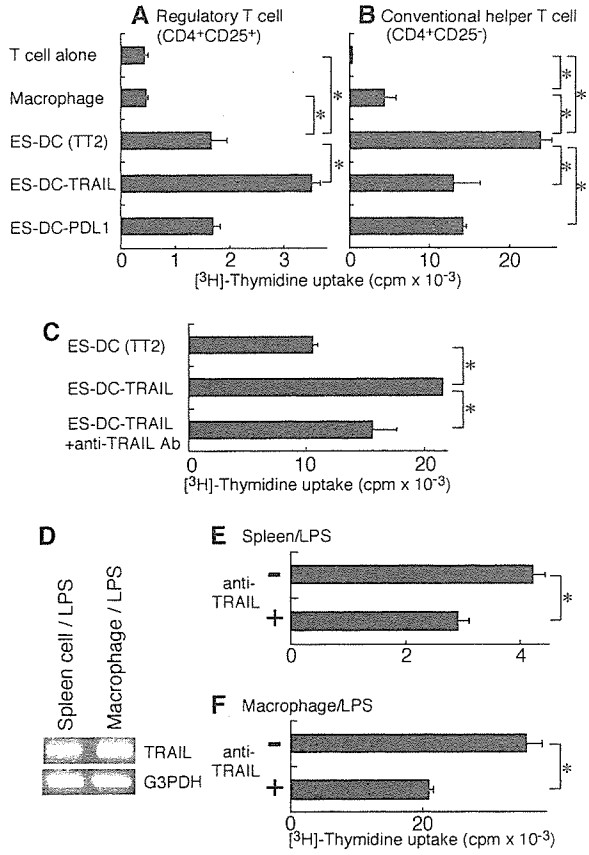

FIGURE 6. Enhanced capacity of APC expressing TRAIL to induce the proliferation of naive $CD4^+CD25^+$ Tregs in vitro. *A*, $CD4^+CD25^+$ T cells (1×10^4) or (*B*) $CD4^+CD25^-$ T cells (1×10^4) isolated from the spleen of the naive CBF_1 mice were cultured alone or cocultured with irradiated stimulators (1×10^4), syngeneic splenic macrophages, nontransfectant ES-DC (TT2), ES-DC-TRAIL, or ES-DC-PDL1, for 3 days in the presence of anti-CD3 mAb (0.1 μg/ml) and human IL-2 (10 U/ml). *C*, $CD4^+CD25^+$ T cells (1×10^4) isolated from the spleen of the naive CBF_1 mice were cultured alone or cocultured with irradiated stimulators (1×10^4), nontransfectant ES-DC (TT2), or ES-DC-TRAIL, for 3 days in the presence of anti-CD3 mAb (2.5 μg/ml) and human IL-2 (10 U/ml) with or without anti-mouse TRAIL mAb (2.5 μg/ml). *D*, The expression of TRAIL in spleen cells or macrophages stimulated with LPS was confirmed by RT-PCR. The naive Treg isolated from a spleen were cocultured for 3 days with 1×10^5 of irradiated LPS-stimulated spleen cells (*E*) or 1×10^4 of irradiated LPS-stimulated macrophages (*F*) in the presence of anti-CD3 mAb (1 μg/ml) and IL-2 (30–100 U/ml) with or without anti-mouse TRAIL mAb (5 μg/ml) for 3 days. The proliferation of responder T cells was quantified by measuring the [^{3}H]thymidine incorporation in the last 12 h of the culture. *, Statistical significance ($p < 0.01$). The results were expressed as the mean of a triplicate assay ± SD. The data are each representative of more than three independent experiments with similar results.

type of ES-DC. In contrast, the magnitude of proliferation of $CD4^+CD25^-$ conventional Th cells was decreased by the expression of TRAIL by ES-DC (Fig. 6*B*). In addition, anti-TRAIL blocking mAb decreased the proliferation of Treg cocultured with ES-DC-TRAIL (Fig. 6*C*). These results suggest that TRAIL expressed on ES-DC has an inhibitory effect on the proliferation of conventional $CD4^+$ T cells and a stimulating effect on that of $CD4^+CD25^+$ Treg.

Anti-TRAIL blocking mAb decreased the proliferation of $CD4^+CD25^+$ Treg responding to LPS-treated natural APCs

The data presented so far suggest that TRAIL expressed on ES-DC has an effect to augment proliferation of $CD4^+CD25^+$ Treg. Lastly, we investigate the effect of TRAIL expressed on natural APCs on proliferation of Treg. Recent studies reported that LPS-stimulation enhanced the expression of TRAIL on spleen cells and bone marrow-derived DC (20–22). We thus examined the proliferation of Treg cocultured with LPS-stimulated whole spleen cells or splenic macrophages in the presence or absence of anti-TRAIL-blocking mAb. Treg isolated from spleen of naive CBF_1 mice were cocultured with LPS-treated APC in the presence of anti-CD3 mAb and a low dose of human IL-2 with or without anti-TRAIL mAb (5 μg/ml). As shown in Fig. 6, *D–F*, anti-TRAIL mAb partially decreased the proliferation of $CD4^+CD25^+$ Treg. These results indicate that TRAIL naturally expressed on APC as well as that expressed on genetically modified ES-DC has a stimulating effect on proliferation of $CD4^+CD25^+$ Treg.

Discussion

In the present study, we found that the severity of not only MOG- but also MBP-induced EAE was reduced by treatment with ES-DC expressing MOG peptide along with TRAIL (Figs. 1 and 2). We obtained several lines of evidences suggesting a possibility that the observed disease-preventive effect is mediated by propagation of MOG-reactive Treg by ES-DC-TRAIL/MOG. Another possible explanation for this phenomenon may be as follows: MOG-reactive T cells might be activated by so-called epitope spreading even in MBP-induced EAE and they might play a major role in the pathogenesis; pretreatment with ES-DC-TRAIL/MOG may abrogate MOG-reactive T cells, thus resulting in a reduction of the disease severity. However, we consider this possibility to be less likely because ES-DC-PDL1/MOG, which showed a MOG-induced EAE-preventive effect similar to that induced by ES-DC-TRAIL/MOG, had no effect on MBP-induced EAE (Fig. 1, *B* and *C*, and Table I). In addition, in previous studies of EAE, the autoreactivity directed to multiple myelin Ags caused by epitope spreading have been observed in chronic or relapsing phases over 4 wk after the immunization. In contrast, the inhibitory effect on MBP-induced EAE, which we observed in the present study, occurred within 2 wk after the immunization. We therefore considered the possibility that MOG-specific T cells with some regulatory activity may have been induced or stimulated by ES-DC-TRAIL/MOG. The results of adoptive transfer experiments demonstrating that $CD4^+$ T cells isolated from ES-DC-TRAIL/MOG-treated donor mice acted to protect the recipient mice from EAE (Fig. 3, *A–C*, and Table II) strongly support this notion.

So far, several types of T cells involved in the negative regulation of immune responses have been identified, such as IL-10-producing Tr1 cells and $CD4^+CD25^+$ Treg (17, 19, 23). Kohm et al. (24) reported that the adoptive transfer of relatively large number of $CD4^+CD25^+$ Treg (2×10^6) isolated from naive mice protected the recipient mice from MOG-induced EAE. It was recently reported that immature DC induced Tr1 cells producing high amounts of IL-10 (25), and also that the proliferation of $CD4^+CD25^+$ Treg was efficiently promoted by DC (17). We thus attempted to characterize the T cells with regulatory activity induced by ES-DC expressing TRAIL and involved in the protection from EAE. We quantified IL-10, IFN-γ, and IL-4 produced by spleen cells isolated from ES-DC-treated mice upon in vitro stimulation with MOG peptide, by ELISA. No significant change in the amount of these cytokines produced by spleen cells of ES-DC-TRAIL/MOG, ES-DC-PDL1/MOG, or ES-DC-MOG-treated mice was observed (data not shown). We thus considered it less likely that the disease-prevention effect was mediated by IL-10-producing Tr1 cells or Th2 cells, although we cannot totally rule out this possibility.

We next assessed the possibility that ES-DC-TRAIL/MOG had propagated or activated $CD4^+CD25^+$ Treg in vivo. Adoptive transfer experiments showed the presence of $CD4^+CD25^+$ T cells with a capacity to prevent not only MOG- but also MBP-induced EAE in ES-DC-TRAIL/MOG-treated mice (Fig. 3, *D–F*, and Table II). In addition, when $CD4^+CD25^+$ T cells were depleted by the pretreatment of mice with anti-CD25 mAb, the protective effect of ES-DC-TRAIL/MOG against MBP-induced EAE was totally abrogated (Fig. 4 and Table I), the observation further supporting the notion that $CD4^+CD25^+$ T cells play a role in the prevention of MBP-induced EAE by treatment with ES-DC-TRAIL/MOG. Recently, Kohm et al. (26) reported that the effect of anti-CD25 Ab is not the depletion but functional inactivation of Treg. Our findings that preventive effect of MBP-induced EAE with ES-DC-TRAIL/MOG was diminished by the injection of anti-CD25 mAb into mice also may be interpreted as inactivation of Treg. Several groups recently reported that $CD4^+CD25^-$ T cells converted to Treg functionality in particular condition (27, 28). It is possible that $CD4^+CD25^+$ Tregs suppressing EAE observed in our study were also those converted from conventional $CD4^+$ T cells.

