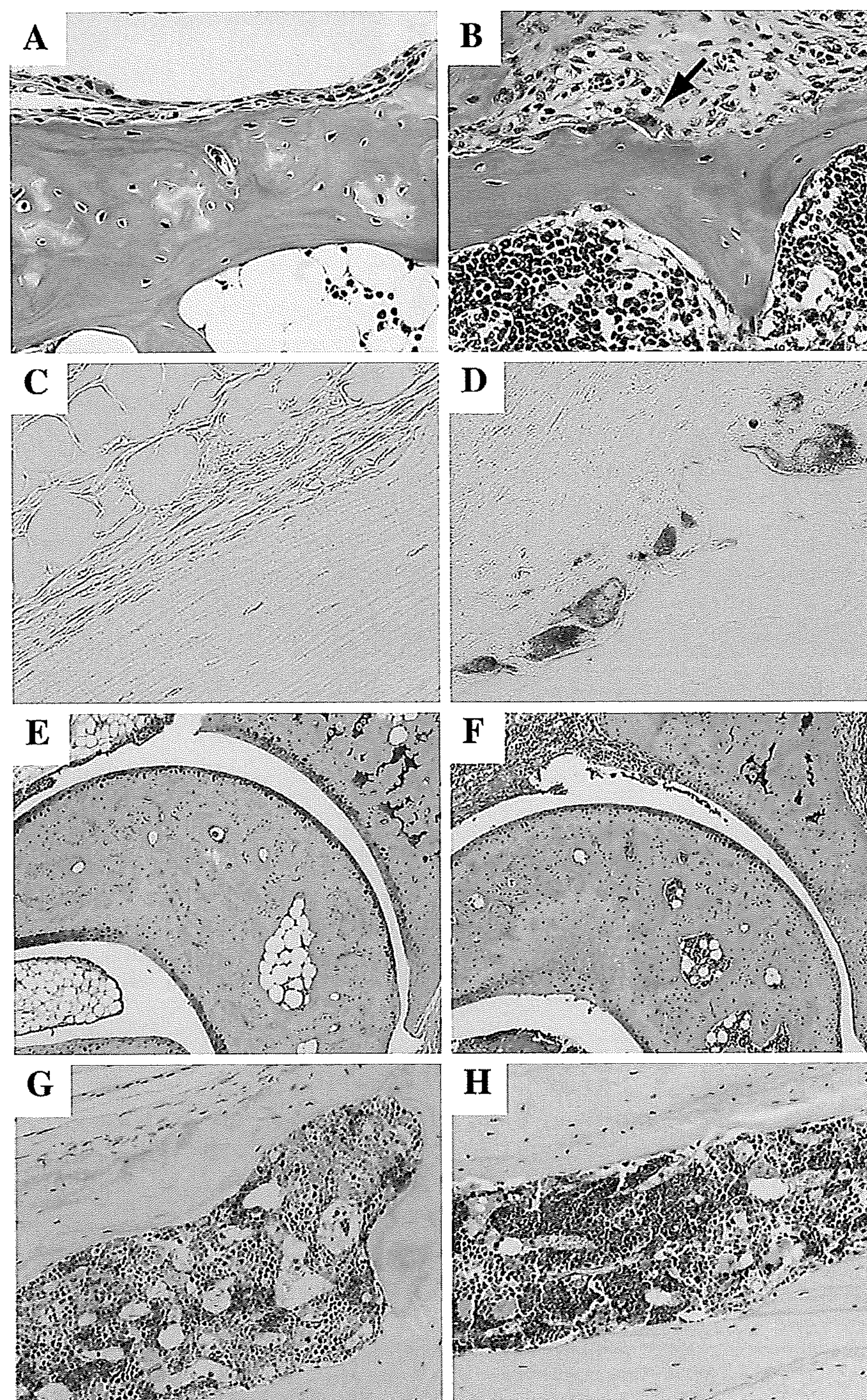

Fig. 6. Bone and cartilage destruction in athymic nude mice transferred with SKG T cell clones. High magnification of H&E-stained sections of a nude mouse recipient of dengue 2F7 (A) or 35S (B), showing bone erosion by pannus and BM activation (×400). Multinuclear cells (osteoclasts) (arrow) are also observed. Tartrate-resistant acid phosphatase-positive cells (osteoclasts) are detected in a 35S recipient (D) but not in a 2F7 recipient (C) (×400). By Safranin-O staining, proteoglycan stained red decreases in the articular cartilage matrix of a recipient of 35S (F) but not in a recipient of 2F7 (E) (×100). By immunohistochemistry, Gr-1-positive cells increase in the BM of a 35S recipient (H) but not in a 2F7 recipient (G) (×200). (A, C, E and G) 12 months after transfer; (B, D, F and H) 10 months after transfer.

Discussion

In this study, we have established two distinct $CD8^+$ T cell clones from arthritic lesions of SKG mice. Interestingly, both exhibited *in vitro* autoreactivity against not only synoviocytes but also a variety of MHC-matched cell lines and elicited both arthritis and interstitial pneumonitis when transferred to

