Published November 19, 2007

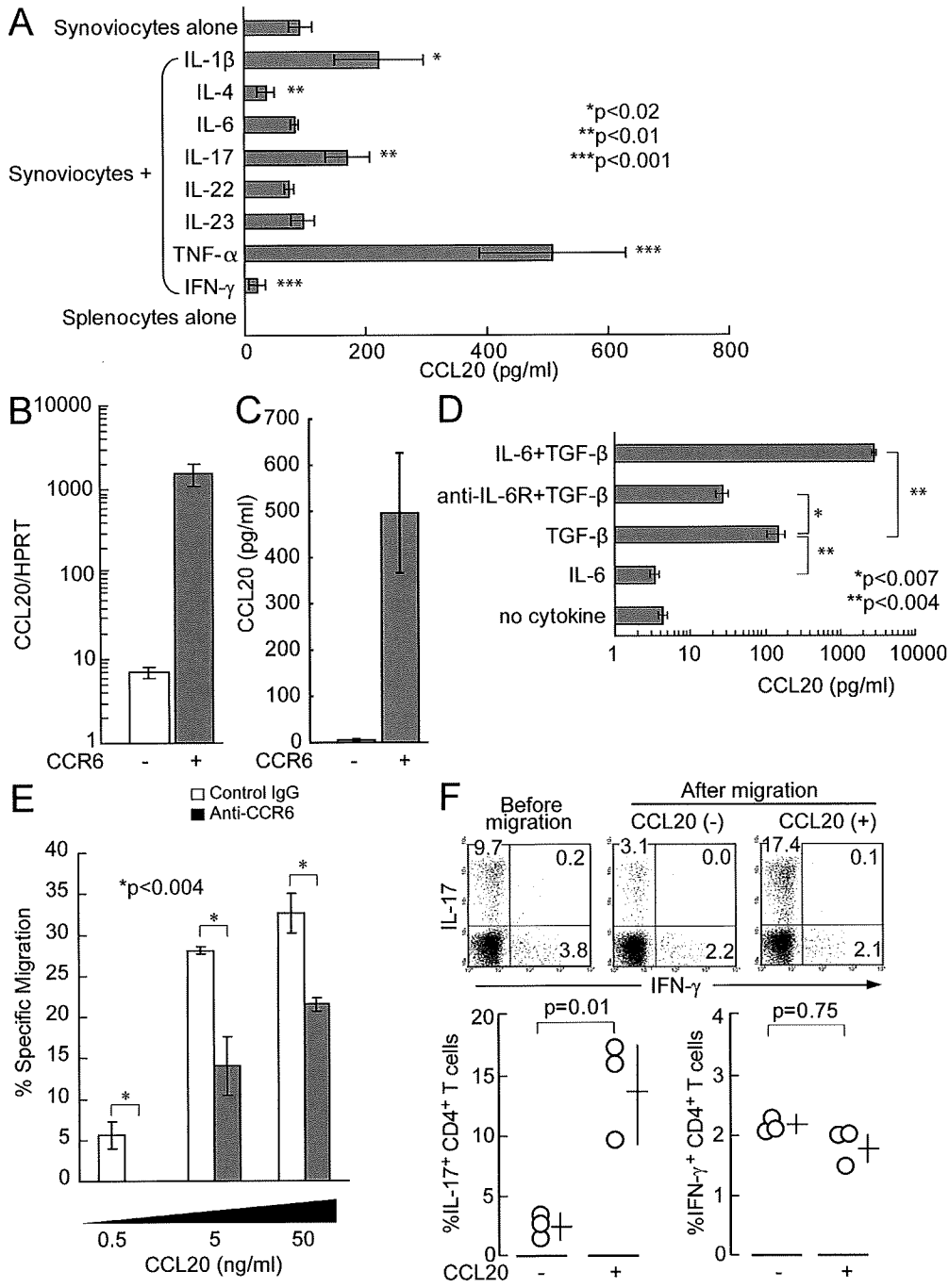

Figure 3. Cross-regulation of synoviocyte CCL20 production by cytokines and preferential migration of Th17 cells in response to CCL20. (A) 2.5 × 10^4 adherent synoviocytes, prepared as previously described (reference 10), were cultured with 10 ng/ml of the indicated cytokine for 24 h. The amounts of CCL20 in culture supernatants were measured by ELISA. Data are shown as the mean ± SD of triplicate wells. (B and C) Purified $CCR6^+$ or $CCR6^-CD4^+$ T cells from SKG mice were stimulated with PMA/ionomycin for 3 h; CCL20 messenger RNA was assessed by quantitative RT-PCR (B), and CCL20 protein was measured by ELISA (C). Data are shown as the mean ± SD of three independent experiments. (D) $CD4^+$ T cells were in vitro driven to differentiate to Th17 cells in the presence of IL-6 and TGF-β, as previously described (reference 21). Cells were restimulated with PMA/ionomycin for 3 h on day 4, and the amounts of CCL20 were measured by ELISA. (E) SKG LN cells were placed with 100 μg/ml anti-CCR6 antibody or control IgG on the upper well of a Transwell system. The migration assay was performed in the presence of designated concentrations of CCL20 added to the bottom well. See Materials and methods for details of the experiments. Data are shown as the mean ± SD of triplicate wells. (F) The migration assay was performed in the absence or the presence of 50 ng/ml CCL20, as in E. $CD4^+$ cells before being added to the upper well and $CD4^+$ T cells that had migrated to the lower well were stained for intracellular IL-17 and IFN-γ (top). Percentages of $IL\text{-}17^+$ or $IFN\text{-}\gamma^+$ cells among $CD4^+$ cells in the lower wells in three independent migration assays with (+) or without (–) CCL20 are shown (bottom). Vertical bars represent the means ± SD. Results in A, D, and E represent three independent experiments.

Published November 19, 2007

small (~15%) fraction of IL-17$^+$ cells, whereas addition of IL-23, TNF-α, or IL-21 did not; the percentage further increased to ~30% by neutralization of both IFN-γ and IL-4 (Fig. 2 C).

Collectively, these results indicate that, although IL-6 and TGF-β together can induce RORγt at an amount sufficient for directing IL-17 production in vitro (7), a higher amount of RORγt or additional cytokines including IL-1 may be required for CCR6 expression. Our results also suggest that IFN-γ and IL-4, both of which inhibit the differentiation of naive T cells to Th17 cells (3), may also suppress CCR6 expression in Th17 cells.

CCL20 production by inflamed synoviocytes and Th17 cells

CCL20 is so far known to be the sole ligand for CCR6 and able to direct the migration of CCR6$^+$ cells (15–19). To assess the possible production of CCL20 in inflamed synovial tissue to attract arthritogenic Th17 cells, collagenase-digested synovial tissues from swollen ankle or wrist joints of SKG mice were in vitro cultured, as previously described (10), and the amounts of CCL20 in culture supernatants were measured by ELISA. The dispersed granulocytes and monocytes from the inflamed synovial tissue, or the splenocytes of arthritic mice, did not produce CCL20 without stimulation (Fig. 3 A and not depicted). Notably, adherent synovial cells, mainly fibroblast-like synoviocytes, spontaneously produced CCL20 (Fig. 3 A). Adding recombinant IL-1β, IL-17, or TNF-α to the culture at the doses used for in vitro Th17 cell induction (21) augmented CCL20 production by the adherent synoviocytes, whereas the addition of recombinant IFN-γ or IL-4 inhibited production (Fig. 3 A). Other Th17 cell–associated cytokines, such as IL-6, IL-22, and IL-23, had no significant effect on the production at the doses used for controlling Th17 cell differentiation (Fig. 3 A) (21–23).

In addition to synoviocytes, purified CCR6$^+$CD4$^+$ SKG T cells actively transcribed CCL20 messenger RNA and secreted a large amount of the CCL20 protein compared with very low transcription or secretion by CCR6$^-$CD4$^+$ SKG T cells (Fig. 3, B and C). This concurred with the result of the microarray analysis, which showed a high expression of CCL20 by SKG FR4$^-$CD4$^+$ T cells and BALB/c IFN-γ$^{-/-}$CD4$^+$ T cells after homeostatic proliferation (Fig. 1 E). In addition, Th17 cells that were induced in vitro from normal BALB/c CD4$^+$ T cells by TCR stimulation in the presence of IL-6 and TGF-β produced a large amount of CCL20 (Fig. 3 D).

Functionally, in vitro migration assays showed that CCR6$^+$CD4$^+$ SKG T cells migrated in response to CCL20 in a dose-dependent manner and that the addition of an anti-CCR6 mAb, which blocks the binding of CCL20 to CCR6, effectively inhibited the migration at such low doses of CCL20 as those secreted by cultured synoviocytes in Fig. 3 A (Fig. 3 E). Furthermore, CD4$^+$ SKG T cells that had migrated in the presence of CCL20 were significantly enriched for IL-17$^+$ CD4$^+$ T cells, but not for IFN-γ$^+$ CD4$^+$ T cells,