Furthermore, we observed an increased number of $Foxp3^+$ cells and the ratio of $Foxp3^+$ cells to $CD4^+$ cells in the spinal cords in mice treated with ES-DC-TRAIL/MOG (Fig. 5). In contrast, we could not detect any significant increase in the expression of Foxp3 mRNA in the spleen of ES-DC-TRAIL/MOG-treated mice (data not shown). Also in flow cytometry analysis the proportion of $Foxp3^+$ cells to $CD4^+$ cells in the spleen and inguinal lymph nodes was not increased in mice treated with ES-DC-TRAIL/MOG as compared with control mice (the proportion of $Foxp3^+$ cells to $CD4^+$ cells was 9.7 ± 0.7% in spleen of control mice, 9.8 ± 0.8% in spleen of ES-DC-TRAIL/MOG-treated mice, 9.2 ± 0.9% in inguinal lymph nodes of control mice, and 9.5 ± 0.1% in inguinal lymph nodes of ES-DC-TRAIL/MOG-treated mice). Probably the increase in the number of MOG-reactive $Foxp3^+$ cells was too small to be detected in total $CD4^+$ T cells of spleen and inguinal lymph nodes. In addition, to investigate whether the ES-DC-TRAIL/MOG were acting at the level of priming or within the target organ (CNS), we tested the primary proliferative response to MBP of the T cells of mice treated with ES-DC-TRAIL/MOG before immunization with MBP for EAE induction (as shown in Fig. 1*A*). The inguinal lymph node cells were harvested on day 19 after the immunization and cultured in the presence of MBP (whole protein, 0, 12.5, 25, 50 μg/ml) for 3 days. In this experiment, the treatment with ES-DC-TRAIL/MOG did not reduce the proliferative response to MBP of T cells in inguinal lymph node (data not shown). This result suggests that treatment with ES-DC-TRAIL/MOG did not act at the level of priming. It may be more likely that the treatment with ES-DC-TRAIL/MOG acted in the target organ (CNS), as shown in Fig. 7.

The $CD4^+CD25^+$ T cells induced by ES-DC-TRAIL/MOG and responsible for disease prevention were probably specific to MOG, because ES-DC expressing TRAIL along with irrelevant Ag (OVA) had no effect on MBP-induced EAE (Figs. 1*D* and 3*F* and Tables I and II).

Consistent with recent reports on the stimulation of $CD4^+CD25^+$ Treg as well as conventional T cells by splenic or BM-derived DC (17, 29), in vitro experiments showed that ES-DC also have the capacity to induce proliferation of both $CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ Treg, when stimulated with anti-CD3 mAb in the presence of low-dose IL-2. The expression of TRAIL on ES-DC enhanced the capacity to induce proliferation of $CD4^+CD25^+$ Treg, but not of $CD4^+CD25^-$ conventional T cells (Fig. 6, *A* and *B*). In addition, anti-TRAIL-blocking mAb decreased the proliferation of

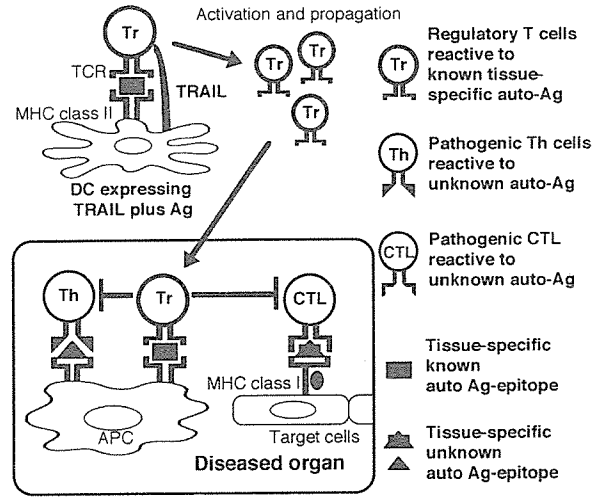

FIGURE 7. Promotion of Tregs with DC expressing TRAIL plus certain tissue-specific autoantigen as a novel strategy for the treatment of the organ-specific autoimmune diseases. DC expressing TRAIL and simultaneously presenting known tissue-specific autoantigen (auto-Ag) in the context of their MHC class II can activate or propagate T cells with a regulatory function (Tr cells) reactive to the autoantigen. In the focus of autoimmune disease, the promoted-Tr cells were retriggered by the same autoantigen expressed in the tissue. Then, the Tr cells inhibited the pathogenic T cells responding to unknown autoantigen. This strategy is applicable, even if target autoantigens are unidentified or if multiple Ags are recognized as targets by the pathogenic autoreactive T cells.

Treg cocultured with ES-DC-TRAIL or natural APC, such as LPS-treated spleen cells or macrophage (Fig. 6, *C–F*). The results of these in vitro experiments suggest that the in vivo transfer of ES-DC-TRAIL/MOG induced proliferation of MOG-reactive Treg which protected the recipient mice from EAE.

Based on the results obtained in the current study, we consider that the inhibition of autoimmunity by TRAIL-expressing ES-DC may be attributed to the promotion of $CD4^+CD25^+$ T cells by TRAIL, in addition to the induction of apoptosis of pathogenic T cells as suggested by our previous study (1). Mi et al. (30) reported that TRAIL inhibited the proliferation of diabetogenic T cells isolated from NOD mice by suppressing IL-2 production and upregulating the expression of $p27^{kip1}$. It may be possible that the effects of Treg were involved in their observations. In addition, Herbeuval et al. (31) reported that a level of TRAIL was elevated in plasma of HIV-1-infected patients and in vitro exposure to HIV-1 induced the expression of TRAIL in APCs. Andersson et al. (32) reported expression of Foxp3 to be enhanced in the lymphoid tissue of HIV-infected patients. These two findings may also be related to our findings.

DC modified by some way to enhance their tolerogenic characteristics is regarded as a promising therapeutic means to negatively manipulate the immune response for the treatment of autoimmune and allergic diseases and also for the induction of transplantation tolerance (1, 23, 33–36). In the clinically manifest phase of autoimmune diseases, such as multiple sclerosis or type I diabetes, it is presumed that multiple tissue-specific Ags are recognized as targets by deregulated immunity due to epitope spreading (37). As a result, the induction of a mere deletion or anergy of pathogenic T cells specific to primarily recognized autoantigens may not be sufficient to control these diseases. The promotion of the immune-suppressive T cells reactive to organ-specific self Ags by treatment with genetically modified DC may be a promising therapeutic modality for subjects with autoimmune diseases (Fig. 7). This strategy may also be useful for the induction of transplantation tolerance.

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Disclosures

The authors have no financial conflict of interest.

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Research article

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Interactions between IL-32 and tumor necrosis factor alpha contribute to the exacerbation of immune-inflammatory diseases

Hirofumi Shoda[1], Keishi Fujio[1], Yumi Yamaguchi[1], Akiko Okamoto[1], Tetsuji Sawada[1], Yuta Kochi[2] and Kazuhiko Yamamoto[1]

[1]Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[2]Laboratory for Rheumatic Diseases, SNP Research Center, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

Corresponding author: Keishi Fujio, kfujio-tky@umin.ac.jp

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Abstract

IL-32 is a newly described cytokine in the human found to be an *in vitro* inducer of tumor necrosis factor alpha (TNFα). We examined the *in vivo* relationship between IL-32 and TNFα, and the pathologic role of IL-32 in the TNFα-related diseases – arthritis and colitis. We demonstrated by quantitative PCR assay that IL-32 mRNA was expressed in the lymphoid tissues, and in stimulated peripheral T cells, monocytes, and B cells. Activated T cells were important for IL-32 mRNA expression in monocytes and B cells. Interestingly, TNFα reciprocally induced IL-32 mRNA expression in T cells, monocyte-derived dendritic cells, and synovial fibroblasts. Moreover, IL-32 mRNA expression was prominent in the synovial tissues of rheumatoid arthritis patients, especially in synovial-infiltrated lymphocytes by *in situ* hybridization. To examine the *in vivo* relationship of IL-32 and TNFα, we prepared an overexpression model mouse of human IL-32β (BM-hIL-32) by bone marrow transplantation. Splenocytes of BM-hIL-32 mice showed increased expression and secretion of TNFα, IL-1β, and IL-6 especially in response to lipopolysaccharide stimulation. Moreover, serum TNFα concentration showed a clear increase in BM-hIL-32 mice. Cell-sorting analysis of splenocytes showed that the expression of TNFα was increased in resting F4/80$^+$ macrophages, and the expression of TNFα, IL-1β and IL-6 was increased in lipopolysaccharide-stimulated F4/80$^+$ macrophages and CD11c$^+$ dendritic cells. In fact, BM-hIL-32 mice showed exacerbation of collagen-antibody-induced arthritis and trinitrobenzen sulfonic acid-induced colitis. In addition, the transfer of hIL-32β-producing CD4$^+$ T cells significantly exacerbated collagen-induced arthritis, and a TNFα blockade cancelled the exacerbating effects of hIL-32β. We therefore conclude that IL-32 is closely associated with TNFα, and contributes to the exacerbation of TNFα-related inflammatory arthritis and colitis.

Introduction

Tumor necrosis factor alpha (TNFα) is a potent proinflammatory cytokine and is related to several inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel diseases (IBDs). RA is a persistent inflammatory arthritis and is thought to be an autoimmune disease. Inflammation of the joints results in the destruction of cartilage and bone early in the course of the disease. Although the pathogenesis of RA is still unclear and may be heterogeneous, several proinflammatory cytokines participate in promoting the inflammation of the joints. TNFα facilitates arthritis and the destruction of bone [1-4]. TNFα is secreted by several kinds of inflammatory cells, including macrophages, monocytes, T cells, and synovial fibroblasts. TNFα induces other inflammatory cytokines and promotes osteoclastogenesis to destroy the bones. TNFα transgenic mice develop inflammatory arthritis spontaneously [1]. Moreover, TNFα inhibition decreases the severity of arthritis, and both monoclonal antibodies to TNFα and a soluble

BM-hIL-32 = overexpression model of human IL-32β model by bone marrow transplantation; Con A = concanavalin A; ELISA = enzyme-linked immunosorbent assay; FCS = fetal calf serum; GFP = green fluorescent protein; H & E = hematoxylin and eosin; hIL-32 = human interleukin-32; IBD = inflammatory bowel disease; IL = interleukin; LPS = lipopolysaccharide; mAb = monoclonal antibody; MACS = magnetic-activated cell sorting; MHC = major histocompatibility comprex; MoDC = monocyte-derived dendritic cell; PBMC = peripheral blood mononuclear cell; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RA = rheumatoid arthritis; RT = reverse transcriptase; TNBS = trinitrobenzen sulfonic acid; TCR = T-cell receptor; TNFα = tumor necrosis factor alpha.

tumor necrosis factor receptor analog have been used as effective therapies for RA and for other types of inflammatory arthritis [5-8]. In addition, other cytokines, such as IL-1 and IL-6, are also known to be important participants, and the inhibition of these cytokines has been a part of the effective therapies for RA in clinical practice [4].