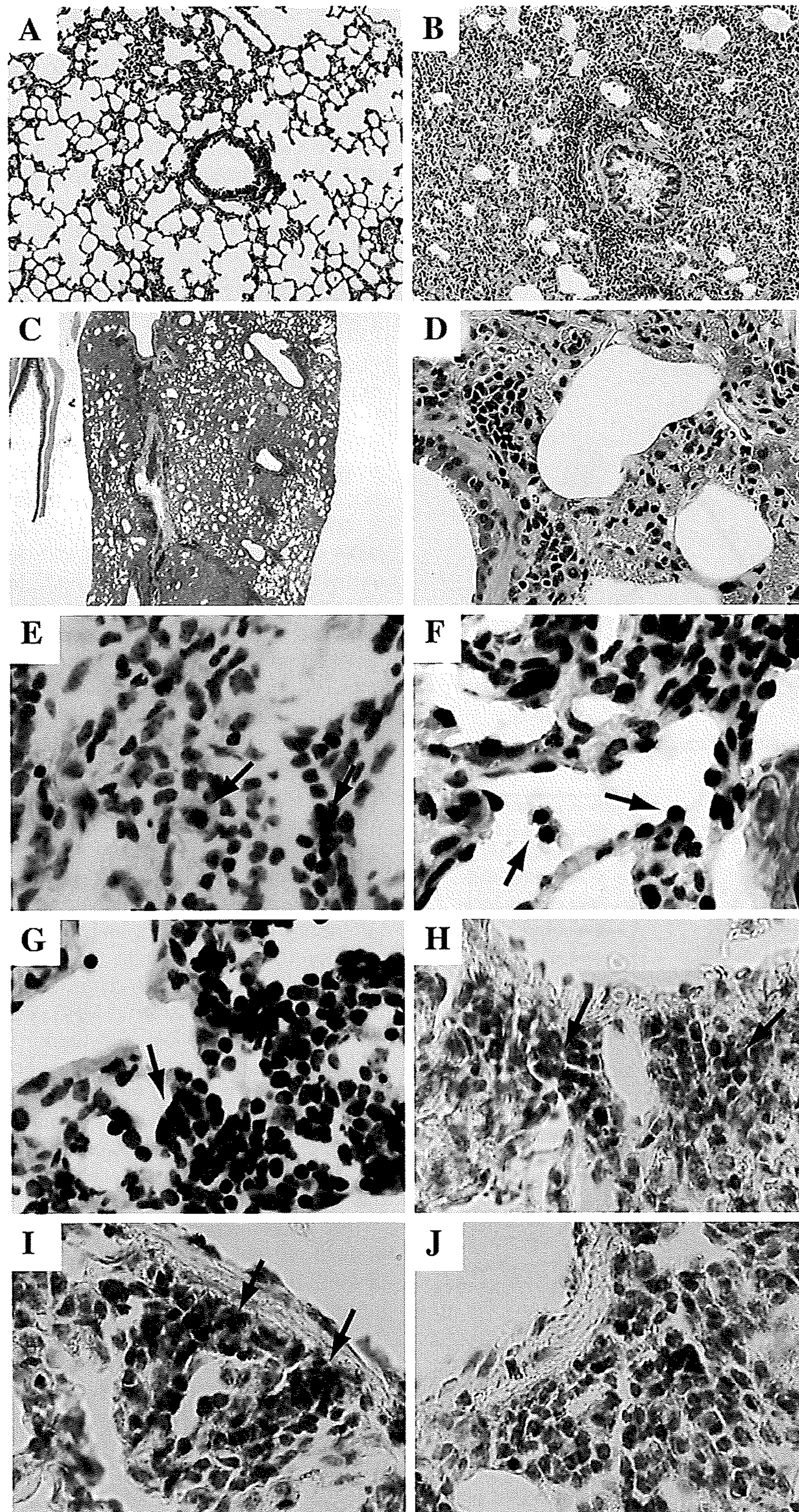

Fig. 7. Interstitial pneumonitis induced by the transfer of SKG T cell clones. (A–D) H&E-stained sections of the lungs of the recipients of control dengue 2F7 clone (A) or 73S clone (B–D) (A–B, ×100). Lower (C, ×10) and higher (D, ×400) magnification of the lung of 73S clone recipient show thickening of alveolar walls diffusely in the lung. (E–J) Serial sections of a lung of a 73S recipient mouse were stained for Ly-6G (Gr-1) (E), F4/80 (F), B220/CD45R (G), CD8a (H) or CD4 (I), with staining control (J) (×400). Typically positive cells in these stainings are arrowed. (A–J) 6 months after transfer.

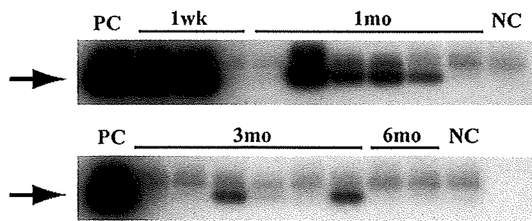

Fig. 8. Detection of a clone-specific TCR message of 35S clone in spleens by RT-PCR amplification and Southern blot analysis. After transfer of 1 × 10^7 clone cells to BALB/c nude mice, RNA was extracted from spleens at indicated days. PC, positive control (RNA from 35S clone diluted to 1%); NC, negative control (RNA from a 6-month-old non-treated BALB/c athymic nude mouse). The separate lanes represent individual mice.

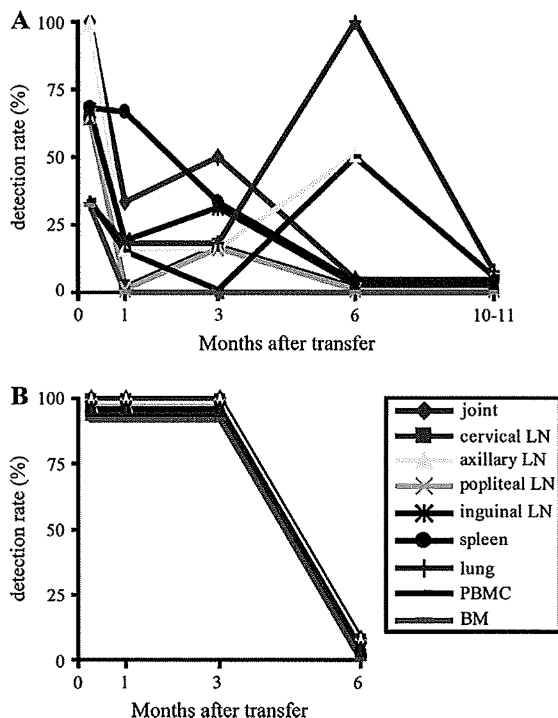

Fig. 9. Detection of TCR mRNA of the transferred clones in recipient BALB/c nude mice. 35S (A) or dengue 2F7 (B) in the recipients were detected by Southern blot analysis using primers and probes specific for TCR V and J region and CDR3 sequences of each clone. All mice with at least one positive signal out of four joints were considered to be positive. (A) n = 3 at 1 week; n = 6 at 1 month; n = 6 at 3 months; n = 2 at 6 months; n = 2 at 10–11 months. (B) n = 2 in every group. No signal was detected in control 6- or 11-month old BALB/c nude mice in each Southern blot analysis (data not shown).

histocompatible T cell-deficient mice. Furthermore, the arthritic and pulmonary lesions chronically progressed irrespective of the decline in the number of transferred T cell clones to hardly detectable levels in either lesion.