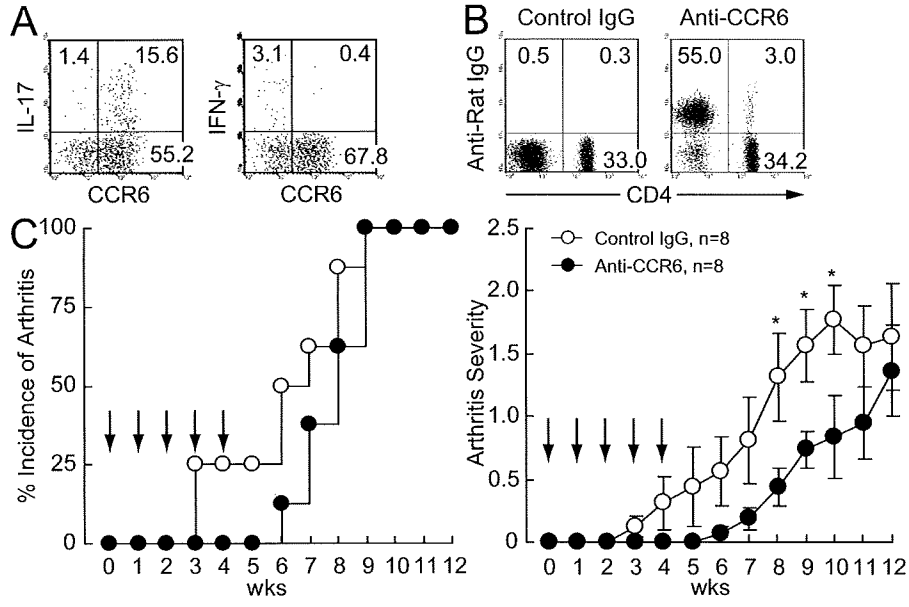

Figure 4. Contribution of CCR6/CCL20 to trafficking of Th17 cells in SKG arthritis. (A) CD4$^+$ T cells infiltrating arthritic joints were prepared from collagenase-digested ankle joints of SKG mice, and stained for CCR6 and intracellular IL-17 or IFN-γ. (B) SKG mice received an i.v. injection of 100 μg anti-CCR6 antibody or control IgG; LN cells were stained by anti-rat IgG and CD4 48 h after injection. The percentage of cells in each quadrant is shown in A and B. (C) SCID mice received an i.v. injection of 10^6 SKG CD4$^+$ T cells, and then injections of 100 μg anti-CCR6 mAb or control IgG once a week for 5 wk. The arrows indicate i.v. injections of anti-CCR6 mAb. The incidence and severity of arthritis was scored every week, as previously described (reference 8). Vertical bars represent the means ± SEM. The disease curves of arthritis scores are significantly different between the two groups ($P < 0.005$ according to the analysis of covariance test). The differences in scores are statistically significant (according to the Mann-Whitney U test) in the 8th (*, $P = 0.04$), 9th (*, $P = 0.02$), and 10th (*, $P = 0.04$) wk. Results in A and B represent three independent experiments.

Published November 19, 2007

indicating that Th17 cells preferentially migrated in response to CCL20, whereas Th1 cells did not (Fig. 3 F).

Collectively, these results indicate that both activated synoviocytes and CCR6$^+$Th17 cells themselves secrete CCL20 and further recruit other CCR6-expressing Th17 cells to the site of Th17 cell–mediated joint inflammation.

The effect of CCR6 blockade on the initial phase of Th17 cell–mediated arthritis

We previously reported that Th17 cells predominantly infiltrated into the arthritic joints of SKG mice (6). These infiltrating Th17 cells indeed expressed CCR6, in contrast with infiltrating Th1 cells, which were CCR6$^-$ (Fig. 4 A). To investigate the role of CCR6 on SKG autoimmune arthritis, we initially examined the effect of in vivo anti-CCR6 mAb treatment on lymphocytes. The anti-CCR6 mAb, which was used for in vitro CCR6 blockade (Fig. 3 E), was not cell depleting in vivo: after one injection into SKG mice, it bound to CCR6 on the cell surface of B cells and a population of CD4$^+$ T cells, and the binding persisted for at least 8 d (Fig. 4 B and Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20071397). To assess the effect of this CCR6 blockade

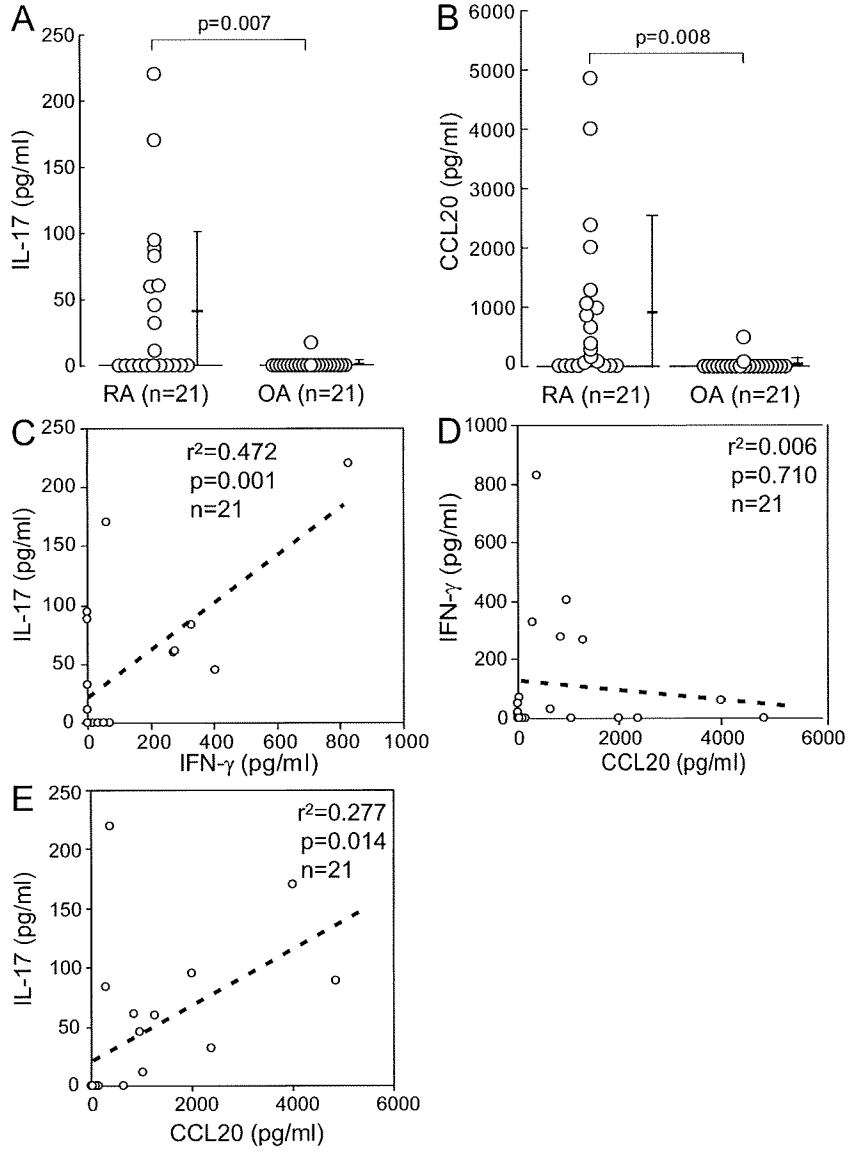

Figure 5. Production of IL-17 and CCL20 in RA joints. (A and B) The concentrations of IL-17 and CCL20 in synovial fluid from RA or OA patients were measured by ELISA. Vertical bars represent the means ± SD. (C–E) Scatter plots show the correlation between IL-17 and IFN-γ (C), CCL20 and IFN-γ (D), or CCL20 and IL-17 (E) in the synovial fluid of RA patients. RA and OA patients are 66.6 ± 11.6 and 74 ± 7.4 yr old, respectively. r^2 values of Pearson's product-moment correlation and p-values of their null hypothesis are shown. Similar analyses performed only on the samples with the amounts of IL-17, IFN-γ, or CCL20 detectable by ELISA yielded the following statistics: IL-17 versus CCL20, $r^2 = 0.147$ and $P = 0.177$ ($n = 14$); IFN-γ versus CCL20, $r^2 = 0.0034$ and $P = 0.481$ ($n = 17$); IL-17 versus IFN-γ, $r^2 = 0.359$ and $P = 0.003$ ($n = 13$).

Published November 19, 2007

on the development of arthritis, we transferred SKG $CD4^+$ T cells to syngeneic SCID mice and i.v. injected mAb once a week for 5 wk after cell transfer (Fig. 4 C). The treatment significantly suppressed the onset and severity of arthritis in an early phase: 3 wk after cell transfer, when control antibody–treated mice started to show joint swelling, much smaller number of T cells infiltrated into the joints of anti-CCR6–treated mice than control antibody–treated mice (Fig. S6). Thus, the blockade of CCL20 binding to CCR6 could suppress the development of Th17 cell–mediated autoimmune arthritis, at least in the initial phase of disease progression, presumably by interfering with the trafficking of Th17 cells (6).

Production of IL-17 and CCL20 in arthritic joints of human RA

We extended our analysis of IL-17 and CCR6/CCL20 in SKG arthritis to human RA. Synovial fluid of RA patients contained significantly higher amounts of IL-17 and CCL20 compared with osteoarthritis (OA) patients, in accord with other reports (Fig. 5, A and B) (24–26). A significant correlation was observed between the amounts of IL-17 and IFN-γ (Fig. 5 C). Notably, however, the level of CCL20 in RA joints was well correlated with that of IL-17 but not of IFN-γ (Fig. 5, D and E). As in mice, intracellular staining of IL-17 and IFN-γ or IL-4 of peripheral $CD4^+$ T cells in normal healthy individuals showed that human Th17 cells were distinct from Th1 or Th2 cells and expressed CCR6, whereas $CD4^+$ T cells producing IFN-γ or IL-4 were $CCR6^-$ (Fig. S7, available at http://www.jem.org/cgi/content/full/jem.20071397), similar to the result recently reported on human Th17 cells in individuals with infectious diseases (27). Collectively, these findings suggest that Th17 cell trafficking via CCR6/CCL20 may contribute to RA pathology.

We have thus shown that CCR6 and CCL20 are expressed by Th17 cells and are required for the migration of Th17 cells to initiate self-destructive immune reactions in the joints, leading to the development of autoimmune arthritis such as RA. Once synovial inflammation occurs, synoviocytes may further recruit Th17 cells through CCL20 production, which is enhanced by proinflammatory cytokines produced by activated synoviocytes, such as IL-17, IL-1β, and TNF-α (9), and dampened by IFN-γ or IL-4. Thus, Th1, Th2, and Th17 cell–produced cytokines, collectively with those produced by synoviocytes, form a cytokine milieu to cross-regulate not only the development of Th17 cells but also the trafficking of $CCR6^+$ Th17 cells via controlling the production of CCL20. It has been shown that joint infiltration of $CCR6^+$ T cells is associated with human RA, and high expression of CCL20 in the central nervous system is observed in an animal model of multiple sclerosis (25, 26, 28–30). It remains to be determined whether intervention in Th17 cell trafficking via CCR6/CCL20 is useful to treat and prevent Th17 cell–mediated autoimmune diseases, including RA and multiple sclerosis. Such treatments include blocking CCR6 on Th17 cells at both the initial and chronic phases of disease progression and reducing the production of CCL20 by changing the cytokine milieu at inflammation sites.