TNFα plays a pivotal role in the pathogenesis of IBDs including Crohn's disease. The murine model of IBD, trinitrobenzen sulfonic acid (TNBS)-induced colitis, is exacerbated in TNFα transgenic mice [9], and is ameliorated in tumor necrosis factor receptor 2-knockout mice [10]. In the clinical setting, TNFα blockade by infliximab is demonstrated as a useful therapy for Crohn's disease [11]. The mechanisms of TNFα production in these inflammatory diseases, however, remain to be clarified.

Human IL-32 (hIL-32) has been reported as a novel cytokine. IL-32 was cloned as a gene induced by IL-18 and was formerly known as natural killer cell transcript 4 [12,13]. IL-32 induces TNFα secretion in human monocyte and mouse macrophage cell lines. hIL-32 has four splice variants, IL-32α, IL-32β, IL-32γ, and IL-32δ. IL-32α is present in intracellular locations, and IL-32β is secreted from the cells. IL-32α and IL-32β are thought to be the major expressed variants. The sequences of IL-32β and IL-32γ are quite similar. A mouse homolog of IL-32 has not so far been reported.

IL-32 is expressed in lymphoid tissues, such as the thymus, the spleen, and the intestines. Human natural killer cells increase the secretion of IL-32 by IL-18 + IL-12 stimulation, and human peripheral blood mononuclear cells (PBMCs) also secrete IL-32 after stimulation with concanavalin A (Con A). The fact that the IL-32-related cytokines, TNFα and IL-18, show a close correlation with arthritis [14,15] implies that IL-32 has a pathologic role in inflammatory diseases. Indeed, the expression of IL-32 is increased in synovial tissues from RA patients, and the administration of recombinant IL-32γ into mice joints provokes cellular infiltration in the joint spaces [16]. We choose IL-32β for our assay, because IL-32β was reported as a dominant variant and as a secreted protein from the cells, and the sequences of IL-32β and IL-32γ were basically similar [13].

We demonstrated that IL-32 is expressed in various lymphoid cells, and in the synovial-infiltrated lymphocytes of RA patients. *In vivo*, we prepared overexpression model mice of human IL-32β by bone marrow transplantation (BM-hIL32). The expression and secretion of TNFα were increased in resting F4/80$^+$ splenic macrophages of BM-hIL-32 mice, and the expression and secretion of TNFα, IL-1β, and IL-6 were increased in F4/80$^+$ splenic macrophages and CD11c$^+$ splenic dendritic cells after lipopolysaccharide (LPS) stimulation. In fact, the murine models of TNFα-related diseases, TNBS-induced colitis and collagen antibody-induced arthritis, were exacerbated in BM-hIL-32 mice. Furthermore, hIL-32β-transduced CD4$^+$ T cells showed marked exacerbation of collagen-induced arthritis, an effect that was, in part, cancelled by TNFα blockade. Our data indicate that IL-32 is closely associated with TNFα and that it plays a role in the exacerbation of inflammatory diseases.

Materials and methods

Mice

DBA/1J mice and C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). All mice were used at 6–8 weeks of age. All animal experiments were conducted in accordance with institutional and national guidelines.

Collagen-induced arthritis and collagen antibody induced arthritis

Collagen-induced arthritis was induced as described previously [17]. In short, bovine type II collagen (Chondrex, Redmond, WA, USA) was emulsified with an equal volume of Complete Freund's adjuvant (Chondrex). DBA/1J mice were immunized with 50 μg bovine type II collagen intradermally at the base of the tail on day 0 and day 21. Collagen antibody-induced arthritis was induced by intravenous injection of 2 mg arthrogen mAb cocktail to type II collagen, and 3 days later by intraperitoneal injection of 50 μg LPS (Chondrex), as described previously [18]. The arthritis score was determined by erythema, swelling, or ankylosis per paw, as described elsewhere [19]. In some experiments, 50 μg/day etanercept (Wyeth, Madison, NJ, USA) was administered intraperitoneally for 14 days after CD4$^+$ T-cell transfer. The antiarthritic effect of human tumor necrosis factor receptor Fc fusion protein (etanercept) was demonstrated in collagen-immunized mice [8]. Sacrifice was performed 40 days after the first immunization in collagen-induced arthritis mice.

Trinitrobenzen sulfonic acid-induced colitis

TNBS (Wako, Osaka, Japan) was diluted to a final concentration of 1.75% with 50% ethanol and PBS. C57BL/6 mice were anesthetized with 500 μg nembutal (Dainippon Pharmaceutical, Osaka, Japan) by intraperitoneal injection, and 100 μl (1.75 mg) TNBS was administered into the rectum through a 4 cm inserted catheter, as previously described [10]. The body weight was measured daily, and mice were sacrificed 4 days after induction for further analysis. One group of BM-hIL-32 mice were administered 200 μg/day etanercept (Wyeth) intraperitoneally after induction of colitis; other mice were administered the same volume of PBS each day.

Cytokines and cell lines

Recombinant human TNFα, IL-12, IL-18, IL-23, granulocyte-macrophage colony-stimulating factor, and IL-4 were obtained from R&D Systems (Minneapolis, MN, USA). The human 293T cell line and the mouse macrophage cell line, Raw 267.4, were obtained from ATCC (Manassas, VA, USA). Cell lines and primary cells were cultured with RPMI 1640 medium supplemented with 10% FCS, 2 mM γ-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^{-5}M 2-mercapto ethanol. Recombinant human cytokines were added to the culture

medium as follows: 50 ng/ml human TNFα, 50 ng/ml hIL-23, 50 ng/ml IL-18, and 10 ng/ml IL-12 (R&D Systems).

Monoclonal antibodies and flow cytometry

Monoclonal antibodies to mouse CD3, CD4, CD8, CD11c, CD19, and F4/80 were obtained from BD Biosciences (San Jose, CA, USA). Cell sorting was performed on a FACSVantage system (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA), and analysis was performed on an EPICS flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Synovial tissue samples from rheumatoid arthritis patients

Synovial membranes and synovial fibroblasts were obtained from patients with RA satisfying the diagnostic criteria of the American College of Rheumatology [20]. We sampled pathological joint synovial tissues from individuals with RA who underwent arthroplasty surgery. Informed consent was obtained from all patients. Synovial fibroblasts were isolated as formally described [21]. In brief, the collected synovial tissues were digested with collagenase type IV, hyaluronidase, and DNase I (Sigma-Aldrich Corporate, St. Louis, MO, USA), and were passed through a metal screen to prepare isolated cells.

Peripheral blood mononuclear cells

Human PBMCs were isolated from the leukocytes of a healthy donor by Ficoll-Paque (Amersham Pharmacia, Dübendorf, Switzerland). In some experiments, PBMCs were subjected to negative selection with MACS (magnetic-activated cell sorting) using anti-human CD3 mAb (Miltenyi Biotec, Auburn, CA, USA). PBMCs were stimulated with Con A or plate-coated anti-human CD3 antibodies and anti-human CD28 antibodies (R&D Systems). The stimulated cells were incubated for 24 hours and were separated by MACS with anti-human CD4 mAb, anti-human CD8 mAb, anti-human CD14 mAb, and anti-human CD20 mAb (BD PharMingen, San Diego, CA, USA).

Human monocyte-derived dendritic cells (MoDCs) were isolated and cultured as previously described [22]. Briefly, $CD14^+$ cells were isolated from human PBMCs by the MACS procedure and were cultured with 50 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor and IL-4. After 7 days of incubation, MoDCs were cultured with 25 ng/ml LPS (Sigma) or 50 ng/ml human TNFα for 24 hours.

Preparation of retroviral constructs of IL-32β

hIL-32β cDNA was isolated from the human cDNA library according to the reported nucleotide sequence (GenBank: NM_001012631) [13]. The full-length fragments were subcloned into the retrovirus vector pMIG [23]. In some experiments, a cell line was cultured with 1 ml of the supernatant of hIL-32β or mock-transfected (pMIG-transfected) 293T cells in the presence of 5 μg/ml polymixin B (Pfizer, New York, NY, USA) for 24 hours [24].

Production of retroviral supernatants and retroviral transduction

Total splenocytes were cultured for 48 hours in the presence of Con A (10 μg/ml) and mIL-2 (50 ng/ml) (R&D Systems). Retroviral supernatants were obtained by transfection of pMIG or pMIG-hIL-32β into PLAT-E packaging cell lines using FuGENE 6 transfection reagent (Roche Diagnostic System, Somerville, NJ, USA) [25]. For the detection of green fluorescent protein (GFP)-positive cells, we used an EPICS flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Gene transduction to mouse splenocytes and adoptive transfer

Retroviral gene transduction was performed as described [26,27]. Briefly, Falcon 24-well plates (BD Biosciences) were coated with the recombinant human fibronectin fragment CH296 (Retronectin; Takara, Otsu, Japan). The viral supernatant was preloaded into each well of the CH296-coated plate, and the plate was spun at 2400 rpm for 3 hours at room temperature. This procedure was repeated three times. The viral supernatant was washed away, and Con A-stimulated splenocytes were placed into each well (1×10^6 per well). Cells were cultured for 48 hours to allow infection to occur [23,28].

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 intravenously (1×10^7) 23 days after the first immunization of bovine type II collagen.