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

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

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

highly sensitive to pro-inflammatory cytokines, for example systemic overproduction of transgenic TNF-α or IL-1 almost exclusively produces chronic arthritis even in mice deficient of both T and B cells (37–39); similarly, systemic deficiency of the IL-1R antagonist, and resulting overproduction of IL-1, or systemic alteration of signal transduction via IL-6 receptor results in predominant development of arthritis with no inflammatory damage to other tissues (40, 41). These findings collectively indicate that synoviocytes are much more sensitive to the SKG self-reactive T cell clones (at least to those secreting pro-inflammatory cytokines) than other tissue cells, even if the common self-antigens recognized by the clones are ubiquitously expressed. In addition, synoviocytes are unique in that they are the target cells and also the mediators of autoimmunity, i.e. upon stimulation (e.g. by cytokines or via cell contact stimulation by self-reactive T cells), they proliferate and secrete pro-inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) and other inflammatory substances (matrix metalloproteinases and prostaglandins), mediating inflammation and tissue damage (42). It is likely that the cells composing the alveolar walls, in particular the alveolar macrophages, are sensitive and responsive to T cell self-reactivity in a similar manner as synoviocytes and that excessively and chronically activated macrophages might mediate alveolitis and interstitial inflammation. A similar mechanism might also be responsible for the development of colitis in SKG mice (Table 1).

We do not assert, however, that SKG arthritis and pneumonitis are solely mediated by T cells recognizing a ubiquitous common self-antigen. We have previously shown that SKG mice spontaneously produce IgG isotype auto-antibody specific for joint-rich type II collagen or IgG antibody cross-reactive with hsp-70 of Tuberculosis bacilli (14). This indicates that helper CD4$^+$ T cells that specifically react with these self-antigens may also be induced in SKG mice either primarily or secondarily to joint damage. Moreover, we have recently shown that some self-reactive T cells in SKG mice may not be arthritogenic but can polyclonally stimulate antigen-presenting cells in the spleen and lymph nodes to secrete IL-6 and other cytokines, which in turn facilitate differentiation of potentially arthritogenic self-reactive T cells to T_h17 effector T cells that mediate synovitis (19). In addition to our current approach to the characterization of antigen specificity of SKG autoimmune T cells by preparing T cell clones, efforts are being made to further characterize infiltrating T cells *in situ* at a single-cell level by amplifying their TCR message.

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

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

Supplementary data

Supplementary figure is available at *International Immunology* Online.

Funding

Grants-in-Aid from the Ministry of Education, Sports and Culture, the Ministry of Human Welfare of Japan; Japan Science and Technology Agency.

Acknowledgements

The authors thank Z. Fehervari for critically reading the manuscript and the members of our laboratories for valuable comments.

Disclosures

The authors declare no conflicting interests.

Abbreviations

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

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

Yumi Yamaguchi,* Keishi Fujio,[2]* Hirofumi Shoda,* Akiko Okamoto,* Nelson H. Tsuno,[†] Koki Takahashi,[†] and Kazuhiko Yamamoto*

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

*Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and [†]Department of Transfusion Medicine and Immunohematology, University of Tokyo, Tokyo, Japan

Received for publication April 12, 2007. Accepted for publication September 5, 2007.

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[1] This study was supported by Program and Project Grant funding from Japan Society for the Promotion of Science; Ministry of Health, Labour and Welfare; and Ministry of Education, Culture, Sports, Science and Technology.

[2] Address correspondence and reprint requests to Dr. Keishi Fujio, Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. E-mail address: kfujio-tky@umin.ac.jp

[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.

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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,

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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.

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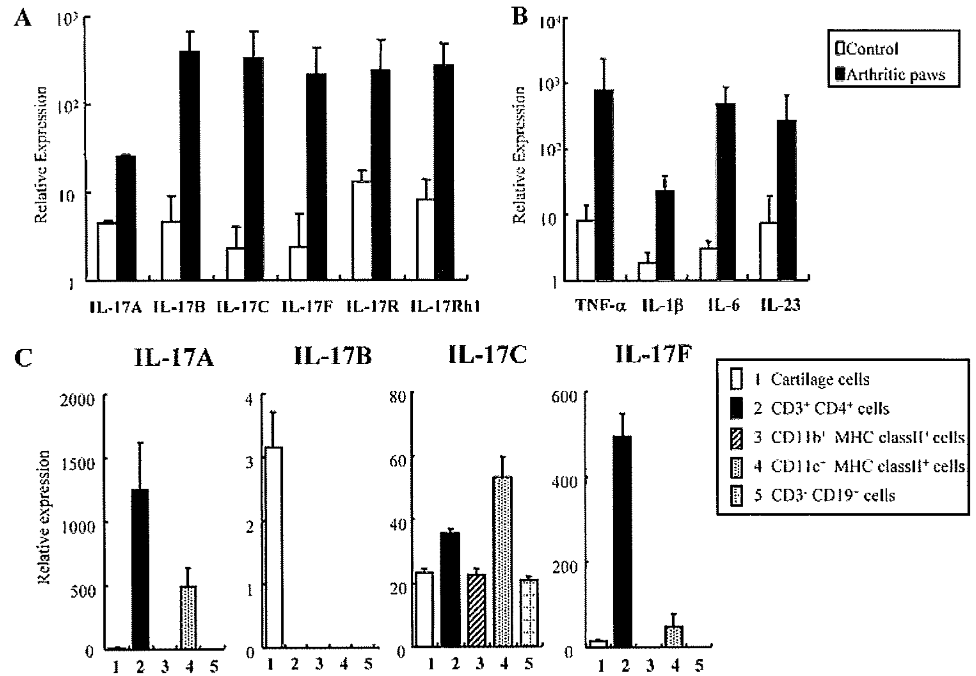

FIGURE 1. The expression of IL-17 family members and IL-17R genes in the arthritic paws of CIA mice. *A*, The expressions of IL-17 family genes and IL-17R genes were examined in the arthritic paws of CIA mice (■; $n = 3$) and in control mice (□; $n = 3$) by quantitative PCR. *B*, The expressions of inflammatory 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