MATERIALS AND METHODS

Mice. BALB/c and SCID mice were purchased from Japan Clea. BALB/c IFN-γ $^{-/-}$ mice were purchased from the Jackson Laboratory. IL-$6^{-/-}$ mice were backcrossed to BALB/c mice more than eight times. $RAG2^{-/-}$ BALB/c mice were a gift from Y. Shinkai (Kyoto University, Kyoto, Japan) and were crossed to IL-$6^{-/-}$ mice to generate IL-$6^{-/-}$ $RAG2^{-/-}$ BALB/c mice. These mice were maintained in our animal facility under specific pathogen-free conditions and treated in accordance with the institutional guidelines for animal care at the Institute for Frontier Medical Sciences of Kyoto University. The animal experiments were approved by the animal ethics committee of the Institute for Frontier Medical Sciences.

Antibodies. The following reagents were purchased from BD Biosciences: anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD16/CD32 (2.4G2), anti-TCRαβ (H57-597), anti-CCR6 (140706), anti–IL-4 (11B11), anti–IFN-γ (XMG1.2), anti–IL-17 (TC11-18H10.1), PE-labeled goat anti–rat Ig, and isotype control IgG. Purified anti–IL-6R (MR16-1) was a gift from N. Nishimoto (Osaka University, Osaka, Japan). Purified anti-CCR6 (140706) was a gift from BD Biosciences. Anti-FR4 (Th6), which is of the rat IgG2a isotype, were purified from the culture supernatant of the hybridoma and labeled in our laboratory, as previously described (12).

Intracellular cytokine staining. LN or spleen cells were stimulated with 20 ng/ml PMA and 1 μM ionomycin in the presence of GolgiStop (BD Biosciences) for 5 h, stained for surface antigens, fixed, and permeabilized using Cytofix/Cytoperm (BD Biosciences), followed by anti–IL-17 and anti–IFN-γ or IL-4 staining.

Measurement of cytokines and chemokines. Mouse IL-17 and CCL20 were measured by ELISA using Quantikine M (R&D Systems), with a detection limit of 11 pg/ml and 3.9 pg/ml, respectively. Human IL-17, IFN-γ (both from eBioscience), and CCL20 (R&D Systems) in synovial fluid were measured by ELISA, with a detection limit of 4, 8, and 8 pg/ml, respectively.

Gene microarray analysis. Total RNA was extracted using the RNeasy Micro Kit (QIAGEN) and was subjected to gene microarray (GeneChip Mouse Genome 430 2.0 Array; Affymetrix). Analysis of gene expression was performed by GeneSpring software (Agilent Technologies). Microarray data are available from the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE9316.

Retroviral constructs and transduction. Complementary DNA (cDNA) encoding full-length mouse *Rorgt* was amplified by RT-PCR from the cDNA of SKG $CD4^+$ T cells and cloned into the pMxs-IRES-GFP vector. Retroviral transduction was performed as previously described (7). The pMxs-IRES-GFP vector was a gift from T. Kitamura (The University of Tokyo, Tokyo, Japan).

Preparation of synoviocytes. Synovial tissues from inflamed ankle joints were digested with 400 Mandl U/ml of Liberase Blendzyme II (Roche) in plain RPMI 1640 medium for 1 h at 37°C to prepare single-cell suspensions. Synovial cells were cultured in RPMI 1640 medium containing 20% FBS, and synoviocytes were prepared from the adherent cells.

Chemotaxis assay. Cell migration was evaluated using the 24-well, 5-μm pore size Transwell system (Costar). 10^6 LN cells were placed on the top of the Transwell in RPMI 1640 containing 10 mM Hepes buffer. CCL20 was added to the bottom of the Transwell system in RPMI 1640 containing 10 mM Hepes buffer and 1% FBS. After 4 h of incubation at 37°C, the number of cells that had migrated into the lower well was analyzed by counting $CCR6^+$ $CD4^+$ cells for 3 min using a flow cytometer (FACSCalibur; BD Biosciences).

Published November 19, 2007

Analysis of synovial fluid. Synovial fluid was collected from RA patients, fulfilling the revised classification criteria of the American College of Rheumatology for RA, or OA patients during orthopedic operation under written informed consent. The experiments were approved by the ethics committee of the Kyoto University Graduate School and Faculty of Medicine.

Statistical analysis. The Student's *t* test was used for statistical analyses, unless indicated otherwise. $P \leq 0.05$ was considered significant.

Online supplemental material. Fig. S1 presents the 23 genes, among 29 genes commonly up-regulated in the two sets of analyses in Fig. 1 D, that are not encoding cytokines, chemokines, or their receptors. Fig. S2 shows the expression of CCR6 on BALB/c or SKG $CD4^+$ T cells and the induction of CCR6 after homeostatic proliferation. Fig. S3 exhibits the expression of IL-1R1 on Th17 cells. Fig. S4. shows that $CCR6^+CD4^+$ T cells include not only IL-17–producing cells but also TNF-α–producing cells and $Foxp3^+$ cells. Fig. S5 shows that anti-CCR6 is not a cell-depleting antibody. Fig. S6 shows that the treatment of anti-CCR6 antibody inhibits the migration of $CCR6^+CD4^+$ cells into the joints of the SCID mice that received cell transfer. Fig. S7 demonstrates the expression of CCR6 on human Th17 cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20071397.

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

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, and the Japan Science and Technology Agency.

The authors declare no competing interests.

Submitted: 9 July 2007
Accepted: 25 October 2007

REFERENCES

1. Harris, E.D. 1997. Rheumatoid Arthritis. W.B. Saunders Co., Philadelphia. 417 pp.
2. Langrish, C.L., Y. Chen, W.M. Blumenschein, J. Mattson, B. Basham, J.D. Sedgwick, T. McClanahan, R.A. Kastelein, and D.J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201:233–240.
3. Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* 6:1123–1132.
4. Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* 6:1133–1141.
5. Nakae, S., S. Saijo, R. Horai, K. Sudo, S. Mori, and Y. Iwakura. 2003. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc. Natl. Acad. Sci. USA.* 100:5986–5990.
6. Hirota, K., M. Hashimoto, H. Yoshitomi, S. Tanaka, T. Nomura, T. Yamaguchi, Y. Iwakura, N. Sakaguchi, and S. Sakaguchi. 2007. T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17^+ Th cells that cause autoimmune arthritis. *J. Exp. Med.* 204: 41–47.
7. Ivanov, I.I., B.S. McKenzie, L. Zhou, C.E. Tadokoro, A. Lepelley, J.J. Lafaille, D.J. Cua, and D.R. Littman. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 126:1121–1133.
8. Sakaguchi, N., T. Takahashi, H. Hata, T. Nomura, T. Tagami, S. Yamazaki, T. Sakihama, T. Matsutani, I. Negishi, S. Nakatsuru, and S. Sakaguchi. 2003. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature.* 426:454–460.
9. Hata, H., N. Sakaguchi, H. Yoshitomi, Y. Iwakura, K. Sekikawa, Y. Azuma, C. Kanai, E. Moriizumi, T. Nomura, T. Nakamura, and S. Sakaguchi. 2004. Distinct contribution of IL-6, TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J. Clin. Invest.* 114:582–588.
10. Yoshitomi, H., N. Sakaguchi, K. Kobayashi, G.D. Brown, T. Tagami, T. Sakihama, K. Hirota, S. Tanaka, T. Nomura, I. Miki, et al. 2005. A role for fungal β-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J. Exp. Med.* 201:949–960.
11. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6:345–352.
12. Yamaguchi, T., K. Hirota, K. Nagahama, K. Ohkawa, T. Takahashi, T. Nomura, and S. Sakaguchi. 2007. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. *Immunity.* 27:145–159.
13. Sutton, C., C. Brereton, B. Keogh, K.H. Mills, and E.C. Lavelle. 2006. A crucial role for interleukin (IL) 1 in the induction of IL-17–producing T cells that mediate autoimmune encephalomyelitis. *J. Exp. Med.* 203: 1685–1691.
14. Zheng, Y., D.M. Danilenko, P. Valdez, I. Kasman, J. Eastham-Anderson, J. Wu, and W. Ouyang. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature.* 445:648–651.
15. Liao, F., R.L. Rabin, C.S. Smith, G. Sharma, T.B. Nutman, and J.M. Farber. 1999. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. *J. Immunol.* 162:186–194.
16. Charbonnier, A.S., N. Kohrgruber, E. Kriehuber, G. Stingl, A. Rot, and D. Maurer. 1999. Macrophage inflammatory protein 3α is involved in the constitutive trafficking of epidermal Langerhans cells. *J. Exp. Med.* 190:1755–1768.
17. Dieu-Nosjean, M.C., C. Massacrier, B. Homey, B. Vanbervliet, J.J. Pin, A. Vicari, S. Lebecque, C. Dezutter-Dambuyant, D. Schmitt, A. Zlotnik, and C. Caux. 2000. Macrophage inflammatory protein 3α is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J. Exp. Med.* 192:705–718.
18. Iwasaki, A., and B.L. Kelsall. 2000. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3α, MIP-3β, and secondary lymphoid organ chemokine. *J. Exp. Med.* 191:1381–1394.
19. Le Borgne, M., N. Etchart, A. Goubier, S.A. Lira, J.C. Sirard, N. van Rooijen, C. Caux, S. Ait-Yahia, A. Vicari, D. Kaiserlian, and B. Dubois. 2006. Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8+ T cell crosspriming in vivo. *Immunity.* 24:191–201.
20. Kleinewietfeld, M., F. Puentes, G. Borsellino, L. Battistini, O. Rotzschke, and K. Falk. 2005. CCR6 expression defines regulatory effector/memory-like cells within the CD25(+)CD4+ T-cell subset. *Blood.* 105:2877–2886.
21. Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* 24:179–189.
22. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* 441:235–238.
23. Mangan, P.R., L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, R.D. Hatton, S.M. Wahl, T.R. Schoeb, and C.T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature.* 441:231–234.
24. Kotake, S., N. Udagawa, N. Takahashi, K. Matsuzaki, K. Itoh, S. Ishiyama, S. Saito, K. Inoue, N. Kamatani, M.T. Gillespie, et al. 1999. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J. Clin. Invest.* 103:1345–1352.
25. Matsui, T., T. Akahoshi, R. Namai, A. Hashimoto, Y. Kurihara, M. Rana, A. Nishimura, H. Endo, H. Kitasato, S. Kawai, et al. 2001. Selective

Published November 19, 2007

recruitment of CCR6-expressing cells by increased production of MIP-3 alpha in rheumatoid arthritis. *Clin. Exp. Immunol.* 125:155–161.