Bone marrow precursor cell isolation, infection, and transfer

Bone marrow precursor cell isolation, retrovirus infection, and transfer were performed as described previously [29]. In brief, DBA/1J mice or C57BL/6 mice were treated with 5 mg/body 5-fluorouracil (Sigma) dissolved in PBS. After 5 days, bone marrow cells were harvested and cultured with 50 ng/ml mIL-3, mIL-6, and mouse stem cell factor (R&D Systems) for 48 hours. The bone marrow cells were then spin-infected with the retrovirus supernatants using 16 μg/ml polybrene for 90 minutes at 2400 rpm and 25°C. Recipient mice, which were the same strain as the donor mice, were treated by 700 rad whole-body radiation and were injected with 1×10^6 bone marrow cells intravenously. To avoid wasting of the recipient mice due to the overexpression of inflammatory cytokine, the GFP-positive cells among the bone marrow cells were adjusted to around 10% before transplantation. Recipient mice were maintained for 6–9 weeks until analysis. In some experiments, splenocytes derived from bone marrow transplantation DBA/1J mice were cultured for 48 hours with RPMI 1640 medium containing 10% FCS and 1 μg/ml LPS (Sigma) for further analysis.

RT-PCR and quantitative PCR

RNA of the cells was extracted using the RNeasy Micro Kit and RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA from the tissues was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using ISOGEN (Nippon Gene, Tokyo, Japan). RNA was reverse-transcribed to cDNA with random primers (Invitrogen, Carlsbad, CA, USA) and Superscript III according to the manufacturer's protocol (Invitrogen). Quantitative real-time PCR analysis was performed by the Assay-on-Demand TaqMan probe (Hs00992441_m1 for natural killer cell transcript 4) using the ABI PRISM 7900 system (Applied Biosystems, Branchburg, NJ, USA) in the analysis of tissue expression, and using the iCycler system (Bio-rad, Hercules, CA, USA) in the analysis of cellular expression. The TaqMan gene expression assay was performed according to the manufacturer's protocol; a 20 μl reaction mixture contained 1 μl of 20 TaqMan gene expression assay, 9 μl cDNA template, and 10 μl of 2x TaqMan universal master mix. For analyzing cellular expression, the PCR mixture consisted of 25 μl SYBR Green Master Mix (Qiagen), 15 pmol forward and reverse primers, and cDNA samples for a total volume of 50 μl. The results of real-time PCR are shown in terms of relative expression compared with β-actin. The primers used in the real-time PCR are presented in Table 1. The indicated primers and probes for IL-32 were designed for detecting all known isoforms of hIL-32.

Immunoassays of mouse cytokines

Concentrations of mouse TNFα, IL-1β, and IL-6 in sera and culture supernatants were measured by sandwich ELISA according to the manufacturer's protocol (BD Pharmingen). An automatic microplate reader (Bio-rad 550; Bio-rad) was used to measure the optical density.

Histopathology

Tissue samples of RA patients and sacrificed 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, by pannus formation, and by cartilage erosion as described previously [30]. Inflammation of the colon was graded by the extent, cellular infiltration, ulceration, and regeneration as described elsewhere [10].

In situ hybridization

In situ hybridization of the synovial tissue samples was performed as previously described [31]. Single-stranded sense and antisense probes were generated by *in vitro* transcription from the cDNA encoding hIL-32β, nucleotides 30–340 (311 base pairs), which was marked by digoxinogen using the DIG RNA Labeling Mix (Roche, Basel, Switzerland). The sequence of the hIL-32 probe was complementary to the unique sequence of hIL-32β, because IL-32β is the dominant secreting isoform of IL-32. This probe could detect the cDNA of hIL-32β and IL-32γ, but not of IL-32α or IL-32δ, by Southern hybridization (data not shown). Hybridization was performed with probes at a concentration of 100 ng/ml at 60°C for 16 hours. Anti-DIG AP conjugate (Roche) was used as the detection antibody, and coloring reactions were performed with BM purple AP substrate (Roche). The sections were counterstained with Kernechtrot stain solution (Mutoh, Tokyo, Japan), were dehydrated, and were mounted with Malinol (Mutoh). We

Table 1

Primers used in the real-time PCR

Human IL-32	Sense	5'-TGAGGAGCAGCACCCAGAGC-3'
	Antisense	5'-CCGTAGGACTGGAAAGAGGA-3'
Human TNFα	Sense	5'-GTCTCCTACCAGACCAAG-3'
	Antisense	5'-CAAAGTAGACCTGCCCAGACTC-3'
Human β-actin	Sense	5'-TTCCTGGGCATGGAGTCCT-3'
	Antisense	5'-AGGAGGAGCAATGATCTTGATC-3'
Mouse TNFα	Sense	5'-CATCTTCTCAAAATTCGAG-3'
	Antisense	5'-TGGGAGTAGACAAGGTACAACCC-3'
Mouse IL-1β	Sense	5'-CAACCAACAAGTGATATTCTCCATG-3'
	Antisense	5'-GATCCACACTCTCCAGCTGCA-3'
Mouse IL-6	Sense	5'-CACTTCACAAGTCGGAGGCTTA-3'
	Antisense	5'-GCAAGTGCATCATCGTTGTTG-3'
Mouse β-actin	Sense	5'-AGAGGGAAATCGTGCGTGAC-3'
	Antisense	5'-CAATAGTGATGACCTGGCCGT-3'

TNFα, tumor necrosis factor alpha.

also examined control probes, which yielded no specific hybridization (data not shown).

Statistical analysis

Data are expressed as the mean ± standard deviation. All results were obtained from at least three independent experiments. Statistical significance was determined by the Mann-Whitney U test, and $P < 0.05$ was considered significant.

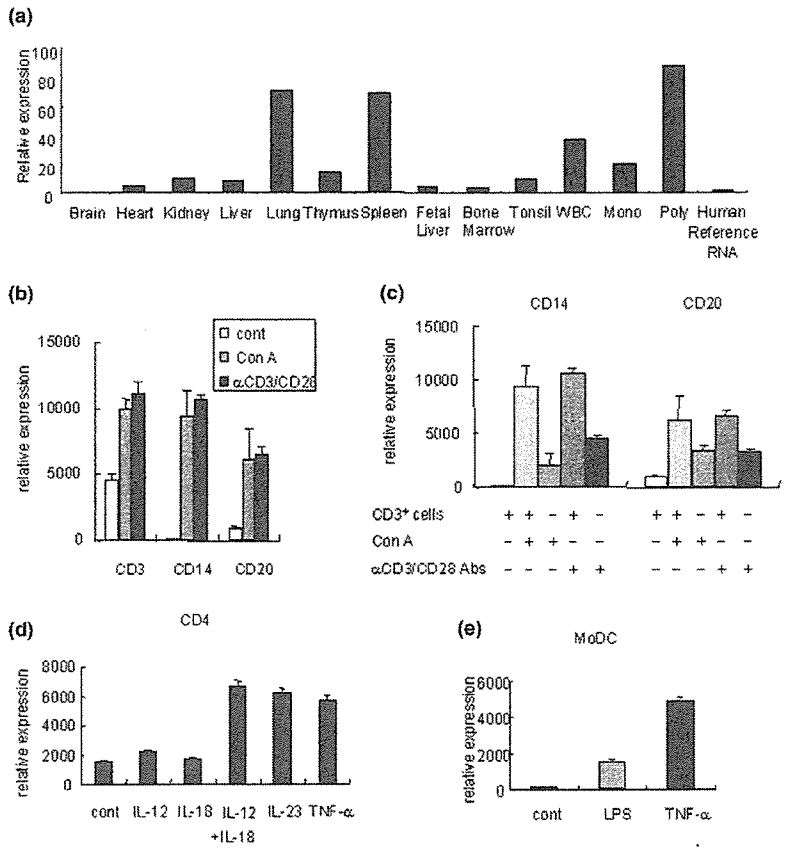
Results

Increased IL-32 expression in activated human peripheral blood mononuclear cells

A previous study showed that IL-32 was expressed in the thymus, the spleen, the intestines, and Con A-stimulated PBMCs by northern blotting and electrochemiluminescence [13]. At first we examined the tissue and cellular expression of IL-32 by quantitative real-time PCR. The tissue expression of IL-32 was prominent in the spleen, the lung, and the peripheral white blood cells (Figure 1a). IL-32 was therefore expressed mainly in the lymphoid tissues and leukocytes.

Since human PBMCs secrete IL-32 by means of the stimulation of Con A [13], we investigated which components of PBMCs expressed IL-32 during both the resting and activated states. $CD3^+$ T cells expressed significant amounts of IL-32 without stimulation, and $CD3^+$ T cells, $CD14^+$ monocytes, and $CD20^+$ B cells increased IL-32 expression after Con A stimulation (Figure 1b). The cellular IL-32 expression was essentially the same in the case of anti-CD3 antibody and anti-CD28 antibody stimulation, which stimulated T cells specifically (Figure 1b). Monocytes or B cells, however, had lower IL-32 expression when they were cultured without $CD3^+$ T cells (Figure 1c). Activated T cells therefore have the capability of inducing IL-32 expression in monocytes and B cells.

Figure 1



Examination of tissue and cell expression of IL-32 by quantitative real-time PCR. **(a)** Tissue expression of IL-32. WBC, white blood cells. **(b)** Human peripheral blood mononuclear cells (PBMCs) expressed IL-32. PBMCs were cultured with or without concanavalin A. PBMCs were also stimulated by immobilized anti-human CD3 and anti-human CD28 antibodies. Cont, control. **(c)** IL-32 expression of monocytes and B cells after the depletion of $CD3^+$ cells. **(d)** Peripheral $CD4^+$ T cells were cultured with the indicated inflammatory cytokines for 24 hours. **(e)** Human monocyte-derived dendritic cells (MoDCs) were cultured with lipopolysaccharide (LPS) or tumor necrosis factor alpha (TNFα) for 24 hours to induce maturation. The data are representative of at least three independent studies.