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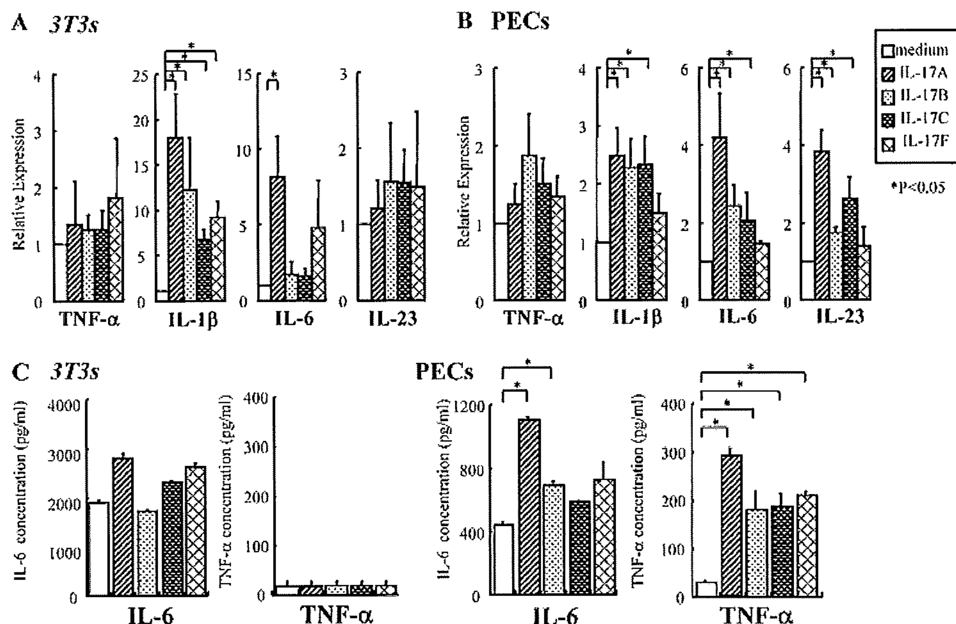

FIGURE 2. The proinflammatory effects of IL-17 family members on mouse fibroblasts and macrophages. *A*, Relative expression of the cytokine genes in *3T3* cell. The mouse fibroblast cell line *3T3* was cultured with each of mIL-17A, mIL-17B, mIL-17C, or mIL-17F for 24 h, and the expressions of inflammatory 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; *, $p < 0.05$.

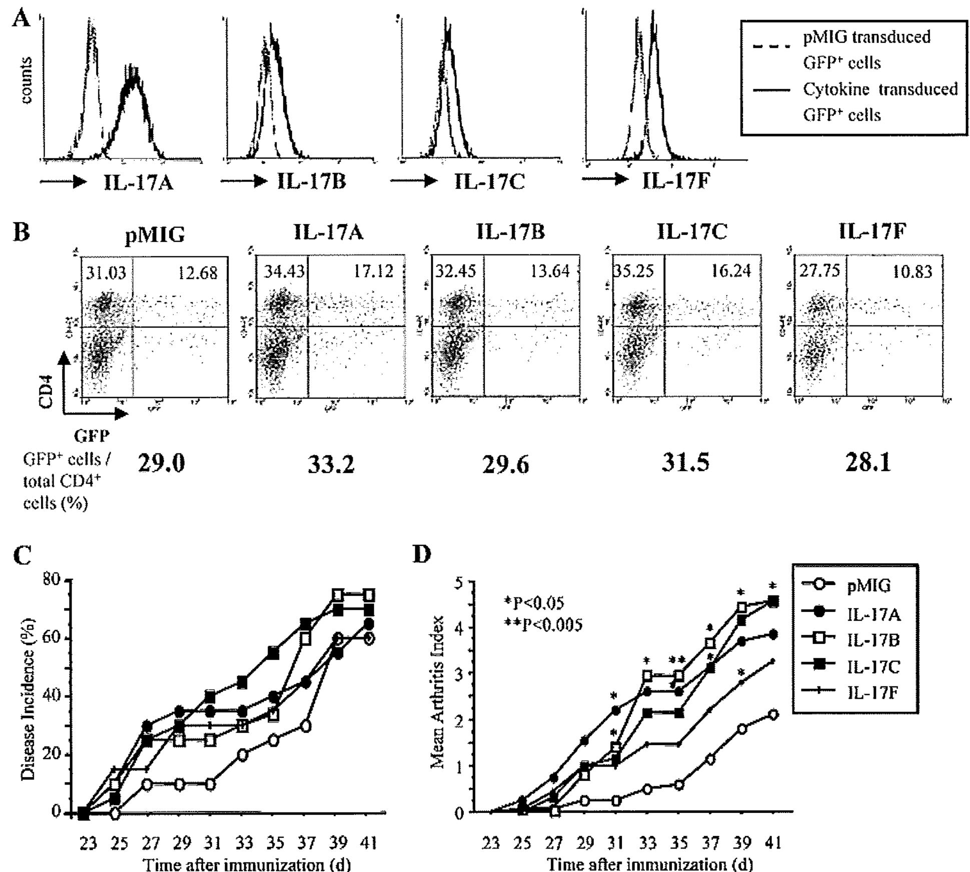

FIGURE 3. The effects of transfer of IL-17 family-transduced CD4$^+$ T cells on CIA. *A*, Intracellular IL-17 family expressions in Ba/F3 cells retrovirally transduced with each IL-17 family member. GFP-gated IL-17 family-transduced (mIL-17A, mIL-17B, mIL-17C, or mIL-17F) Ba/F3 cells were analyzed for 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.

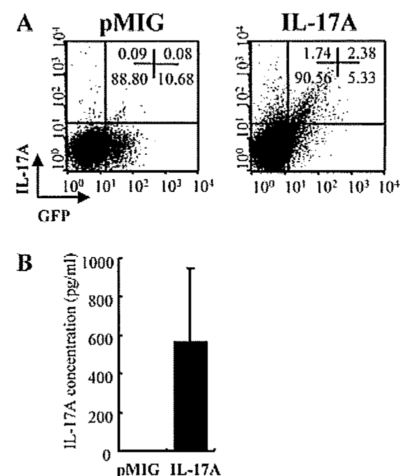

FIGURE 4. Generation of IL-17 family chimeric mice by BM transplantation of gene-transduced BM cells. Each of IL-17 family genes was transduced to BM cells with retrovirus vector and transferred to lethally irradiated mice. *A*, The intracellular expression of IL-17A protein in the spleen of IL-17A BM 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.