26. Ruth, J.H., S. Shahrara, C.C. Park, J.C. Morel, P. Kumar, S. Qin, and A.E. Koch. 2003. Role of macrophage inflammatory protein-3alpha and its ligand CCR6 in rheumatoid arthritis. *Lab. Invest.* 83:579–588.
27. Acosta-Rodriguez, E.V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8:639–646.
28. Serafini, B., S. Columba-Cabezas, F. Di Rosa, and F. Aloisi. 2000. Intracerebral recruitment and maturation of dendritic cells in the onset and progression of experimental autoimmune encephalomyelitis. *Am. J. Pathol.* 157:1991–2002.
29. Ambrosini, E., S. Columba-Cabezas, B. Serafini, A. Muscella, and F. Aloisi. 2003. Astrocytes are the major intracerebral source of macrophage inflammatory protein-3alpha/CCL20 in relapsing experimental autoimmune encephalomyelitis and in vitro. *Glia.* 41:290–300.
30. Kohler, R.E., A.C. Caon, D.O. Willenborg, I. Clark-Lewis, and S.R. McColl. 2003. A role for macrophage inflammatory protein-3 alpha/CC chemokine ligand 20 in immune priming during T cell-mediated inflammation of the central nervous system. *J. Immunol.* 170: 6298–6306.

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.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[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.

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

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

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

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

Materials and Methods

Animals

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

Collagen-induced arthritis

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

Cytokines and cell lines

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

Murine PECs

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

Preparation of retroviral constructs of mIL-17 family cDNAs

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

Production of retroviral supernatants and retroviral transduction

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

Gene transduction to mouse splenocytes and adoptive transfer

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

BM precursor cell isolation, infection, and transfer

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

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

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

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

Immunoassays of cytokines and anti-type II collagen Ab

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

Isolation of cartilage

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

Cell purification

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

Intracellular cytokine staining and flow cytometry

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

Anti-IL-17B Ab treatment in CIA mice

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

Histopathology

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

Statistical analysis

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

Results

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

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

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

IL-17 family induced several proinflammatory cytokines

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

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

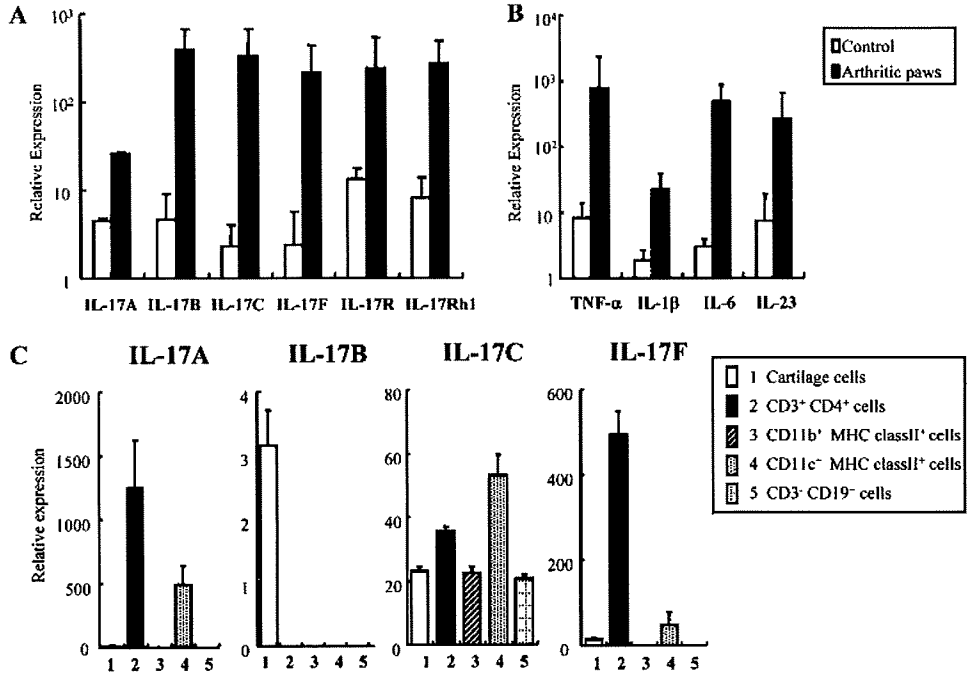

FIGURE 1. The expression of IL-17 family members and IL-17R genes in the arthritic paws of CIA mice. *A*, The expressions of IL-17 family genes and IL-17R genes were examined in the arthritic paws of CIA mice (■; $n = 3$) and in control mice (□; $n = 3$) by quantitative PCR. *B*, The expressions of inflammatory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

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

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

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

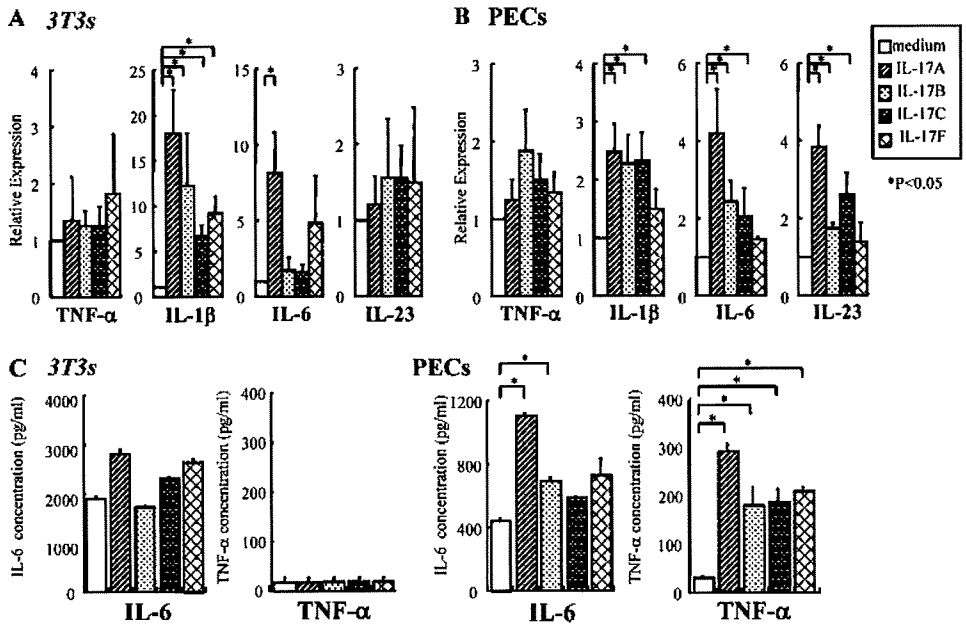

FIGURE 2. The proinflammatory effects of IL-17 family members on mouse fibroblasts and macrophages. *A*, Relative expression of the cytokine genes in *3T3* cell. The mouse fibroblast cell line *3T3* was cultured with each of mIL-17A, mIL-17B, mIL-17C, or mIL-17F for 24 h, and the expressions of inflammatory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

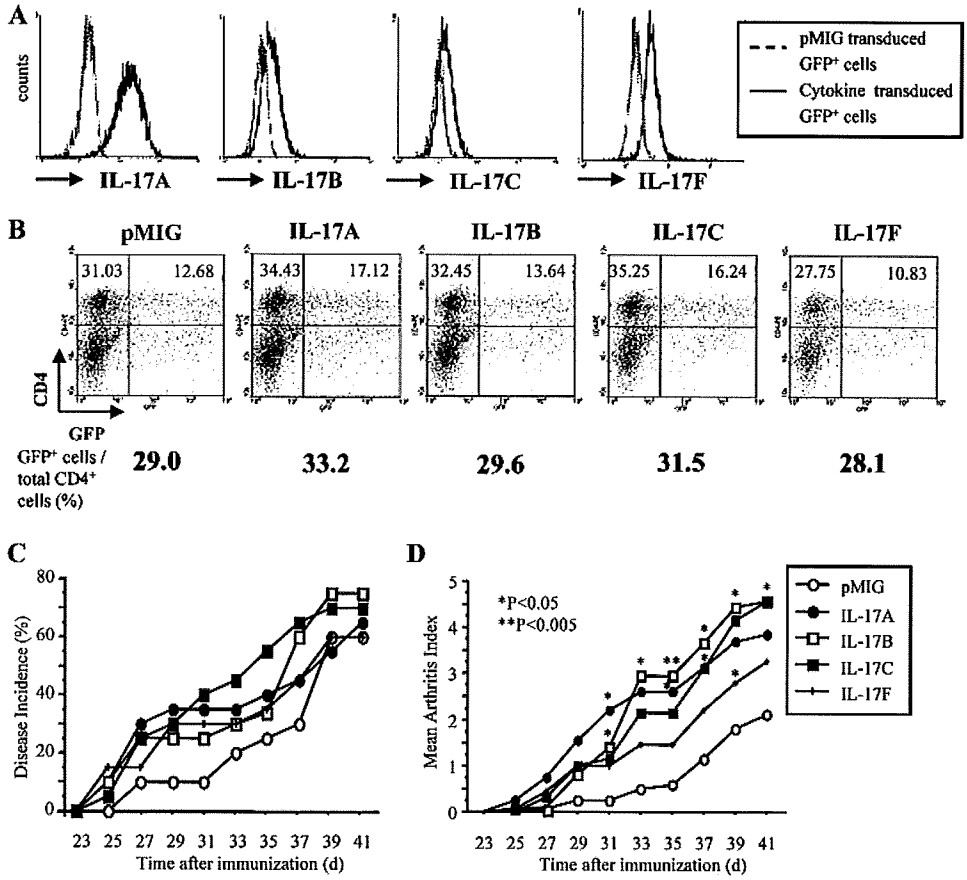

FIGURE 3. The effects of transfer of IL-17 family-transduced CD4$^+$ T cells on CIA. *A*, Intracellular IL-17 family expressions in Ba/F3 cells retrovirally transduced with each IL-17 family member. GFP-gated IL-17 family-transduced (mIL-17A, mIL-17B, mIL-17C, or mIL-17F) Ba/F3 cells were analyzed for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