Several dendritic cell-derived cytokines, such as IL-12, IL-18, and IL-23, are known activators of T cells and important cytokines in the pathogenesis of autoimmune diseases. $CD4^+$ T cells increased IL-32 expression in response to IL-12 + IL-18 and IL-23 stimulation (Figure 1d). In contrast, $CD8^+$ T cells did not increase IL-32 expression (data not shown). Moreover, TNFα also increased IL-32 expression significantly in $CD4^+$ T cells (Figure 1d).

We also generated human MoDCs from $CD14^+$ PBMCs. Although immature control MoDCs hardly expressed IL-32, LPS-stimulated MoDCs and, especially, TNFα-stimulated MoDCs showed a significant increase of IL-32 expression (Figure 1e). In this way, several kinds of immune cells, including T cells, B cells, monocytes, and dendritic cells, were shown to express IL-32, especially in activated states. Moreover, reciprocal IL-32 induction by TNFα was observed in $CD4^+$ T cells and MoDCs.

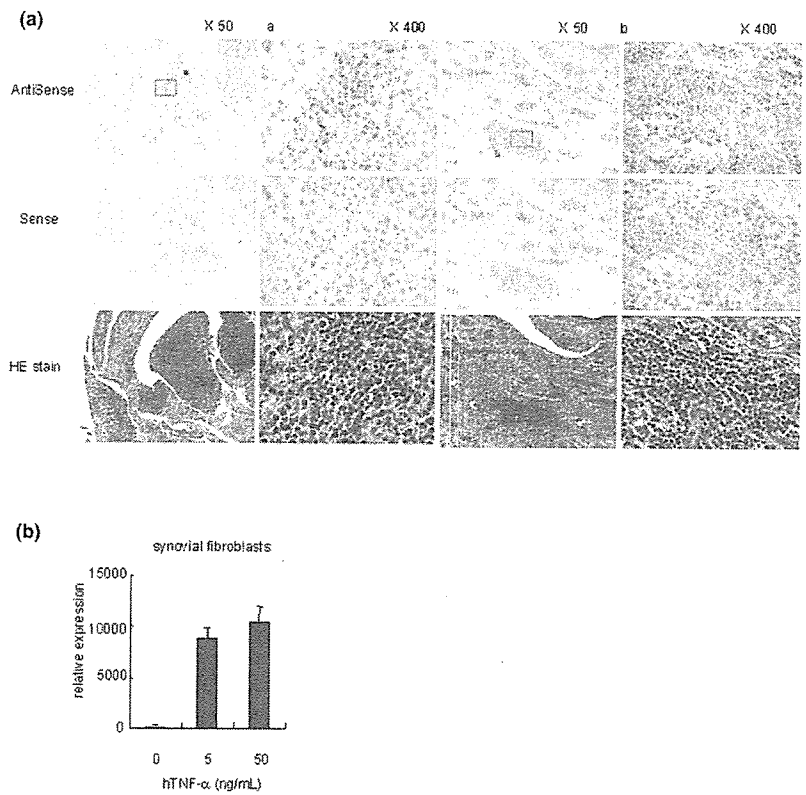
Abundant IL-32 expression in the synovial-infiltrated lymphocytes of rheumatoid arthritis patients

To examine the pathological roles of IL-32 in RA, we tested IL-32 expression in the synovial tissues of RA patients by *in situ* hybridization (Figure 2a). We detected abundant IL-32 expression in the synovial-infiltrated lymphocytes of RA patients rather than in the synovial lining cells. We could not detect the IL-32 expression in the synovial lining layers, where monocytes and synovial fibroblasts usually exist. Synovial fibroblasts produce cytokines and proteases, which play an important role in joint inflammation [32]. We examined the IL-32 expression of the synovial fibroblasts derived from four RA patients *in vitro*. The synovial fibroblasts expressed IL-32 significantly after the stimulation of TNFα (Figure 2b). This result suggested the potential contribution of IL-32 to the joint inflammation mediated by synovial fibroblasts.

Cytokine expression of the bone marrow chimera mice of IL-32β

Activated macrophages are known to be important sources of the inflammatory cytokines in the joints of arthritis patients. hIL-

Figure 2



IL-32 was abundantly expressed in the synovial tissues of rheumatoid arthritis patients. **(a)** *In situ* hybridization of the synovial tissues from rheumatoid arthritis (RA) patients. IL-32β was expressed in the synovial-infiltrated lymphocytes of RA patients. HE stain, hematoxylin and eosin stain. We examined the tissue samples from four RA patients, and show representative examples. **(b)** IL-32 expression of the synovial fibroblasts derived from four RA patients in response to human tumor necrosis factor alpha (hTNFα).

32 was reported to induce TNFα in the mouse macrophage cell line Raw 267.4 [13]. We next confirmed the function of IL-32 with our retroviral construct, MIG-hIL-32β. We choose IL-32β for our assay because IL-32β was reported as a dominant variant and a secreted protein from the cells [13]. The mouse macrophage cell line Raw 267.4 was cultured with the supernatants of MIG-hIL-32β-transfected cells. After 24 hours, the mRNA expression of TNFα was increased by the stimulation of hIL-32β (Figure 3a). In addition, the protein levels of TNFα were increased in the supernatants of hIL-32β-stimulated cells (Figure 3a).

To examine the proinflammatory effect of constitutively expressed IL-32 *in vivo*, we prepared BM-hIL-32 mice. Six weeks to 9 weeks after the bone marrow transplantation, approximately 15% of the cells were GFP-positive in the thymus and the spleen of the BM-hIL-32 mice (Figure 3b). The GFP expression of $CD4^+$ cells, $CD8^+$ cells, $CD11c^+$ cells, $CD19^+$ cells, and $F4/80^+$ cells was also analyzed. There was no significant difference in specific cellular components or the percentage of GFP expression between mock mice and BM-hIL-32 mice (data not shown). hIL-32β expression in the spleen of BM-hIL-32 mice was also confirmed by quantitative real-time PCR and *in situ* hybridization (data not shown).

In accordance with the data of cell lines, freshly isolated splenocytes of BM-hIL-32 mice showed increased expression and secretion of TNFα, compared with those of BM-Mock mice (Figure 3c). We observed no increased expression and secretion of IL-1β or IL-6 in freshly isolated splenocytes of BM-hIL-32 mice. The serum concentration of TNFα protein was elevated significantly in BM-hIL-32 mice (Figure 3d). The serum concentration of IL-1β or IL-6 protein was not detected in BM-hIL-32 mice, in BM-Mock mice, or in control mice. Cell sorting analysis of splenocytes of BM-hIL-32 mice revealed that the expression of TNFα was increased in freshly isolated $F4/80^+$ macrophages (Figure 3e). Other cellular components (that is, $CD4^+$ cells, $CD8^+$ cells, $CD11c^+$ cells, or $CD19^+$ cells) did not show any significant change of the expression of TNFα (data not shown). Although the serum TNFα concentration of BM-hIL-32 mice was comparable with that reported for human TNFα transgenic mice [1], no evident inflammation was observed in histological examination of the spleen, the joint, the intestine, the kidney, and the liver (data not shown).

We next examined the response of splenocytes of BM-hIL-32 mice to LPS stimulations. When cultured with LPS for 2 days, splenocytes of BM-hIL-32 mice showed markedly increased expression and secretion of TNFα and IL-1β (Figure 3c). Among $F4/80^+$ macrophages, $CD11c^+$ dendritic cells, $CD19^+$ B cells, $CD4^+$ T cells, and $CD8^+$ T cells from the spleen, both $F4/80^+$ macrophages and $CD11c^+$ dendritic cells showed an increased expression of TNFα and IL-1β after LPS stimulation in the splenocytes of BM-hIL-32 mice (Figure 3f and data not shown). We also observed that LPS-stimulated splenocytes of BM-hIL-32 mice showed an increased secretion of IL-6 protein (Figure 3c), and $F4/80^+$ macrophages showed an increased expression of IL-6 (Figure 3f). Notably, purified splenic $CD4^+$ T cells from BM-hIL-32 mice did not show any change in cytokine expression, including TNFα, IFN-γ, IL-1β, IL-4, IL-6, and IL-17A (data not shown). In addition, splenocyte proliferation induced by LPS or anti-CD3 antibody was no different between BM-hIL-32 mice and BM-Mock mice (data not shown).

These results suggested that the function of *in vivo* expressed IL-32β was focused on the induction of TNFα production, especially in the macrophages. Our results also suggested that *in vivo* expressed IL-32β collaborated with TLR4 signaling to induce IL-1β and IL-6 production in macrophages and dendritic cells.