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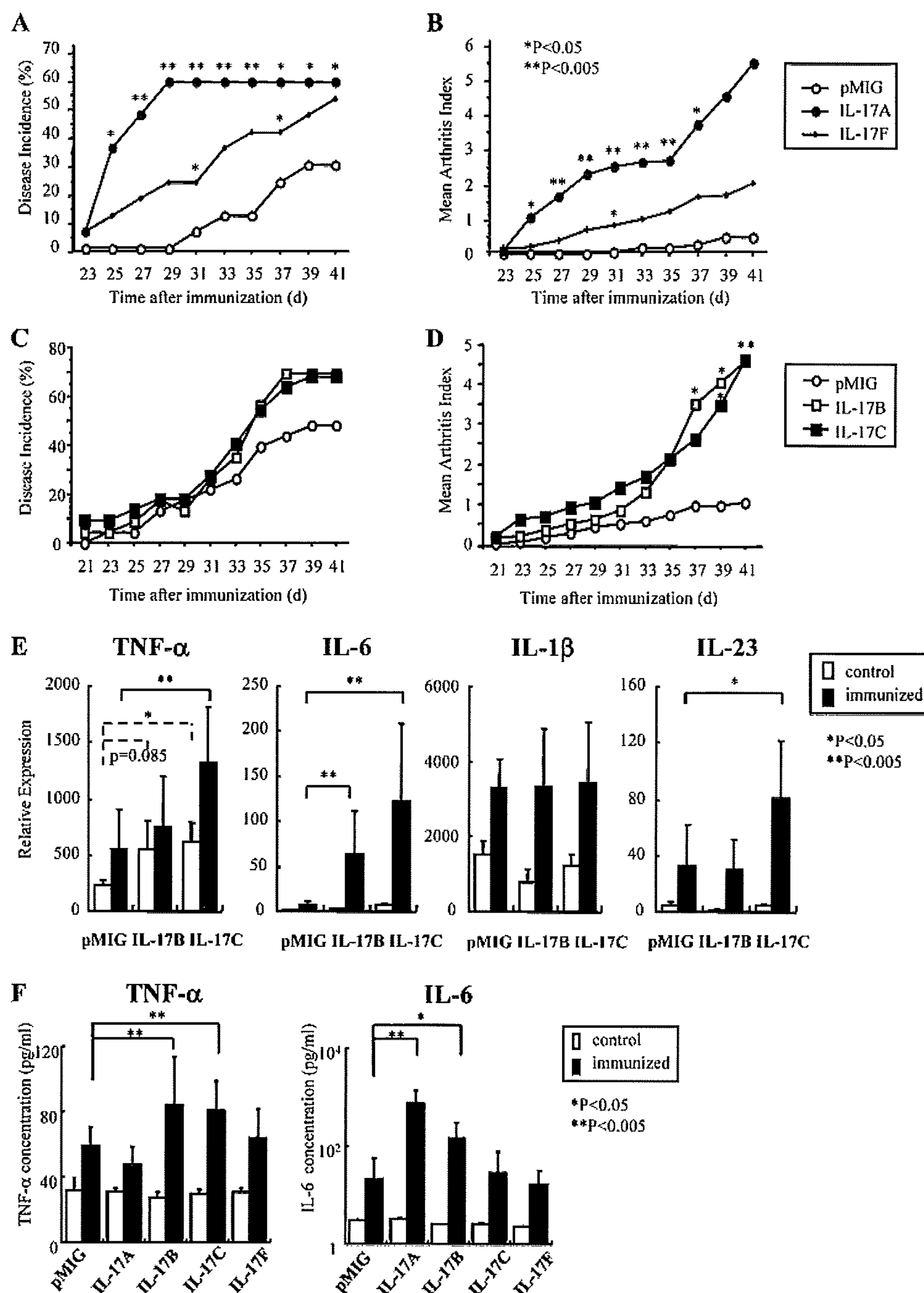

FIGURE 5. Incidence of CIA and arthritis scores in IL-17 family BM chimeric mice. Incidence of CIA and arthritis scores in IL-17A and IL-17F BM chimeric mice (*A* and *B*), and in IL-17B and IL-17C BM chimeric mice (*C* and *D*). Mice were immunized with BCII 8 wk after the BM transplantation. Values 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

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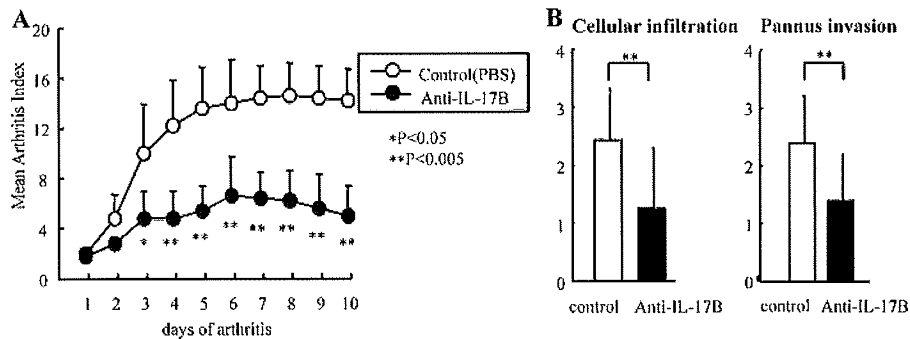

FIGURE 6. Effect of anti-IL-17B Ab treatment in CIA mice. *A*, CIA mice received i.p. injection of anti-mouse IL-17B Abs after the first clinical signs of arthritis (arthritis score between 1 and 2). As a control, PBS was injected. The arthritis score was shown. *B*, Histological score of the inflammatory 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

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(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.

Acknowledgments

We are grateful to Yayoi Tsukahara and Kayako Watada for their excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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Mod Rheumatol (2009) 19:366–371
DOI 10.1007/s10165-009-0174-0

ORIGINAL ARTICLE

Altered peptide ligands regulate type II collagen-induced arthritis in mice

Ei Wakamatsu · Isao Matsumoto · Yohei Yoshiga · Taichi Hayashi · Daisuke Goto · Satoshi Ito · Takayuki Sumida

Received: 4 March 2009 / Accepted: 2 April 2009 / Published online: 12 May 2009
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Abstract We reported that peripheral blood mononuclear cells from HLA-DRB1*0101 Japanese patients with rheumatoid arthritis (RA) were highly reactive to 256–271 peptide of type II collagen (CII). Similar to RA, T cells reactive to CII (AA256–271) play a crucial role in the generation of arthritis in CII-induced arthritis mouse (I-A^q). In the present study, we regulated the CII reactivity of T cells from CIA mouse with I-A^q by altered peptide ligand (APL). Eight different APLs were designed and screened for their antagonistic activity using CII reactive cytokine production assay. Four APLs of CII 256–271 exhibited antagonistic activity in CII-reactive T cells. Moreover, intraperitoneally injected APL-5 (G262A) significantly suppressed CII-induced arthritis in mice, whereas the other three APLs did not. Compared with the control, APL-5 suppressed interleukin (IL)-17 production by T cells from CII-induced arthritis mice. These results suggest that CII APL is a potentially suitable therapeutic strategy for the control of RA.