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

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

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

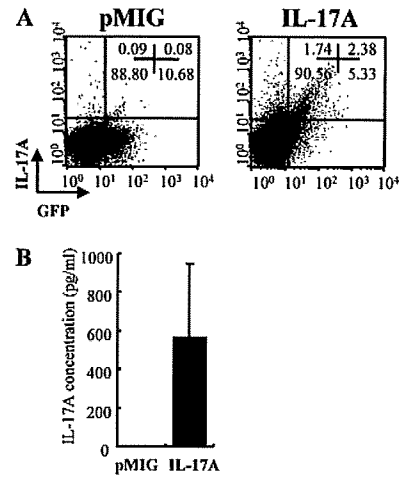

FIGURE 4. Generation of IL-17 family chimeric mice by BM transplantation of gene-transduced BM cells. Each of IL-17 family genes was transduced to BM cells with retrovirus vector and transferred to lethally irradiated mice. *A*, The intracellular expression of IL-17A protein in the spleen of IL-17A BM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

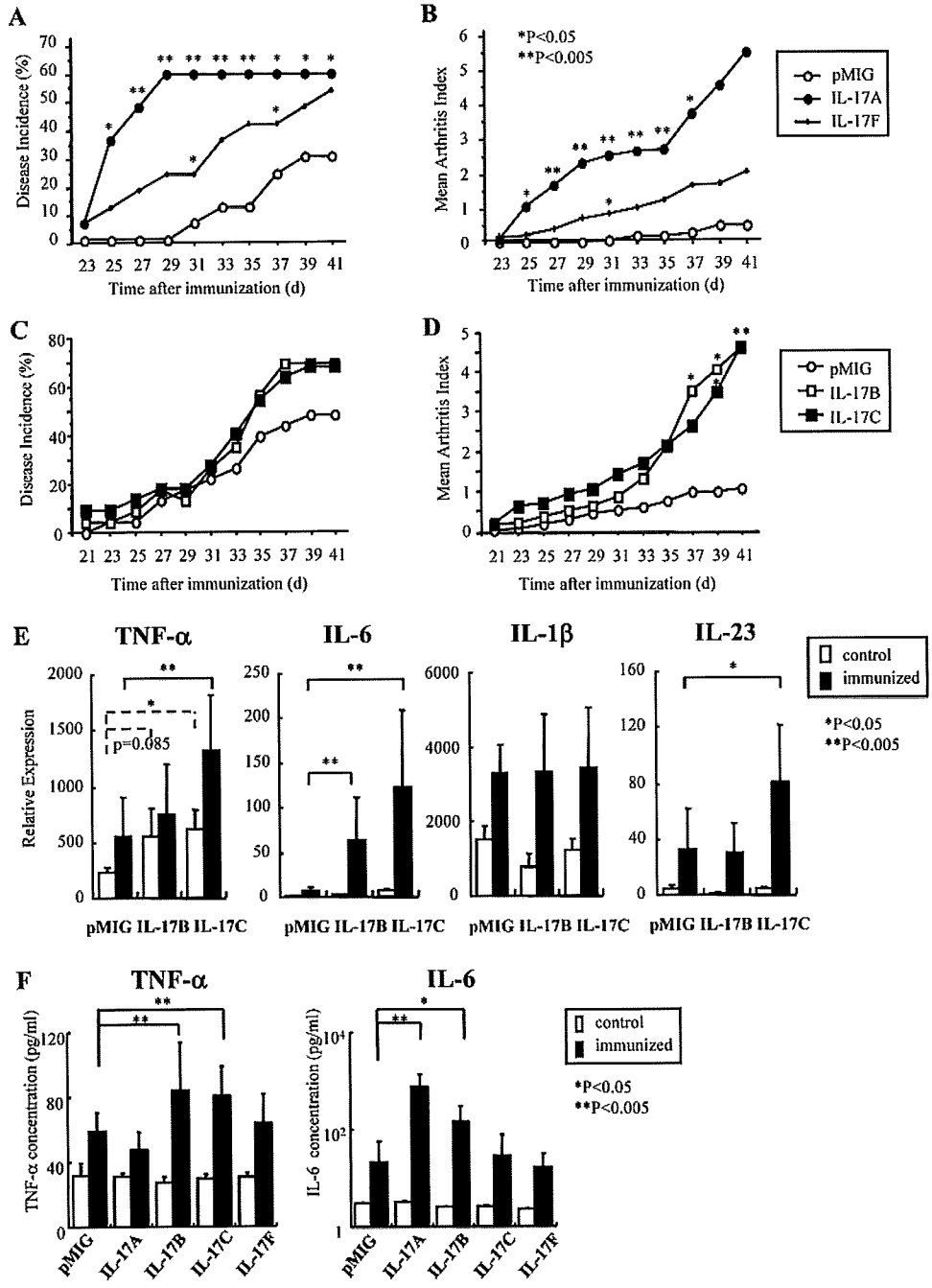

FIGURE 5. Incidence of CIA and arthritis scores in IL-17 family BM chimeric mice. Incidence of CIA and arthritis scores in IL-17A and IL-17F BM chimeric mice (*A* and *B*), and in IL-17B and IL-17C BM chimeric mice (*C* and *D*). Mice were immunized with BCII 8 wk after the BM transplantation. Values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

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

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

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

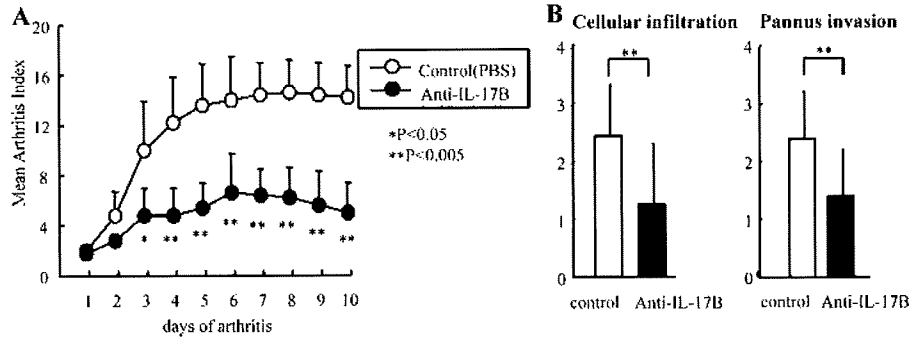

FIGURE 6. Effect of anti-IL-17B Ab treatment in CIA mice. *A*, CIA mice received i.p. injection of anti-mouse IL-17B Abs after the first clinical signs of arthritis (arthritis score between 1 and 2). As a control, PBS was injected. The arthritis score was shown. *B*, Histological score of the inflammatory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

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

Discussion

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

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

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

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

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

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

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

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

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

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

Acknowledgments

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

Disclosures

The authors have no financial conflict of interest.