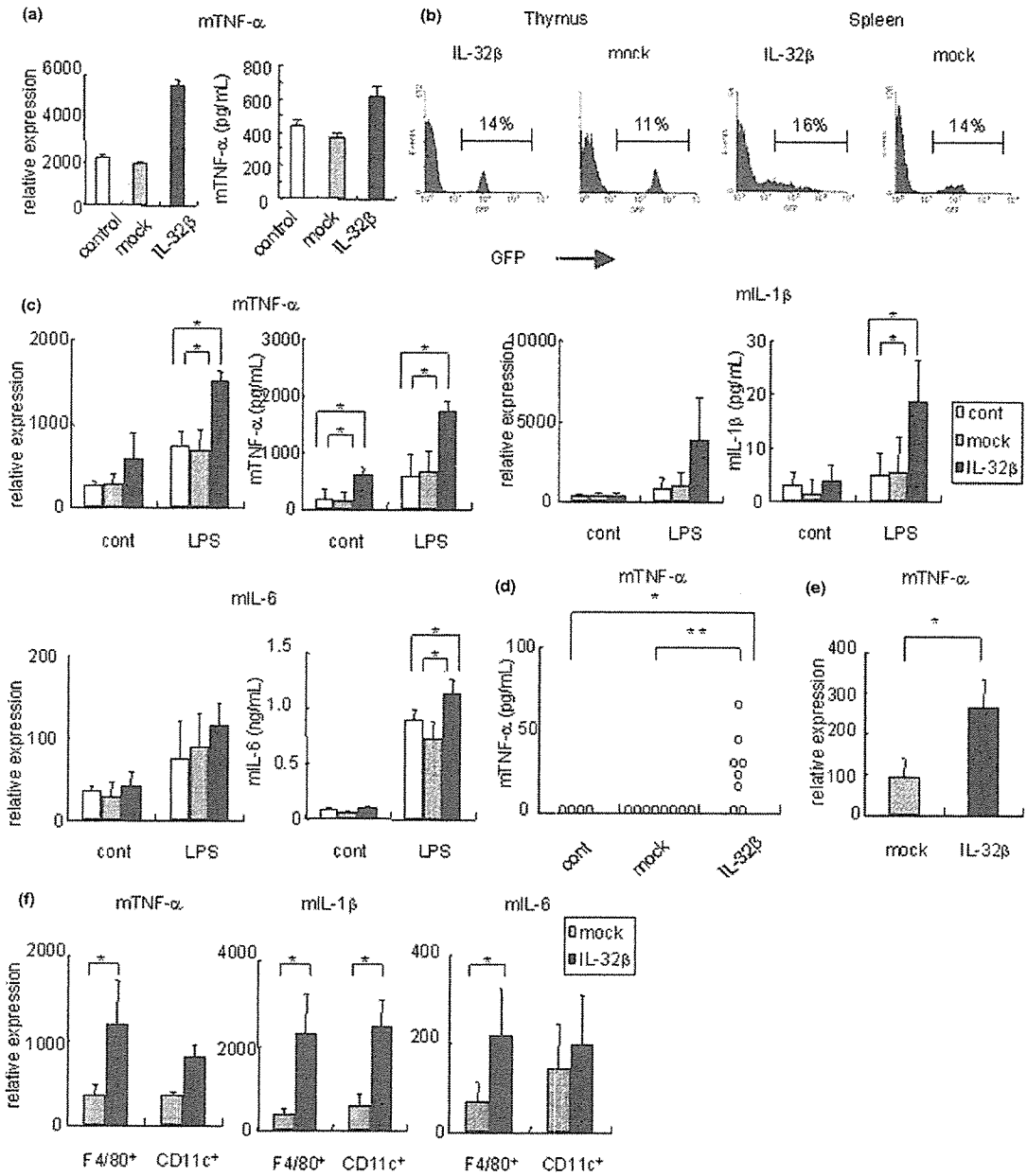
Exacerbation of TNFα-related inflammation in BM-hIL-32 mice

We next examined the association of *in vivo* expressed IL-32β with TNFα-related inflammation. We prepared two kinds of murine models of inflammatory diseases – collagen antibody-induced arthritis and TNBS-induced colitis. We induced arthritis by administration of monoclonal antibodies to type II collagen and administration of LPS to BM-hIL-32 mice. After administration of LPS, more severe arthritis developed in BM-hIL-32 mice than in BM-Mock mice in the early phase of the disease (Figure 4a). This result was consistent with the *in vitro* data, which showed that LPS stimulation induced a larger amount of TNFα from splenocytes of BM-hIL-32 mice.

TNBS-induced colitis is a model of IBDs, in which TNFα plays an important role. BM-hIL-32 mice showed more severe loss of body weight than BM-Mock mice after the administration of TNBS (Figure 4b). The histological scores were significantly higher in BM-hIL-32 mice than in BM-Mock mice (Figure 4c). The expression of hIL-32β mRNA was clearly increased in the inflamed intestinal lesions of BM-hIL-32 mice but could not be detected in BM-Mock or control mice by quantitative PCR (data not shown).

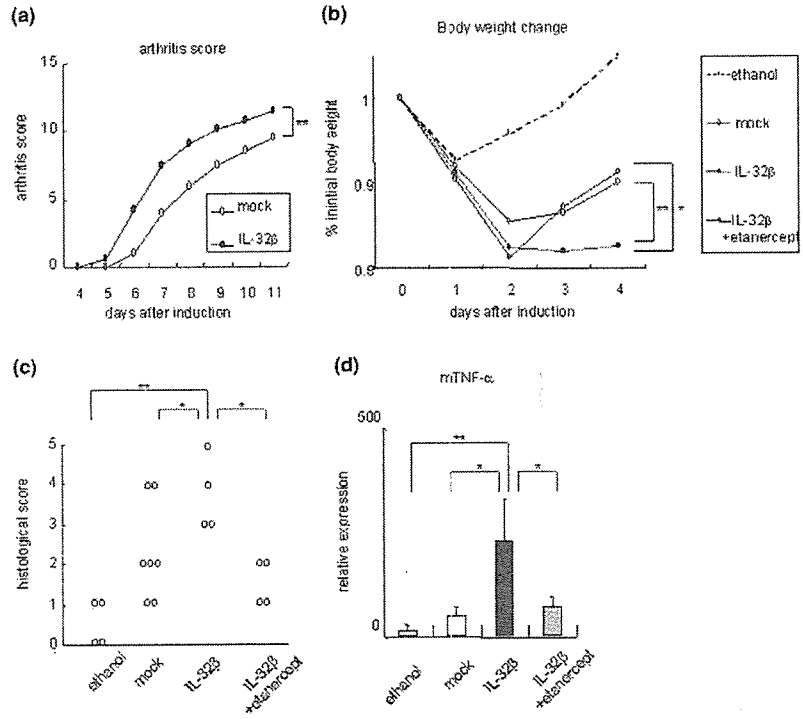
Human TNF receptor p80 Fc fusion protein, known as etanercept, neutralized the action of mouse TNFα and ameliorated disease progression in collagen-immunized mice [8,33]. Although etanercept is reported as less effective in treating Crohn's disease, the efficacy of etanercept in treating refractory Crohn's disease patients has been demonstrated [34,35]. We confirmed the efficacy of an increased dose of etanercept to TNBS-induced colitis C57BL/6 mice as a preliminary study (data not shown). When etanercept was administered to TNBS-treated BM-hIL-32 mice just after the onset of colitis, the severity of body weight loss was ameliorated (Figure 4b). *In vivo* expressed IL-32 was therefore supposed to play an important role in the exacerbation of colitis, in part through the TNFα-inducing effect. The expression of TNFα was markedly

Figure 3



Inflammatory cytokines were induced by human IL-32β in mice. **(a)** Raw 267.4 was cultured with the supernatant of human IL-32β (h IL-32β) or mock-transfected mammalian cells (293T) for 24 hours. Left, relative expression of mouse tumor necrosis factor alpha (mTNFα), compared with β-actin; right, secreted TNFα protein level measured by ELISA. **(b)** We generated hIL-32β overexpressed mice by transplantation of hIL-32β-transduced bone marrow cells. The expression of green fluorescent protein, was analyzed by flow cytometry 6–9 weeks after transplantation. **(c)** Expression of mTNFα, mIL-1β and mIL-6 in the cultured splenocytes of the control group (white bars; $n = 3$), or bone-marrow chimeric mice of the mock group (BM-Mock mice) (gray bars; $n = 4$), or hIL-32β (BM-hIL-32) (black bars; $n = 4$) with or without 1 μg/ml lipopolysaccharide (LPS). Concentrations of indicated cytokines of the cultured supernatants are shown in the right-hand figures. **(d)** Serum concentration of mTNFα determined in control mice ($n = 4$), in BM-Mock mice ($n = 8$), and in BM-hIL-32 mice ($n = 8$). **(e)** Expression of mTNFα in splenic F4/80$^+$ CD11c$^-$ macrophages of BM-Mock mice (gray bars; $n = 4$) and in BM-hIL-32 mice (black bars; $n = 4$). **(f)** Expression of mTNFα, mIL-1β, and mIL-6 in LPS-stimulated splenic F4/80$^+$ CD11c$^-$ macrophages and CD11c$^+$, CD3$^-$, and CD19$^-$ dendritic cells in BM-Mock mice (gray bars; $n = 4$), and in BM-hIL-32 mice (black bars; $n = 4$). Data are representative of at least three independent studies. *$P < 0.05$, **$P < 0.01$, BM-hIL-32 mice versus BM-Mock mice or control mice.

Figure 4



Exacerbation of murine models of tumor necrosis factor alpha-related inflammatory diseases in BM-hIL-32 mice. **(a)** Collagen-antibody-induced arthritis was induced in bone-marrow chimeric human IL-32β mice (BM-hIL-32) ($n = 6$) and bone-marrow chimeric mice of the mock group (BM-Mock) ($n = 4$). Mean arthritis scores are shown. **(b)** Body weight change after induction of trinitrobenzen sulfonic acid (TNBS)-induced colitis in BM-Mock mice ($n = 7$), in BM-hIL-32 mice ($n = 4$), and in BM-hIL-32 mice + 200 μg/day intraperitoneal administration of etanercept ($n = 4$). Control mice ($n = 5$) were administered only 50% ethanol with PBS. Percentage of initial body weight is shown. **(c)** Histological scores of TNBS-induced colitis. **(d)** Relative expression of mouse tumor necrosis factor alpha (mTNFα) in the colon of TNBS-induced colitis mice. $^*P < 0.05$, $^{**}P < 0.01$, BM-hIL-32 mice versus BM-Mock mice or BM-hIL-32 mice + etanercept.

increased in the rectal tissues of BM-hIL-32 mice, compared with BM-Mock mice or with etanercept-treated mice (Figure 4d). In this way, TNFα-related inflammation was exacerbated by overexpression of hIL-32β in the mouse model, and the proinflammatory effects of hIL-32β were demonstrated in the *in vivo* model.