Keywords Altered peptide ligand · Antagonist · Type II collagen-induced arthritis · T cells

E. Wakamatsu is a research fellow of the Japan Society for the Promotion of Science.

E. Wakamatsu · I. Matsumoto · Y. Yoshiga · T. Hayashi · D. Goto · S. Ito · T. Sumida (✉)
Division of Clinical Immunology,
Doctoral Programs in Medical Sciences,
Major of Advanced Biomedical Applications,
Graduate School Comprehensive Human Science,
University of Tsukuba, 1-1-1 Tenodai,
Tsukuba, Ibaraki 305-8575, Japan
e-mail: tsumida@md.tsukuba.ac.jp

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by persistent inflammatory synovitis leading to various degrees of cartilage destruction, bone erosion, and ultimately joint deformity and loss of joint function. Although the pathogenesis of RA is not clear, there is sufficient evidence to suggest the involvement of T cells in the inflammatory process, such as the infiltration of T cells, especially $CD4^+$ $CD45RO^+$ T cells, in joints of RA patients [1]. Furthermore, the susceptibility to RA is associated with HLA-DRB1 genes [2].

Type II collagen (CII), a molecule abundant in the articular cartilage, is considered one of the target autoantigens in RA. CII-reactive T cell clones have been established in vitro from synovial T cells of RA [3]. Sekine et al. [4] suggested that the expansion of oligoclonal T cells in RA joints is driven by stimulation of CII. Furthermore, the pathology in CII-induced arthritis (CIA) mice is similar to that in RA synovium. The susceptibility to CIA is determined by I-A^q, which is a major histocompatibility complex (MHC) class II molecule, and the immunodominant CII256–271 region of CII could be bound to I-A^q molecules [5].

T cell activation depends on the ability of the T cell receptor (TCR) to recognize 8–20 amino acid peptides that are bound to MHC molecules. The process of recognition of peptides by TCR is flexible. If the amino acid residue of peptide ligands for TCR is substituted for a different amino acid and can still bind to the MHC molecules (altered peptide ligands, APLs), these APLs could regulate the activation of T cells. Several studies [6, 7] have shown that APL can potentially induce differential cytokine secretion, anergy, and antagonism of the response to wild-type antigens. Therefore, it is possible to use APL as a therapeutic

agent against T-cell-mediated diseases such as autoimmune diseases.

Our previous report [8] demonstrated that peripheral blood mononuclear cells from HLA-DRB1*0101 Japanese patients with RA were highly reactive to the 256–271 peptide of CII, and designed APLs suppressed T cell response to the immunodominant epitope (CII256–271) of CII. In the present study, we tried to regulate the CII-reactive T cells from CIA by eight different APLs to CII256–271. The results showed that four APLs could suppress the CII-reactive immune response in vitro and one APL exhibited an inhibitory effect on arthritis in vivo. These results suggest that the application of CII APL is a potentially suitable therapeutic strategy in the control of RA.

Materials and methods

Mice

DBA/1 J mice were purchased from The Charles River Laboratory (Yokohama, Japan). They were maintained in specific pathogen-free conditions in the laboratory animal resource center. All experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* at Tsukuba University.

Induction of arthritis

Mice were immunized intradermally with 100 μg bovine type II collagen (CII: Collagen Research Center, Tokyo, Japan) in Complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA). Each mouse received a booster dose on day 21 by intraperitoneal injection of 100 μg CII.

CII (256–271) peptide and altered peptide ligands

The peptide representing CII (AA256–271) and its altered peptide ligands (APL) containing specific amino acid substitutions were chemically synthesized by solid-phase procedure and purified by high-performance liquid chromatography (OPERON Biotechnologies, Tokyo).

Pre-pulse assay

Mice were immunized with 100 μg CII emulsified with CFA. Twelve days after immunization, the mice were anesthetized and the spleens removed. The spleen was treated with collagenase D (Roche, Mannheim, Germany), and $CD11c^{+}$ cells were isolated by CD11c microbeads (Miltenyi Biotec, Tokyo). The cells were pulsed with 50 μM CII256–271 peptides for 2 h. After washing, they were adjusted to 1×10^{6} cells/ml and pulsed with 200 μM APLs for 12 h. On the other hand, $CD4^{+}$ cells were isolated using CD4 microbeads (Miltenyi Biotec) from splenocytes of mice immunized with CII. Then they were adjusted to 5×10^{5} cells/ml and added to the plate where $CD11c^{+}$ cells were cultured for 12 h. The supernatants were collected 72 h later and interferon-γ (IFN-γ), IL-17, IL-2, IL-4, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) (IL-17 and IL-2; BioLegend, San Diego, CA, IFN-γ, IL-4 and IL-10; eBioscience, San Diego, CA).

Treatment with APLs

Mice were treated with three injections each of 333 μg of APLs intraperitoneally (total 1 mg) on days 24, 26, and 28 after the first immunization with CII. The animals were observed at 3-day intervals and evaluated for the severity of arthritis by scoring each paw. The score ranged from 0 to 3 (0, no swelling or redness; 1, swelling or redness in one joint; 2, involvement of two or more joints; 3, severe arthritis of the entire paw and joints). The score of each animal was the sum of scores for all four paws.

Histopathology

The ankles were removed on day 60 after the first immunization with CII and fixed in 3% buffered formalin. The paws were decalcified in ethylenediaminetetraacetic acid (EDTA) in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Analysis of T cell response

Mice were treated with APLs by the above method. Their splenocytes were removed on day 35 after immunization, and $CD4^{+}$ cells and $CD11c^{+}$ cells were isolated by microbeads as described above. $CD4^{+}$ cells and $CD11c^{+}$ cells (5:1) were mixed and cultured with denatured CII (10 μg/ml), and supernatants were collected 24 h later. The amounts of IL-17 and IFN-γ were measured by ELISA.

Statistical analysis

The Mann–Whitney *U* test was used for statistical analysis. *P* values less than 0.05 denoted significant difference.