References

1. Yao, Z., W. C. Fanslow, M. F. Seldin, A. M. Rousseau, S. L. Painter, M. R. Comeau, J. I. Cohen, and M. K. Spriggs. 1995. Herpesvirus Saimiri encodes a new cytokine: IL-17, which binds to a novel cytokine receptor. *Immunity* 3: 811–821.
2. Yao, Z., S. L. Painter, W. C. Fanslow, D. Ulrich, B. M. Macduff, M. K. Spriggs, and R. J. Armitage. 1995. Human IL-17: a novel cytokine derived from T cells. *J. Immunol.* 155: 5483–5486.
3. Ziolkowska, M., A. Koc, G. Luszczykiewicz, K. Ksiezopolska-Pietrzak, E. Klimczak, H. Chwalinska-Sadowska, and W. Maslinski. 2000. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *J. Immunol.* 164: 2832–2838.
4. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines IL-β and TNF-α, by human macrophages. *J. Immunol.* 160: 3513–3521.
5. Chabaud, M., F. Fossiez, J. L. Taupin, and P. Miossec. 1998. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J. Immunol.* 161: 409–414.
6. Katz, Y., O. Nadiv, and Y. Beer. 2001. Interleukin-17 enhances tumor necrosis factor α-induced synthesis of interleukins 1, 6, and 8 in skin and synovial fibroblasts: a possible role as a "fine-tuning cytokine" in inflammation processes. *Arthritis Rheum.* 44: 2176–2184.
7. Lubberts, E., L. A. Joosten, B. Oppers, L. van den Bersselaar, C. J. Coenen-de Roo, J. K. Kolls, P. Schwarzenberger, F. A. van de Loo, and W. B. van den Berg. 2001. IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis. *J. Immunol.* 167: 1004–1013.
8. Lubberts, E., M. I. Koenders, B. Oppers-Walgreen, L. van den Bersselaar, C. J. Coenen-de Roo, L. A. Joosten, and W. B. van den Berg. 2004. Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. *Arthritis Rheum.* 50: 650–659.
9. Nakae, S., S. Saijo, R. Horai, K. Sudo, S. Mori, and Y. Iwakura. 2003. IL-17 production from activated T cells is required for the spontaneous development of destructive arthritis in mice deficient in IL-1 receptor antagonist. *Proc. Natl. Acad. Sci. USA* 100: 5986–5990.
10. Koenders, M. I., E. Lubberts, F. A. van de Loo, B. Oppers-Walgreen, L. van den Bersselaar, M. M. Helsen, J. K. Kolls, F. E. Di Padova, L. A. Joosten, and W. B. van den Berg. 2006. Interleukin-17 acts independently of TNF-α under arthritic conditions. *J. Immunol.* 176: 6262–6269.
11. Moseley, T. A., D. R. Haudenschild, L. Rose, and A. H. Reddi. 2003. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev.* 14: 155–174.
12. Kolls, J. K., and A. Linden. 2004. Interleukin-17 family members and inflammation. *Immunity* 21: 467–476.
13. Starnes, T., M. J. Robertson, G. Sledge, S. Kelich, H. Nakshatri, H. E. Broxmeyer, and R. Hromas. 2001. Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J. Immunol.* 167: 4137–4140.
14. Aggarwal, S., N. Ghilardi, M. H. Xie, F. J. de Sauvage, and A. L. Gurney. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J. Biol. Chem.* 278: 1910–1914.
15. Happel, K. I., P. J. Dubin, M. Zheng, N. Ghilardi, C. Lockhart, L. J. Quinton, A. R. Odden, J. E. Shellito, G. J. Bagby, S. Nelson, and J. K. Kolls. 2005. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. *J. Exp. Med.* 202: 761–769.
16. Shi, Y., S. J. Ullrich, J. Zhang, K. Connolly, K. J. Grzegorzewski, M. C. Barber, W. Wang, K. Wathen, V. Hodge, C. L. Fisher, et al. 2000. A novel cytokine receptor-ligand pair: identification, molecular characterization, and in vivo immunomodulatory activity. *J. Biol. Chem.* 275: 19167–19176.
17. Li, H., J. Chen, A. Huang, J. Stinson, S. Heldens, J. Foster, P. Dowd, A. L. Gurney, and W. I. Wood. 2000. Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. *Proc. Natl. Acad. Sci. USA* 97: 773–778.
18. Hurst, S. D., T. Muchamuel, D. M. Gorman, J. M. Gilbert, T. Clifford, S. Kwan, S. Menon, B. Seymour, C. Jackson, T. T. Kung, et al. 2002. New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *J. Immunol.* 169: 443–453.
19. Chabaud, M., J. M. Durand, N. Buchs, F. Fossiez, G. Page, L. Frappart, and P. Miossec. 1999. Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. *Arthritis Rheum.* 42: 963–970.
20. Hymowitz, S. G., E. H. Filvaroff, J. P. Yin, J. Lee, L. Cai, P. Risser, M. Maruoka, W. Mao, J. Foster, R. F. Kelley, et al. 2001. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* 20: 5332–5341.
21. Hwang, S. Y., and H. Y. Kim. 2005. Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. *Mol. Cell* 19: 180–184.
22. Nasu, K., H. Kohsaka, Y. Nonomura, Y. Terada, H. Ito, K. Hirokawa, and N. Miyasaka. 2000. Adenoviral transfer of cyclin-dependent kinase inhibitor genes suppresses collagen-induced arthritis in mice. *J. Immunol.* 165: 7246–7252.
23. Trentham, D. E., A. S. Townes, and A. H. Kang. 1977. Autoimmunity to type II collagen an experimental model of arthritis. *J. Exp. Med.* 146: 857–868.
24. Stuart, J. M., A. S. Townes, and A. H. Kang. 1982. Nature and specificity of the immune response to collagen in type II collagen-induced arthritis in mice. *J. Clin. Invest.* 69: 673–683.
25. Gerlag, D. M., L. Ransone, P. P. Tak, Z. Han, M. Palanki, M. S. Barbosa, D. Boyle, A. M. Manning, and G. S. Firestein. 2000. The effect of a T cell-specific NF-κB inhibitor on in vitro cytokine production and collagen-induced arthritis. *J. Immunol.* 165: 1652–1658.
26. Nanki, T., Y. Urasaki, T. Imai, M. Nishimura, K. Muramoto, T. Kubota, and N. Miyasaka. 2004. Inhibition of fractalkine ameliorates murine collagen-induced arthritis. *J. Immunol.* 173: 7010–7016.
27. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139: 834–850.
28. Cheng, E. H., M. C. Wei, S. Weiler, R. A. Flavell, T. W. Mak, T. Lindsten, and S. J. Korsmeyer. 2001. BCL-2. Bcl-x_L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol. Cell* 8: 705–711.
29. Morita, S., T. Kojima, and T. Kitamura. 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* 7: 1063–1066.
30. Fujio, K., Y. Misaki, K. Setoguchi, S. Morita, K. Kawahata, I. Kato, T. Nosaka, K. Yamamoto, and T. Kitamura. 2000. Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the $\alpha\beta$ TCR complex. *J. Immunol.* 165: 528–532.
31. Fujio, K., A. Okamoto, H. Tahara, M. Abe, Y. Jiang, T. Kitamura, S. Hirose, and K. Yamamoto. 2004. Nucleosome-specific regulatory T cells engineered by triple gene transfer suppress a systemic autoimmune disease. *J. Immunol.* 173: 2118–2125.

32. McGaha, T. L., B. Sorrentino, and J. V. Ravetch. 2005. Restoration of tolerance in lupus by targeted inhibitory receptor expression. *Science* 307: 590–593.
33. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\delta\delta C_T)$ method. *Methods* 25: 402–408.
34. Ferreira, I. D., V. E. Rosario, and P. V. Cravo. 2006. Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in *Plasmodium falciparum*. *Malar J.* 5: 1.
35. Corthay, A., A. Johansson, M. Vestberg, and R. Holmdahl. 1999. Collagen-induced arthritis development requires $\alpha\beta$ T cells but not $\gamma\delta$ T cells: studies with T cell-deficient (TCR mutant) mice. *Int. Immunol.* 11: 1065–1073.
36. Glansbeek, H. L., P. M. van der Kraan, F. P. Lafeber, E. L. Vitters, and W. B. van den Berg. 1997. Species-specific expression of type II TGF-β receptor isoforms by articular chondrocytes: effect of proteoglycan depletion and aging. *Cytokine* 9: 347–351.
37. Taniguchi, K., H. Kohsaka, N. Inoue, Y. Terada, H. Ito, K. Hirokawa, and N. Miyasaka. 1999. Induction of the p16INK4a senescence gene as a new therapeutic strategy for the treatment of rheumatoid arthritis. *Nat. Med.* 5: 760–767.
38. Honorati, M. C., R. Meliconi, L. Pulsatelli, S. Cane, L. Frizziero, and A. Facchini. 2001. High in vivo expression of interleukin-17 receptor in synovial endothelial cells and chondrocytes from arthritis patients. *Rheumatology* 40: 522–527.
39. Schwarzenberger, P., V. La Russa, A. Miller, P. Ye, W. Huang, A. Zieske, S. Nelson, G. J. Bagby, D. Stoltz, R. L. Mynatt, et al. 1998. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J. Immunol.* 161: 6383–6389.
40. Lubberts, E., L. van den Bersselaar, B. Oppers-Walgreen, P. Schwarzenberger, C. J. Coenen-de Roo, J. K. Kolls, L. A. Joosten, and W. B. van den Berg. 2003. IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-κB ligand/osteoprotegerin balance. *J. Immunol.* 170: 2655–2662.
41. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-β induces development of the T_H17 lineage. *Nature* 441: 231–234.
42. Hunter, C. A. 2005. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. *Nat. Rev. Immunol.* 5: 521–531.
43. Koenders, M. I., J. K. Kolls, B. Oppers-Walgreen, L. van den Bersselaar, L. A. Joosten, J. R. Schurr, P. Schwarzenberger, W. B. van den Berg, and E. Lubberts. 2005. Interleukin-17 receptor deficiency results in impaired synovial expression of interleukin-1 and matrix metalloproteinases 3, 9, and 13 and prevents cartilage destruction during chronic reactivated streptococcal cell wall-induced arthritis. *Arthritis Rheum.* 52: 3239–3247.
44. Fujino, S., A. Andoh, S. Bamba, A. Ogawa, K. Hata, Y. Araki, T. Bamba, and Y. Fujiyama. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52: 65–70.
45. Haznedaroglu, S., M. A. Ozturk, B. Sancak, B. Goker, A. M. Onat, N. Bukan, I. Ertenli, S. Kiraz, and M. Calguneri. 2005. Serum interleukin 17 and interleukin 18 levels in familial Mediterranean fever. *Clin. Exp. Rheumatol.* 23: S77–S80.
46. Sohn, M. H., S. Y. Noh, W. Chang, K. M. Shin, and D. S. Kim. 2003. Circulating interleukin 17 is increased in the acute stage of Kawasaki disease. *Scand. J. Rheumatol.* 32: 364–366.

Research article

Open Access

Arthritogenic T cell epitope in glucose-6-phosphate isomerase-induced arthritis

Keiichi Iwanami[1], Isao Matsumoto[2], Yoko Tanaka[1], Asuka Inoue[1], Daisuke Goto[1], Satoshi Ito[1], Akito Tsutsumi[1] and Takayuki Sumida[1]

[1]Department of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Science, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8575, Japan
[2]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

Corresponding author: Isao Matsumoto, ismatsu@md.tsukuba.ac.jp

Received: 25 Jul 2008 Revisions requested: 22 Sep 2008 Revisions received: 27 Sep 2008 Accepted: 7 Nov 2008 Published: 7 Nov 2008

Arthritis Research & Therapy 2008, **10**:R130 (doi:10.1186/ar2545)
This article is online at: http://arthritis-research.com/content/10/6/R130

Abstract

Introduction Arthritis induced by immunisation with glucose-6-phosphate isomerase (GPI) in DBA/1 mice was proven to be T helper (Th) 17 dependent. We undertook this study to identify GPI-specific T cell epitopes in DBA/1 mice (H-2q) and investigate the mechanisms of arthritis generation.

Methods For epitope mapping, the binding motif of the major histocompatibility complex (MHC) class II (I-Aq) from DBA/1 mice was identified from the amino acid sequence of T cell epitopes and candidate peptides of T cell epitopes in GPI-induced arthritis were synthesised. Human GPI-primed CD4+ T cells and antigen-presenting cells (APCs) were co-cultured with each synthetic peptide and the cytokine production was measured by ELISA to identify the major epitopes. Synthetic peptides were immunised in DBA/1 mice to investigate whether arthritis could be induced by peptides. After immunisation with the major epitope, anti-interleukin (IL) 17 monoclonal antibody (mAb) was injected to monitor arthritis score. To investigate the mechanisms of arthritis induced by a major epitope, cross-reactivity to mouse GPI peptide was analysed by flow cytometry and anti-GPI antibodies were measured by ELISA. Deposition of anti-GPI antibodies on the cartilage surface was detected by immunohistology.

Results We selected 32 types of peptides as core sequences from the human GPI 558 amino acid sequence, which binds the binding motif, and synthesised 25 kinds of 20-mer peptides for screening, each containing the core sequence at its centre. By epitope mapping, human GPI325–339 was found to induce interferon (IFN) γ and IL-17 production most prominently. Immunisation with human GPI325–339 could induce polyarthritis similar to arthritis induced by human GPI protein, and administration of anti-IL-17 mAb significantly ameliorated arthritis ($p < 0.01$). Th17 cells primed with human GPI325–339 cross-reacted with mouse GPI325–339, and led B cells to produce anti-mouse GPI antibodies, which were deposited on cartilage surface.