Exacerbation of collagen-induced arthritis by transfer of IL-32β-transduced CD4+ T cells

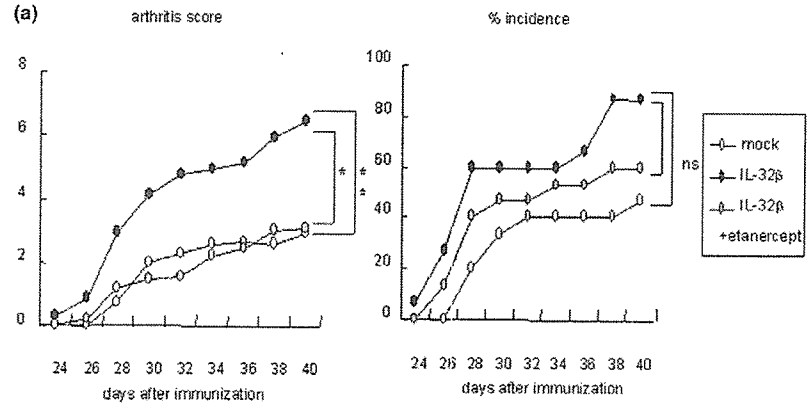
Since synovial-infiltrated lymphocytes strongly expressed IL-32, and peripheral CD4+ T cells significantly expressed IL-32, we supposed CD4+ T cells to be one of the important sources of IL-32 in the pathogenesis of inflammatory arthritis. To examine the proinflammatory effects of IL-32 produced by CD4+ T cells, we transduced the hIL-32β gene to CD4+ T cells with a retrovirus vector. We transferred these cells to bovine type II collagen-immunized mice before the onset of arthritis. The mice group to which the hIL-32β-transduced CD4+ T cells had been transferred developed arthritis earlier than the mock group of mice and showed significantly higher arthritis scores (Figure 5a). Histological investigation of the joints showed significantly severe cell infiltration in the hIL-32β group of mice (Figure 5b). In this way, hIL-32β produced by CD4+ T cells exacerbated arthritis in the mouse model.

In addition, a TNFα blockade by etanercept canceled the proarthritic effects of hIL-32β according to the clinical and pathological scores (Figure 5). IL-32-producing CD4+ T cells were therefore supposed to play an important role in the exacerbation of the inflammatory arthritis, in part through a TNFα-inducing effect. The proinflammatory effects of IL-32 were therefore generally dependent on the TNFα-inducing effect in these mouse models of inflammatory diseases.

Discussion

TNFα is a potent proinflammatory cytokine related to the pathogenesis of inflammatory diseases such as RA and IBDs [5,6,11]. The precise mechanism of TNFα induction in the inflammatory diseases, however, is still unclear. We have shown in the present article that *in vivo* expression of the novel cytokine hIL-32 induced TNFα production, and that overexpressed IL-32β significantly exacerbated the mouse model of

Figure 5



(b)

	Cell infiltration		pannus formation	bone erosion
Mock	1.21 ± 1.31	*	0.93 ± 1.39	0.79 ± 1.05
IL-32β	2.21 ± 0.89	*	1.86 ± 1.35	1.14 ± 0.86
IL-32β +etanercept	1.29 ± 1.44		1.07 ± 1.49	0.79 ± 1.05

Transfer of human IL-32β-transduced CD4+ T cells exacerbated collagen-induced arthritis. Human IL-32β-transduced CD4+ T cells were transferred to collagen-immunized mice before the onset of arthritis (day 23). In one group (IL-32β + etanercept group), 50 μg/day etanercept was administered intraperitoneally for 14 days after transfer of CD4+ T cells. Each group consisted of 14 mice. **(a)** Arthritis scores and the percentage incidence of arthritis. **(b)** Cell infiltration, pannus formation, and bone erosion in CIA mice are quantified. Histological scores are shown as the mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, IL-32β group versus mock group or IL-32β + etanercept group. ns, not significant.

arthritis and colitis. These results suggest that IL-32 plays an important role in the exacerbation of inflammatory diseases.

IL-32 has been reported an inducer of TNFα and other inflammatory cytokines *in vitro* [13]. Joosten and colleagues reported that the magnitude of IL-32 expression in the synovial tissues was related to the RA severity, and that recombinant hIL-32γ induced the joint inflammation in wild-type mice, which was suppressed in TNFα-deficient mice [16]. The *in vivo* effects and targets of IL-32, however, are still under examination. Moreover, the question of whether IL-32 plays a pathological role in animal models other than arthritis has not been addressed. Although the IL-32 receptor or mouse analog of IL-32 have not so far been reported, hIL-32 had biological activities on a mouse cell line and evoked joint inflammation in mice [13,16]. We therefore examined the *in vivo* effects of hIL-32β on bone marrow chimeric mice. We demonstrated the strong association *of in vivo* expressed IL-32 with TNFα production in the splenocytes, especially F4/80+ macrophages. Splenocyte proliferation to the anti-CD3 antibody or LPS stimulation was not affected by the *in vivo* expression of IL-32β (data not shown). The CD4+ T cells did not change cytokine expression in the presence of IL-32β. Therefore IL-32β had effects on macrophages rather on than T cells *in vivo*, and the *in vivo* roles of IL-32β were mainly to induce other inflammatory cytokines rather than to activate the proliferation of the immune cells. In the present study, we also demonstrated that the *in vivo* overexpression of hIL-32β resulted in the exacerbation of other mouse models of TNFα-related diseases – collagen-induced arthritis and hapten-induced colitis. In addition, these exacerbating effects of IL-32 were blocked by TNFα blockage, which was consistent with Joosten and colleagues' work [16].

IL-1 and IL-6 are also crucial cytokines in arthritis [4]. Injection of IL-1 into the normal joints of rabbits has caused severe arthritis [36]. IL-1RA-deficient mice developed chronic inflammatory arthritis [37,38]. Anti-IL-1 antibody and IL-1 deficiency ameliorated the mouse model of arthritis [39-41]. We have shown that the expression and secretion of IL-1β and IL-6 was increased in LPS-stimulated splenocytes from BM-hIL-32β

mice. IL-1β was expressed in CD11c$^+$ dendritic cells, and IL-6 was expressed in F4/80$^+$ macrophages after LPS stimulation. IL-32 therefore induced IL-1β and IL-6 secretion in collaboration with TLR4 stimulation in these cells. In parallel withthe *in vitro* effect of LPS, disease models of BM-hIL-32 mice were exacerbated in response to anticollagen antibodies and LPS stimulation or TNBS administration. These results strongly suggest that IL-32 functions in cooperation with other inflammatory signals *in vivo*. This induction of these proinflammatory cytokines may be one of the important mechanisms of IL-32 leading towards inflammation. Although the previous report did not demonstrate the synergizing effect of hIL-32 with Toll-like receptor signaling *in vitro* [42], our results suggest that continuous exposure to hIL-32 in relatively low concentrations would have an influence on Toll-like receptor signaling of splenocytes *in vivo*. Further studies are needed to clarify the relationship between IL-32 and Toll-like receptor signaling, however, and further studies are necessary for discerning the actual mechanisms of IL-32 in the development of inflammatory diseases.

Moreover, we demonstrated a reciprocal relationship between TNFα and IL-32. TNFα induced the reciprocal expression of IL-32 in various kinds of cells (namely CD4$^+$ T cells, MoDCs, and synovial fibroblasts). We suppose that a positive feedback system between TNFα and IL-32 promotes the tissue inflammation in the synovium and the intestinal epithelium. In this way, IL-32 has a close relationship with the proinflammatory cytokines, especially TNFα, and this relationship may be one of the main mechanisms by which IL-32 promotes inflammation. Indeed, the proinflammatory effects of hIL-32β on collagen-induced arthritis mice and TNBS-induced colitis mice were, in part, canceled by a TNFα blockade.

It was reported that human TNFα transgenic mice spontaneously developed inflammatory arthritis [1]. Although the serum TNFα concentration of BM-hIL-32 mice was comparable with those of reported human TNFα transgenic mice [43], no inflammatory change was observed in histological examination of the joints (data not shown). It is suspected that this different outcome occurred because BM-hIL-32 mice expressed the transduced cytokine only in bone-marrow-derived cells, not in fibroblasts or chondrocytes of the joints. There would therefore be no initiation of inflammation in the joints without the infiltration of bone-marrow-derived cells expressing hIL-32.

The source of IL-32 remains unsolved. In our experiment, T cells played a principal role in IL-32 production. In contrast to B cells and monocytes, T cells expressed IL-32 mRNA in a resting state. Moreover, activated T cells had the capacity to induce IL-32 mRNA expression in B cells and monocytes. In addition to TCR stimulation, IL-32 mRNA expression in CD4$^+$ T cells is induced by various stimuli of inflammatory cytokines related to RA. IL-12 + IL18 stimuli, IL-23 stimuli, and TNFα stimuli increased IL-32 mRNA expression in CD4$^+$ T cells. Since the mRNA expression of IL-32 was induced by either type of stimulation, IL-32 may be associated with the pathological roles of various dendritic cell-derived cytokines (namely IL-12, IL-18, and IL-23) in inflammatory diseases. These results suggested the capacity of CD4$^+$ T cells to produce IL-32 in response to a wide range of stimuli.

T cells are reported as important mediators in inflammatory diseases, such as RA and IBDs. In terms of genetics, MHC class II genes, especially the HLA-DR1 and DR4 subtypes, are associated with RA sensitivity [44], and HLA-DRB1 is associated with Crohn's disease sensitivity [45]. In the mouse model of colitis, Th1 cells were reported as important in the pathogenesis of colitis. The colons of TNBS-treated mice were marked by infiltration of CD4$^+$ T cells exhibiting a Th1 pattern of cytokine secretion. Administration of anti-IL-12 antibodies led to a striking improvement of TNBS-induced colitis [46].

In animal models of arthritis, the transfer of CD4$^+$ T cells from SKG mice and IL-1Ra-deficient mice has evoked arthritis in the recipient mice [38,47]. In particular, IL-17 production from activated T cells is required for the development of destructive arthritis in IL-1Ra-deficient mice [48]. Like the CD4$^+$ T cells from these animal models, IL-32β-producing CD4$^+$ T cells exacerbated collagen-induced arthritis. In RA synovium, IL-32 is principally expressed in infiltrated lymphocytes, which usually contain activated T cells [49]. We therefore speculate that CD4$^+$ T cells play an important role in the exacerbation of inflammatory arthritis by means of IL-32 secretion. Notably, IL-32 mRNA expression was detected in the synovial-infiltrated lymphocytes. We supposed that IL-32-producing lymphocytes infiltrating the inflamed synovium participate in the production of TNFα in the RA synovium.