Results

Antagonistic activity of APLs in vitro

We designed eight different APLs as shown in Fig. 1a. We screened these APLs for their antagonistic activity

(A)

					P1	P2	P3	P4	P5	P6	P7	P8				
CII256-271	G	K	P	G	I	A	G	F	K	G	E	Q	G	P	K	G
APL-1	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-
APL-2					-	D	-	-	-	-	-	-	-	-	-	-
APL-3	-	-	-	-	-	-	D	-	-	-	-	-	-	-	-	-
APL-4	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	-
APL-5	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-
APL-6	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-
APL-7	-	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-
APL-8	-	-	-	-	-	-	-	-	M	-	-	-	-	-	-	-

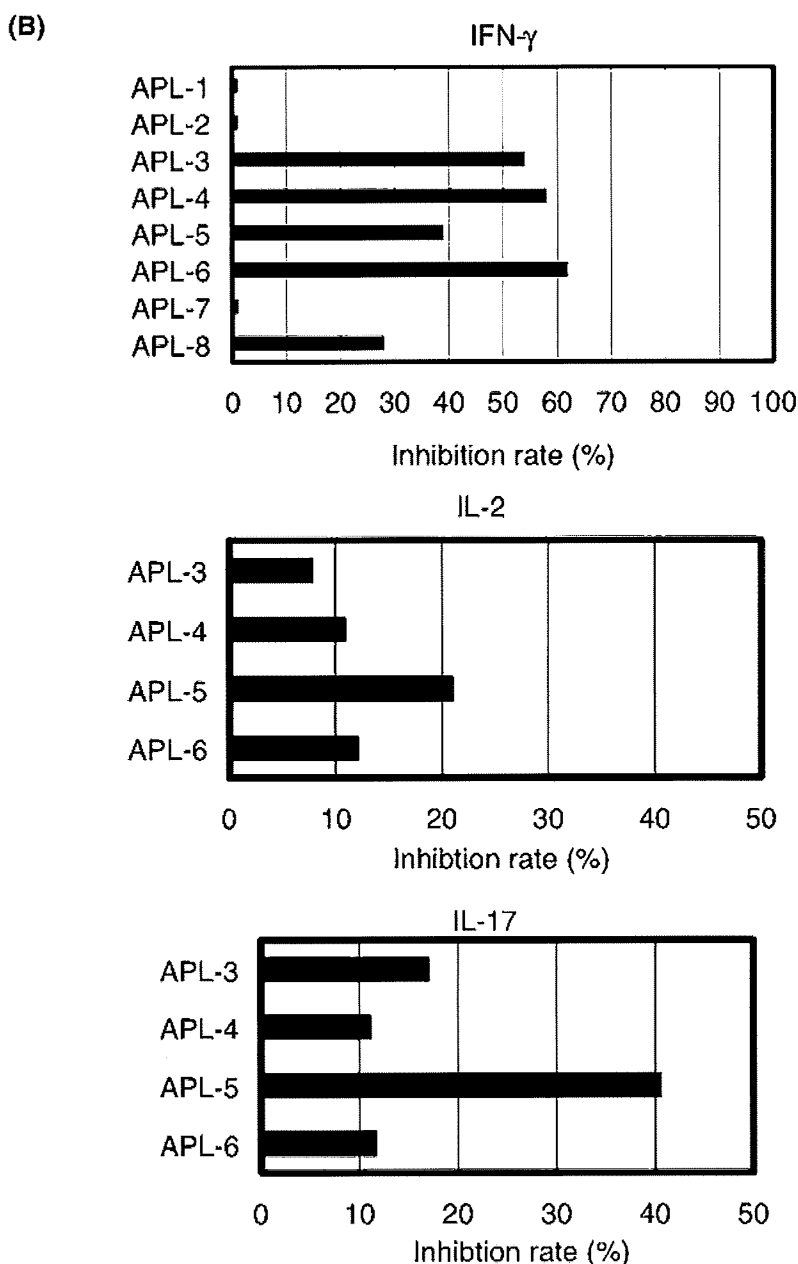

Fig. 1 Screening of APLs with antagonistic activity. **a** Altered peptide ligands were designed based on the I-A^q anchor motif. P1, P4, and P7 were predicted anchor position in CII256–271 peptide. **b** DBA/1 mice were immunized with CII. Antagonistic activity of APLs was investigated using CD4 T cells on day 12 after immunization by pre-pulse assay described in "Materials and Methods." Concentrations of IFN-γ, IL-2, and IL-17 were measured in the culture supernatants by ELISA. Inhibition rates of IFN-γ, IL-2 and IL-17 are expressed as percentage inhibition against the CII 256–271 peptide response. Data are representative of three similar experiments ($n = 3$)

using CII-reactive cytokine production assay. Four APLs (APL-3, -4, -5, and -6) suppressed (by more than 30–60%) production of CII256–271-reactive IFN-γ in vitro (Fig. 1b). We also investigated whether APL-3, -4, -5, and -6 suppressed the production of other cytokines. Similar to IFN-γ, the four APLs suppressed production of IL-2 and IL-17