Conclusions Human GPI325–339 was identified as a major epitope in GPI-induced arthritis, and proved to have the potential to induce polyarthritis. Understanding the pathological mechanism of arthritis induced by an immune reaction to a single short peptide could help elucidate the pathogenic mechanisms of autoimmune arthritis.

Introduction

Rheumatoid arthritis (RA) is characterised by symmetrical polyarthritis and joint destruction. Although the aetiology is considered to be autoimmune reactivity to some antigens, the exact mechanisms are not fully understood. So far, several models of arthritis have been described and analysed to understand the aetiological mechanisms of RA. Glucose-6-phosphate isomerase (GPI)-induced arthritis, a murine model of RA, is induced by immunisation with recombinant human (rh) GPI of DBA/1 mice [1]. We have previously demonstrated

APC: antigen-presenting cell; CIA: collagen-induced arthritis; CII: type II collagen; CTLA-4 Ig: cytotoxic T-lymphocyte antigen 4 immunoglobulin; DAPI: 4',6-diamidino-2-phenyindole, dilactate; ELISA: enzyme-linked immunosorbent assay; FCS: fetal calf serum; GPI: glucose-6-phosphate isomerase; IFN: interferon; IL: interleukin; mAb: monoclonal antibody; MHC: major histocompatibility complex; PBS: phosphate-buffered saline; RA: rheumatoid arthritis; rh: recombinant human; SD: standard deviation; SEM: standard error of the mean; TCR: T cell receptor; Th: T helper.

that the T helper (Th) 17 subset of CD4+ T cells play a central role in the pathogenesis of GPI-induced arthritis; GPI-specific CD4+ T cells were skewed to Th17 at the time of onset, and blockade of interleukin (IL) 17 resulted in a significant amelioration of arthritis [2]. Furthermore, the data that the administration of cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA-4 Ig) in the effector phase ameliorated the progress of arthritis implies the importance of Th17 cells even in the effector phase [3].

In this study, we further explored the epitopes of GPI-specific CD4+ T cells and identified human GPI $(hGPI)_{325-339}$ as a major epitope. Interestingly, the amino acid sequence of $hGPI_{325-339}$ (IWYINCFGCETHAML) was the same as that of bovine (type II collagen) $CII_{256-270}$(GEPGIAGFKGEQGPK), the dominant epitope of collagen-induced arthritis (CIA), at the major histocompatibility complex (MHC) binding sites [4]. Of note is that arthritis similar to GPI-induced arthritis was generated by immunisation with a short 15-mer single peptide in genetically unaltered mice. By analysis of peptide-induced arthritis, we found that $hGPI_{325-339}$-primed Th17 cells reacted with mouse GPI $(mGPI)_{325-339}$ peptide and subsequently lead to the production of anti-mouse GPI antibodies, which deposited over the cartilage surface of inflaming joints. Our findings should be helpful in unravelling the mechanism of autoimmune arthritis.

Materials and methods

Mice

DBA/1 mice were purchased from Charles River Laboratories, Japan. All mice were kept under specific pathogen-free conditions and all experiments were conducted in accordance with the University of Tsukuba ethical guidelines.

GPI and synthetic peptides

Recombinant mouse GPI and rhGPI were prepared as described previously [5,6]. Briefly, human GPI or mouse GPI cDNA was inserted into the plasmid pGEX-4T3 (Pharmacia, Uppsala, Sweden) for expression of glutathione S-transferase-tagged proteins. *Escherichia coli* harboring the pGEX-hGPI plasmid was allowed to proliferate at 37°C, before 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to the medium, followed by further culture overnight at 30°C. The bacteria were lysed with a sonicator and the supernatant was purified with a glutathione-sepharose column (Pharmacia, Uppsala, Sweden). The purity was estimated by SDS-PAGE.

Crude peptides were synthesised for epitope screening by Mimotopes (Melbourne, Victoria, Australia), and peptides with 90% purity were synthesised for a major epitope decision and induction of arthritis by Invitrogen (Carlsbad, CA). Candidate peptides, which were thought to bind the binding motif, were selected with web soft MHCPred (The Jenner Institute, Oxford, UK) [7].

Induction of arthritis

DBA/1 mice were immunised with 300 μg rhGPI for GPI-induced arthritis, or 10 μg or 25 μg synthetic peptide for peptide-induced arthritis in complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The rhGPI and synthetic peptide were emulsified with complete Freund's adjuvant at a 1:1 ratio (v/v). For induction of arthritis, 150 μl of the emulsion was injected intradermally at the base of the tail of the mouse. On days 0 and 2 after immunisation, 200 ng of pertussis toxin was injected intraperitoneally to develop peptide-induced arthritis. The arthritis score was evaluated visually using a score of 0 to 3 for each paw. A score of 0 represented no evidence of inflammation, 1 represented subtle inflammation or localised oedema, 2 represented easily identified swelling but localised to either the dorsal or ventral surface of the paws, and 3 represented swelling in all areas of the paws.

Treatments of arthritis with anti-IL-17 monoclonal antibodies

To neutralise IL-17, mice were injected intraperitoneally with 100 μg of neutralising antibody or isotype control on day 7 or day 6, 8, and 10. Anti-IL-17 mAb MAB421 (IgG2a) was purchased from R&D Systems (Minneapolis, MN, USA). IgG2a isotype control was purchased from eBioscience (San Diego, CA, USA).

Analysis of cytokine production

Mice were sacrificed on the indicated day. Spleens were harvested and haemolysed with a solution of 0.83% NH_4Cl, 0.12% $NaHCO_3$ and 0.004% $EDTA_2Na$ in PBS. Single-cell suspensions were prepared in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FCS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 50 μM 2-mercaptoethanol. CD4+ T cells were isolated by MACS positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the collected cells (>97%) was confirmed by flow cytometry. Splenic feeder cells treated with 50 μg/ml of mitomycin C were used as antigen presenting cells (APCs). The purified CD4+ T cells and APCs were co-cultured with 10 μM of the synthetic peptide at a ratio of 5:1 at 37°C under 5% CO_2 for 24 hours. The supernatants were assayed for interferon (IFN)-γ and IL-17 by Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Intracellular cytokine staining and flow cytometric analysis

Mice were sacrificed on day 5. The draining lymph nodes were harvested and single cell suspensions were prepared as described above. Cells (1×10^6/ml) were stimulated with 10 μM of the synthetic peptides in 96-well round bottom plates (Nunc, Roskilde, Denmark) for 24 hours and GoldiStop (BD PharMingen, San Diego, CA) was added for the last four hours of each culture. Cells were first stained extracellularly, fixed and permeabilised with Cytofix/Cytoperm solution (BD PharMingen, San Diego, CA) and then stained intracellularly.

Samples were acquired on FACSCalibur (BD PharMingen, San Diego, CA) and data were analysed with FlowJo (Tree Star, Ashland, OR).

Analysis of anti-GPI antibody

Sera were taken from immunised mice on day 14 and diluted 1:500 in blocking solution (25% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) in PBS) for antibody analysis. We also prepared 96-well plates (Sumitomo Bakelite, Tokyo, Japan) coated with 5 μg/ml rhGPI or recombinant mouse GPI for 12 hours at 4°C. After washing twice with a washing buffer (0.05% Tween20 in PBS), the blocking solution was used for blocking nonspecific binding for two hours at room temperature. After three washes, 150 μl of the diluted serum was added and incubated for two hoursat room temperature. After three washes, alkaline phosphatase-conjugated anti-mouse IgG was added at a final dilution of 1:5000, for one hour at room temperature. After three washes, colour was developed with substrate solution (1 alkaline phosphatase tablet (Sigma-Aldrich, St. Louis, MO, USA) per 5 ml alkaline phosphatase reaction solution (containing 9.6% diethanolamine and 0.25 mM $MgCl_2$, pH 9.8)). Plates were incubated for 20 minutes at room temperature and optical density was measured by a microplate reader at 405 nm.

Immunohistology

For immunohistology, cryostat sections from ankle joints were prepared with the tape capture technique as described previously [8]. Briefly, ankle joints were taken from immunised mice on day 14 and placed in Tissue-Tek (Sakura Finetek, Torrance, CA) filled with 4% carboxymethyl cellulose compound (Finetec, Tokyo, Japan). Frozen ankles joints in the carboxymethyl cellulose compound were attached to the adhesive Cryofilm (Finetec, Tokyo, Japan) and were cut in the microtome. The sections on the adhesive film were fixed with cold acetone. After blocking with 2% bovine serum albumin and 0.05% Tween in PBS, the sections were stained with Alexa 546-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA) (200 ng/slide), and nuclei were counterstained with 4',6-diamidino-2-phenyindole dilactate (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) (50 ng/slide). Fluorescence was detected with the Leica DMRA2 microscopy (Leica, Wetzlar, Germany). The images were acquired and processed with Leica FW4000 (Leica, Wetzlar, Germany).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). Differences between groups and variables were examined for statistical significance using the Mann-Whitney's U test and the Spearman's rank correlation coefficient, respectively. A $p < 0.05$ denoted the presence of a statistically significant difference.

Results

I-A^q binding motif and epitope candidates

To analyse T cell epitopes, we first investigated the binding motif of I-A^q from T cell epitopes reported in the literature because DBA/1 mice express only I-A^q as MHC class II. Based on the work by Bayrak and colleagues [9], the anchor motif of I-A^q would exist at P1, P4 and P7, therefore we predicted the binding motifs from amino acid sequences of I-A^q restricted epitopes on murine $RNase_{90-105}$ [10], myelin basic $protein_{89-101}$ [11,12], chicken type II collagen $(CII)_{181-209}$ [13], rat $CII_{256-270}$ [14,15], bovine $CII_{256-270}$ [4] and mouse type II collagen [9] (Table 1). Next, we selected 32 types of peptides as core sequences from the human GPI 558 amino acid sequence, which is thought to bind the binding motif (Table 2), and synthesised 25 kinds of 20-mer peptides for screening, each containing the core sequence in its centre (Table 3).