This result does not exclude the possibility that a relatively low amount of IL-32 is expressed in the synovial membranes of RA patients, because the sensitivity of *in situ* hybridization is limited [50]. In the previous study, the synovial lining cells of RA patients were stained by the anti-IL-32 antibody [16]. We assumed that the phase or type of synovial inflammation was different between Joosten and colleagues' patients and our patients. In Joosten and colleagues' report, the synovial samples were obtained by percutaneous needle biopsy, and it is suspected that their RA patients had active disease [16]. Our samples, however, were obtained from patients in the progressed stage during total joint replacement surgery. In the severely damaged joint, the cytokine-producing functions of synovial lining cells are impaired [51]. We therefore suspected that the expression of IL-32 could not be detected in the synovial lining cells by *in situ* hybridization in our study. Another explanation is the difference of types of RA. The distribution of IL-32 expression in synovial tissues may be dependent on the type of RA, a matter that needs to be examined further.

Conclusion

IL-32 mRNA was expressed mainly in the lymphoid tissues and in a broad range of immune cells, including CD4+ T cells. In RA patients, abundant IL-32 mRNA expression was observed in the synovial-infiltrated lymphocytes. Splenic macrophages of hIL-32β-overexpressed mice showed increased expression and secretion of TNFα and IL-6, and splenic dendritic cells showed increased expression and secretion of IL-1β in response to LPS stimulation. Overexpression of hIL-32β also resulted in the exacerbation of collagen antibody-induced arthritis and TNBS-induced colitis. IL-32β-producing CD4+ T cells significantly exacerbated inflammatory arthritis in the mouse model. The effects of IL-32 in different disease models were almost canceled by TNFα blockade.

This is the first study that demonstrated the *in vivo* cytokine-inducing effects of IL-32. In addition, the reciprocal induction between IL-32 and TNFα was also demonstrated in many types of cells. IL-32 therefore has a close relationship with TNFα, contributes to the exacerbation of inflammatory diseases, and could be a new therapeutic target of these inflammatory diseases.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HS carried out the molecular and animal experiments, performed the statistical analysis, and drafted the manuscript. KF supervised the study design, the statistical analysis, and the writing of the manuscript. YY and AO carried out the cell culture experiments. TS prepared the human samples of synovial tissues and cells. YK performed the quantitative PCR. KY supervised the study design and gave valuable advice to HS. All authors read and approved the final manuscript.

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

Keishi Fujio,[2]* Akiko Okamoto,* Yasuto Araki,* Hirofumi Shoda,* Hiroyuki Tahara,* Nelson H. Tsuno,† Koki Takahashi,† Toshio Kitamura,‡ and Kazuhiko Yamamoto*

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,

*Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; †Department of Transfusion Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and ‡Division of Cellular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan

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[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, 113-0033, Japan. E-mail address: kfujio-tky@umin.ac.jp

[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$\times$ 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$\times$ 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).

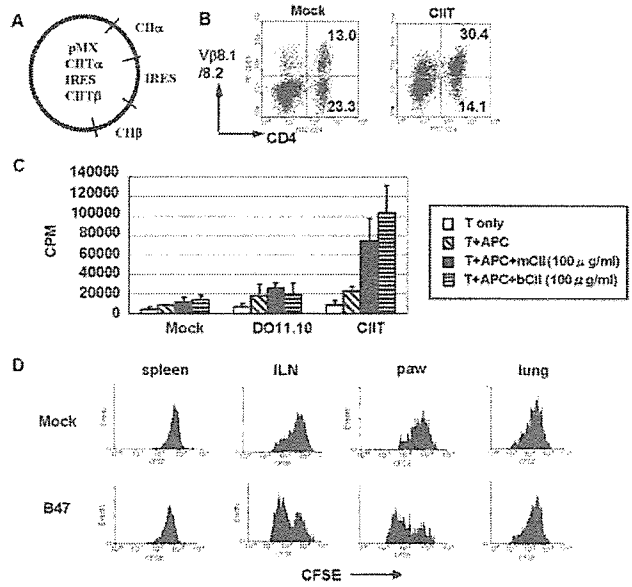

FIGURE 1. Reconstitution of CII-specific TCR, CIIT, on splenic CD4$^+$ T cells of DBA1 mice. *A*, Schematic representation of the bicistronic retrovirus construct of CIIT. *B*, Representative result of retroviral transduction of CIIT in DBA1 splenocytes. The cells were stained for Vβ8.1/8.2 and CD4. *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.

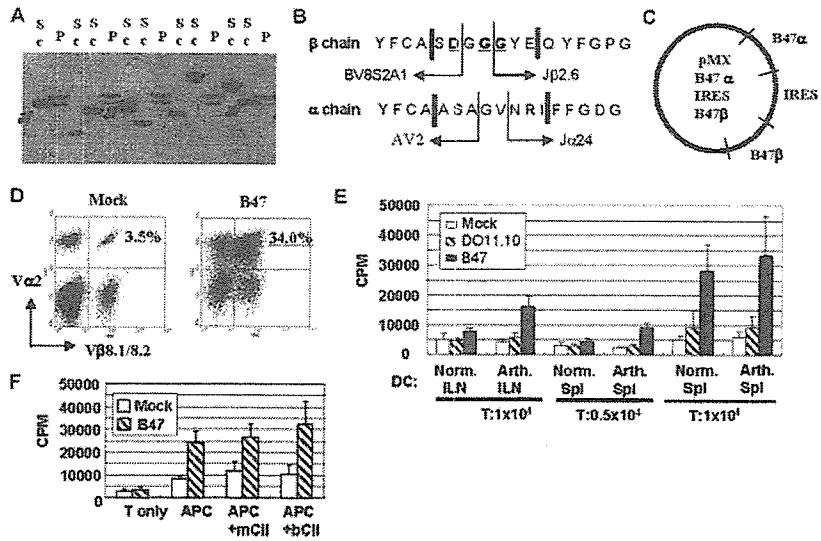

FIGURE 2. Identification and reconstitution of B47 TCR, which was autoreactive and not specific to CII. *A*, An example of identification of an expanded TCR in CIA by the TCR-SSCP method. RT-PCR was performed with Vβ8.1/8.2-specific and Cβ primers for cDNA of sorted single cells and the total paw. PCR 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.

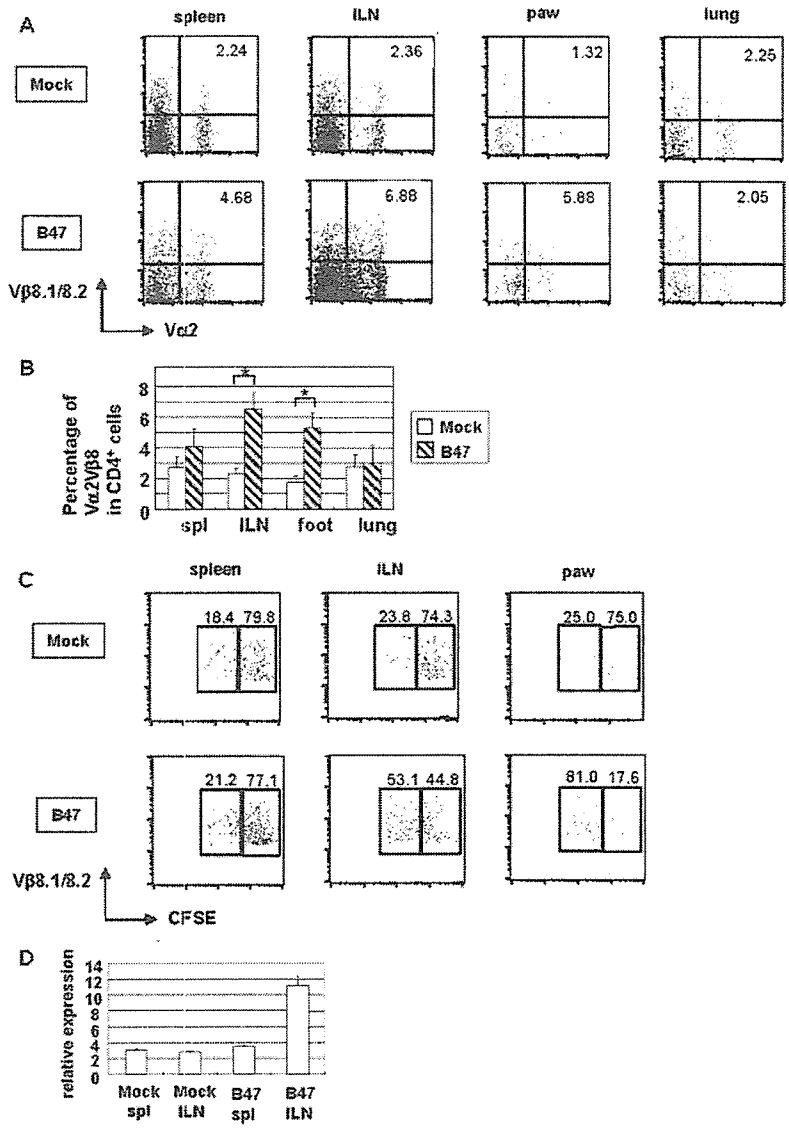

FIGURE 3. Kinetics of B47-transduced T cells in arthritic mice. *A*, Mock- or B47-transduced cells were labeled with CFSE and i.v. transferred to arthritic mice. Five days later, the spleen, ILN, paws, and lungs were analyzed for $V\alpha2^+V\beta8.1/8.2^+CFSE^+CD4^+$ T cells by FACS. *B*, The average 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