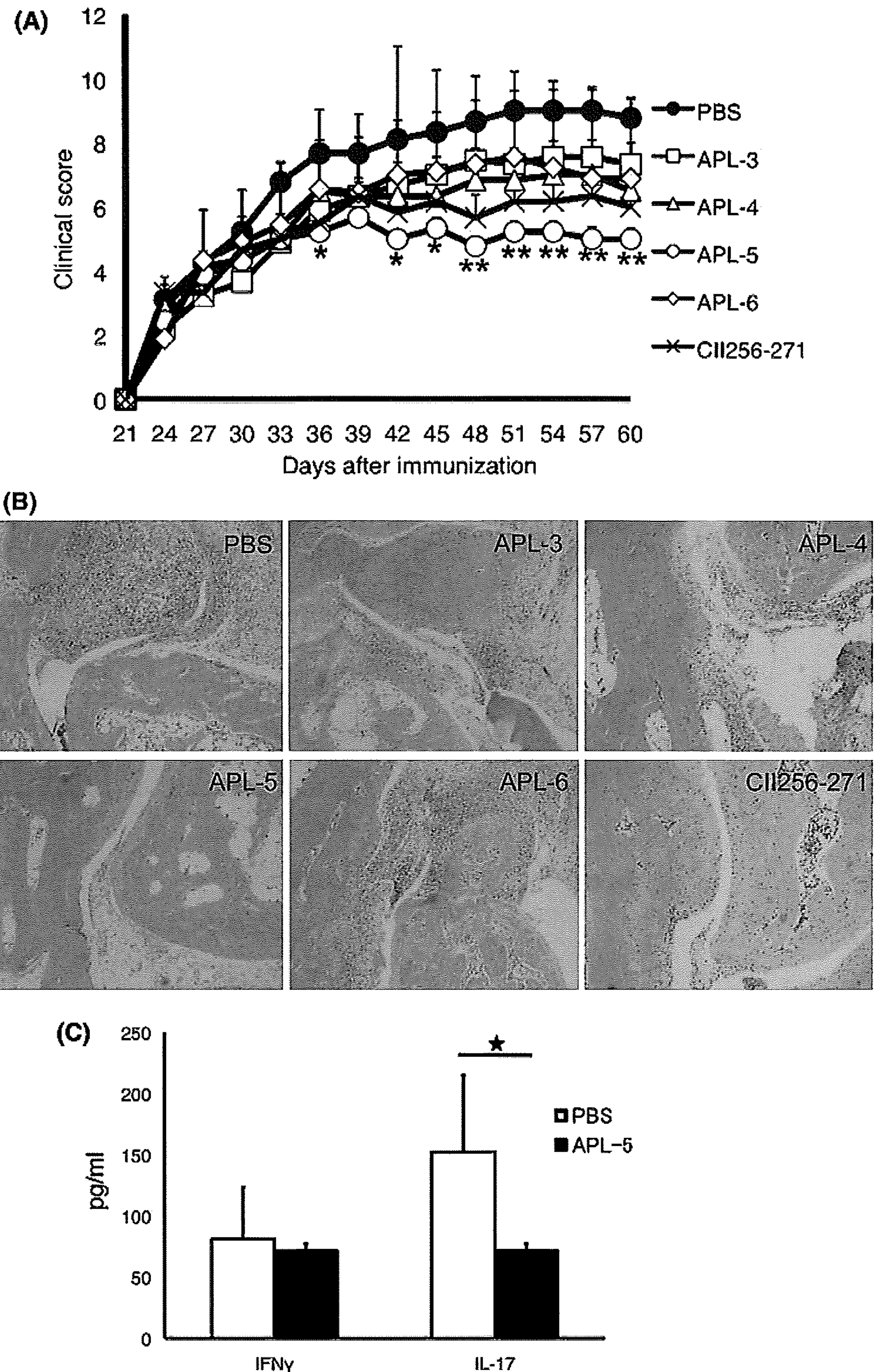

Fig. 2 Therapeutic effect of G262A in CIA. **a** DBA/1 mice were immunized and boosted with CII. Four APLs, CII256–271 peptide, and PBS were administered i.p. on days 24, 26, and 28 after immunization of CII (each $n = 9$), respectively. The clinical score of arthritis is expressed as mean ± standard error of the mean (SEM). $^{*}P < 0.01$, $^{**}P < 0.001$ versus PBS. **b** Each mouse was sacrificed on day 60 after immunization, and the ankles were examined by H&E staining. **c** On day 7 after the administration of APLs, $CD4^{+}$ T cells and dendritic cells were isolated from the spleen and co-cultured for 24 h. IFN-γ and IL-17 concentrations were measured by ELISA. T cell response of mice injected with PBS was assigned as 1. Data are mean ± standard deviation (SD) with triplicate culture. $^{*}P < 0.05$ versus PBS

(Fig. 1b). However, IL-4 and IL-10 were not detected in any samples (data not shown). Thus, we considered these four APLs as candidates for antagonistic APLs and used them in further experiments in vivo.

APL-5 results in significant suppression of arthritis

To investigate the therapeutic effects of the above four APLs on arthritis in vivo, we treated CII mice immunized

with APL intraperitoneally on day 24. CII256–271 peptide and phosphate-buffered saline (PBS) were injected as negative control. The results showed that APL-5 significantly suppressed the development of arthritis compared with the other three APLs ($P < 0.01$) and PBS ($P < 0.001$) (Fig. 2a). These mice were sacrificed on day 60 after immunization of CII, and histological examination was performed. As shown in Fig. 2b, APL-5 inhibited mononuclear cell infiltration compared with the other three APLs and PBS. These results indicate that CII-induced arthritis could be regulated by APL-5. T cell predominant epitope by itself (CII256–271) slightly decreased the severity of arthritis, suggesting activation-induced cell death by the excess dose of dominant epitope.

Effects of APLs on cytokine production

To examine whether APLs suppress CII-reactive T cells in vivo, we investigated the production of IFN-γ and IL-17 from CII-reactive T cells in APLs-treated mice. As shown in Fig. 2c, injection of APL-5 significantly suppressed IL-17 production, but not that of IFN-γ, by CII-reactive T cells ($P < 0.05$). The other APLs and CII256–271 peptides did not have any effects on the production of IFN-γ and IL-17 (data not shown).

Discussion

Several investigators [9–11] demonstrated the protective effects of APL using experimental autoimmune encephalomyelitis (EAE) and CIA. Co-immunization of mice bearing the H-2^u haplotype with an APL and an encephalogenic peptide prevented the development of EAE [9]. Furthermore, Myers et al. [10] showed that APL regulated the onset of CIA in H-2^q mice. Although their reports showed the protective activity of APL, the therapeutic effects of APLs have been hardly reported. Myers et al. [12] reported that administration of their APLs on day 28 after CII immunization decreased the incidence rate of arthritis though they did not show the score of arthritis of those mice. Zhao et al. [13] demonstrated that collagen-induced arthritis in rat was suppressed by oral administration of APLs after the onset of arthritis. These observations support the notion that their APLs might be a therapeutic strategy against arthritis. In the present study, administration of APL-5 after the onset of arthritis suppressed the development of CIA, indicating the therapeutic effect of APL on CIA. In contrast, Myers's study [12] administered APLs to CIA mice at the onset of arthritis. The experiments by Zhao et al. [13] were designed with oral administration and done using rat model. The most important message from this study is that APL-5 is common between CIA mice and patients with RA [8], indicating that clinical trial can be hoped for in the near future.

Several studies have examined the role of Th17 cells in CIA. Development of CIA was regulated in IL-17 knockout mice, suggesting that IL-17 plays a crucial role in arthritis [14]. Severe clinical and histologic CIA was induced in IFN-γ receptor knockout mice [15], whereas CIA was suppressed by the administration of anti-IFN-γ antibodies [16]. Therefore, the role of IFN-γ in the development of CIA is controversial. Our studies showed that APL-5 inhibited CII-reactive IL-17 production in vivo, suggesting the main contribution of IL-17 on the development of CIA.

Our experiments in vivo showed the possibility that each APL had different effect on T cell subset. APL-5 preferentially suppressed IL-17 but not IFN-γ; on the other hand, APL-6 suppressed IFN-γ but not IL-17. Although the precise mechanism is not clarified, these findings suggest that minor variations of the peptide may affect the peptide-binding affinity, or minor change of the physicochemical properties of amino acid residues may involve TCR binding activity.

In conclusion, the present study showed that administration of APL-5 suppressed the development of CIA in mice. CII APL is a potentially suitable therapeutic strategy for the control of RA.

Acknowledgment This work was supported in part by a Research Grant from the Ministry of Health, Labor, and Welfare, and the Research Grant from the Ministry of Education, Culture, Sports, Science, and Technology.

Conflict of interest statement All of the authors confirm that they have no conflicts of interest with regard to this work.

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