Epitope screening

rhGPI-specific $CD4^+$ T cells differentiate into Th1 and Th17 [2], so we analysed IFN-γ and IL-17 production for epitope screening when rhGPI-primed $CD4^+$ T cells were stimulated with each synthetic peptide. The production of both IFN-γ and IL-17 was pronounced when GPI-primed $CD4^+$ T cells were stimulated with number 18 peptide ($hGPI_{327-346}$) and number 25 peptide ($hGPI_{539-558}$). Therefore, we considered that major epitopes exist in either of the two peptides (Figure 1). In the K/BxN mouse model of arthritis, KRN T cell receptor (TCR) transgenic T cells recognise $mGPI_{282-294}$, the dominant epitope of K/BxN mouse, on I-A^{g7} [16]. However, in the GPI-induced arthritis model, it was unlikely that $hGPI_{282-294}$ was the dominant epitope because GPI-specific T cells did not react prominently to number 16 peptide ($hGPI_{280-299}$).

Because the synthetic peptides used for screening were not purified, we re-synthesised the 15-mer peptides with a purity of 90%; these peptides contained each core sequence of

Table 1

I-Aq binding motifs

P1	P2	P3	P4	P5	P6	P7	P8	P9
A			A			E		
F			P			D		
L			F			Q		
I			S			P		
P			V			N		
S			L			I		
V			N					
			R					

The anchor motif of I-A^q would exist at P1, P4 and P7, therefore we predicted the binding motif from amino acid sequences of I-A^q restricted epitopes on murine $RNase_{90-105}$, myelin basic $protein_{89-101}$, chicken type II $collagen_{181-209}$, rat type II $collagen_{256-270}$, bovine type II $collagen_{256-270}$ and mouse type II collagen.

Table 2

Core sequences of glucose-6-phosphate isomerase (GPI) amino acids binding I-A^q

Peptide	Amino acid residues
3–11	ALTRDPQFQ
29–37	LFDANKDRF
41–49	SLTLNTNHG
56–64	SKNLVTEDV
72–80	AKSRGVEAA
80–88	ARERMFNGE
99–107	LHVALRNRS
102–110	ALRNRSNTP
149–157	ITDVINIGI
167–175	VTEALKPYS
173–181	PYSSGGPRV
181–189	VWYVSNIDG
196–204	LAQLNPESS
201–209	PESSLFIIA
210–218	SKTFTTQET
229–237	FLQAAKDPS
230–238	LQAAKDPSA
243–251	FVALSTNTT
253–261	VKEFGIDPQ
285–293	ALHVGFDNF
319–327	LLALLGIWY
328–336	INCFGCETH
337–345	AMLPYDQYL
391–399	FYQLIHQGT
403–411	PCDFLIPVQ
407–415	LIPVQTQHP
426–434	LANFLAQTE
452–460	AGKSPEDLE
489–497	ALVAMYEHK
537–545	SHDASTNGL
540–548	ASTNGLINF
545–553	LINFIKQQR

Thirty-two types of peptides were selected as core sequences from the GPI 558 amino acid sequence, which is thought to bind the binding motif. Amino acid residues that are thought to bind anchors of I-A^q are shown in bold letters.

number 18 peptide ($hGPI_{327-346}$) and number 25 peptide ($hGPI_{539-558}$). Number 18 peptide ($hGPI_{327-346}$) contains two core sequences ($hGPI_{328-336}$ and $hGPI_{337-345}$), so therefore we re-synthesised two peptides ($hGPI_{325-339}$ and $hGPI_{334-348}$). The former sequences of number 25 peptide ($hGPI_{539-558}$) overlapped with number 24 peptide ($hGPI_{533-552}$), which could not stimulate CD4$^+$ T cells primed with GPI. Therefore we re-synthesised two peptides ($hGPI_{542-556}$ and $hGPI_{544-558}$) from the latter sequences of number 25 peptide (Table 4). We analysed IFN-γ and IL-17 production for epitope screening as described above. The peptide ($hGPI_{325-339}$) induced marked stimulation of GPI-primed CD4$^+$ T cells, and was considered a major epitope (Figure 2).

Immunisation with a major epitope induces arthritis similar to GPI-induced arthritis

To test if $hGPI_{325-339}$ is arthritogenic, DBA/1 mice were immunised with 10 μg or 25 μg $hGPI_{325-339}$ instead of GPI protein, and 200 ng of pertussis toxin was injected intraperitoneally on days 0 and 2 after immunisation. Arthritis resembling GPI-induced arthritis could be generated by immunisation with the peptide, including incidence, manifestations and severity. Symmetrical polyarthritis appeared on day 8, showed peak severity on day 14 and subsided gradually thereafter (Figure 3a). The use of different immunisation doses (10 and 25 μg) did not seem to affect the incidence and severity of arthritis. Immunised with 10 μg or 25 μg $hGPI_{325-339}$ without injection of pertussis toxin could also induce arthritis. However, the arthritis was less severe than with pertussis toxin (data not shown). On the other hand, immunisation with neither $hGPI_{539-558}$ nor $hGPI_{544-558}$, which were considered minor epitopes in GPI-induced arthritis, could induce overt arthritis (Figure 3a). Mice immunised with $hGPI_{325-339}$ developed severe swelling of the wrist and ankle joints. Histologically, severe synovitis was noted in the wrists in the forepaws, and at ankles and tarsal joints in the hind paws (Figure 3b and data not shown).

Peptide-induced arthritis is mediated by Th17

GPI-induced arthritis is Th17-mediated [2], so we explored the aetiological role of Th17 in peptide-induced arthritis. Like GPI-induced arthritis, one time administration of anti-IL-17 mAb on day 7 and three times administration on day 6, 8 and 10 significantly ameliorated the arthritis (Figure 4). From these data, the arthritis induced by $hGPI_{325-339}$ was also considered to be Th17 mediated.

Immunisation of human $GPI_{325-339}$ leads Th17 cells to cross-react with mouse $GPI_{325-339}$

We examined the pathogenesis of arthritis induced by $hGPI_{325-339}$ by comparing it with mice immunised with $hGPI_{544-558}$.

First, we speculated that the difference in cross-reactivity to mouse GPI might affect the incidence of arthritis, because $hGPI_{325-339}$ (IWYINCFGCETHAML) has 13/15 amino acids homology to $mGPI_{325-339}$ (IWYINCYGCETHALL) while $hGPI_{544-558}$ (GLINFIKQQREARVQ) has only 9/15 amino

Table 3

Synthetic peptides for screening T cell epitopes

Peptide number	Peptide	Synthetic peptide sequence
1	1–20	H-MAALTRDPQFQKLQQWYREH-OH
2	23–42	H-ELNLRRLFDANKDRFNHFSL-OH
3	37–56	H-FNHFSLTLNTNHGHILVDYS-OH
4	51–70	H-ILVDYSKNLVTEDVMRMLVD-OH
5	71–90	H-LAKSRGVEAARERMFNGEKI-OH
6	96–115	H-RAVLHVALRNRSNTPILVDG-OH
7	145–164	H-TGKTITDVINIGIGGSDLGP-OH
8	162–181	H-LGPLMVTEALKPYSSGGPRV-OH
9	168–187	H-TEALKPYSSGGPRVWYVSNI-OH
10	176–195	H-SGGPRVWYVSNIDGTHIAKT-OH
11	191–210	H-HIAKTLAQLNPESSLFIIAS-OH
12	200–219	H-NPESSLFIIASKTFTTQETI-OH
13	225–244	H-AKEWFLQAAKDPSAVAKHFV-OH
14	238–257	H-AVAKHFVALSTNTTKVKEFG-OH
15	247–266	H-STNTTKVKEFGIDPQNMFEF-OH
16	280–299	H-IGLSIALHVGFDNFEQLLSG-OH
17	313–332	H-EKNAPVLLALLGIWYINCFG-OH
18	327–346	H-YINCFGCETHAMLPYDQYLH-OH
19	386–405	H-NGQHAFYQLIHQGTKMIPCD-OH
20	400–419	H-KMIPCDFLIPVQTQHPIRKG-OH
21	420–439	H-LHHKILLANFLAQTEALMRG-OH
22	445–464	H-ARKELQAAGKSPEDLERLLP-OH
23	484–503	H-PFMLGALVAMYEHKIFVQGI-OH
24	533–552	H-AQVTSHDASTNGLINFIKQQ-OH
25	539–558	H-DASTNGLINFIKQQREARVQ-OH

Listed are 25 20-mer unpurified peptides in which each core sequence were centred around. Amino acid residues constituting the core sequence and those thought to bind anchors of I-A^q are underlined and shown in bold letters, respectively.

acids homology to $mGPI_{544-558}$ (GLISFIKQQRDTKLE). The draining lymph node cells from mice immunised with $hGPI_{325-339}$ or $hGPI_{544-558}$were cultured in the presence of $hGPI_{325-339}$, $mGPI_{325-339}$, $hGPI_{544-558}$ or $mGPI_{544-558}$ for 24 hours. The $hGPI_{325-339}$-primed cells had distinct cross-reactive immune reaction to $mGPI_{325-339}$ by producing IL-17, whereas the $hGPI_{544-558}$ primed cells did not cross-react to $mGPI_{544-558}$ (Figure 5a). As compared with the draining lymph node cells of $hGPI_{325-339}$-immunised mice, IL-17 production was not remarkable in that of $hGPI_{544-558}$-immunised mice even when the corresponding peptide was used as an antigen for *in vitro* stimulation (Figure 5a). The production of IFN-γ was much lower than that of IL-17, and IL-4 production was not detectable independent of immunisation patterns and antigens for *in vitro* stimulation (data not shown).

It has been reported that Th17 cells are not the only cellular sources of IL-17, but $CD8^+$ T cells, natural killer T cells and γδT cells are also capable of producing IL-17 [17-22]. Therefore, we investigated the IL-17 producing cells using flow cytometry. The draining lymph node cells from mice immunised with $hGPI_{325-339}$ or $hGPI_{544-558}$ were stimulated with $hGPI_{325-339}$ and $mGPI_{325-339}$, or $hGPI_{544-558}$ and $mGPI_{544-558}$, respectively. Intracellular cytokine staining was performed without nonspecific stimulants, such as phorbol myristate acetate or ionomycin. We confirmed that immunisation of $hGPI_{325-339}$ induced antigen-specific Th17 cells, which cross-reacted with $mGPI_{325-339}$. However, immunisation of $hGPI_{544-558}$ induced neither $hGPI_{544-558}$-specific Th17 cells nor Th17 cells that can cross-react with $mGPI_{544-558}$ remarkably (Figure 5b). These data indicate that induction of antigen-specific Th17 cells and