

Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model

Keiji Hirota,^{1,2} Hiroyuki Yoshitomi,^{1,3} Motomu Hashimoto,¹ Shinji Maeda,¹ Shin Teradaira,¹ Naoshi Sugimoto,¹ Tomoyuki Yamaguchi,¹ Takashi Nomura,¹ Hiromu Ito,³ Takashi Nakamura,³ Noriko Sakaguchi,¹ and Shimon Sakaguchi^{1,2}

¹Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

²Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi 332-0012, Japan

³Department of Orthopaedic Surgery, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

This report shows that interleukin (IL) 17-producing T helper type 17 (Th17) cells predominantly express CC chemokine receptor (CCR) 6 in an animal model of rheumatoid arthritis (RA). Th17 cells induced *in vivo* in normal mice via homeostatic proliferation similarly express CCR6, whereas those inducible *in vitro* by transforming growth factor β and IL-6 additionally need IL-1 and neutralization of interferon (IFN) γ and IL-4 for CCR6 expression. Forced expression of ROR γ t, a key transcription factor for Th17 cell differentiation, induces not only IL-17 but also CCR6 in naive T cells. Furthermore, Th17 cells produce CCL20, the known ligand for CCR6. Synoviocytes from arthritic joints of mice and humans also produce a large amount of CCL20, with a significant correlation ($P = 0.014$) between the amounts of IL-17 and CCL20 in RA joints. The CCL20 production by synoviocytes is augmented *in vitro* by IL-1 β , IL-17, or tumor necrosis factor α , and is suppressed by IFN- γ or IL-4. Administration of blocking anti-CCR6 monoclonal antibody substantially inhibits mouse arthritis. Thus, the joint cytokine milieu formed by T cells and synovial cells controls the production of CCL20 and, consequently, the recruitment of CCR6⁺ arthritogenic Th17 cells to the inflamed joints. These results indicate that CCR6 expression contributes to Th17 cell function in autoimmune disease, especially in autoimmune arthritis such as RA.

CORRESPONDENCE

Shimon Sakaguchi:
shimon@frontier.kyoto-u.ac.jp

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that primarily affects multiple joints. Autoimmune CD4⁺ T cells are required for the progression of RA, especially in an initial phase of the disease (1). It is obscure, however, how such arthritogenic CD4⁺ T cells are produced in the immune system, become activated, and mediate RA. Recent studies with animal models have suggested that CD4⁺ T cells secreting IL-17 (IL-17A), called Th17 cells, may play a key role in the progression of RA as well as multiple sclerosis (2–6). Hindering the development of Th17 cells or blocking IL-17 activity indeed inhibits autoimmune pathology in these models (2–6). A particular cytokine milieu contributes to this preferential differentiation of naive self-reactive T cells to Th17

effector cells (2–6). In addition, the transcription factor ROR γ t specifically controls Th17 cell differentiation, indicating that Th17 cells form a T cell lineage distinct from Th1 or Th2 cells (7). To further analyze the roles of Th17 cells in autoimmune disease, we have searched for cell-surface molecules that are specifically expressed in Th17 cells and are crucial for their functions, such as their migration to inflamed joints in RA.

The SKG strain of mice, a mutant on the BALB/c background, spontaneously develops T cell-mediated autoimmune arthritis, which clinically and immunologically resembles human RA (8–10). The strain harbors a recessive mutation of the gene encoding an Src homology 2 domain of ζ -associated protein 70, a key signaling molecule in T cells. Impaired signal transduction through SKG ζ -associated protein 70 results in thymic positive selection and failure

K. Hirota and H. Yoshitomi contributed equally to this work.

The online version of this article contains supplemental material.

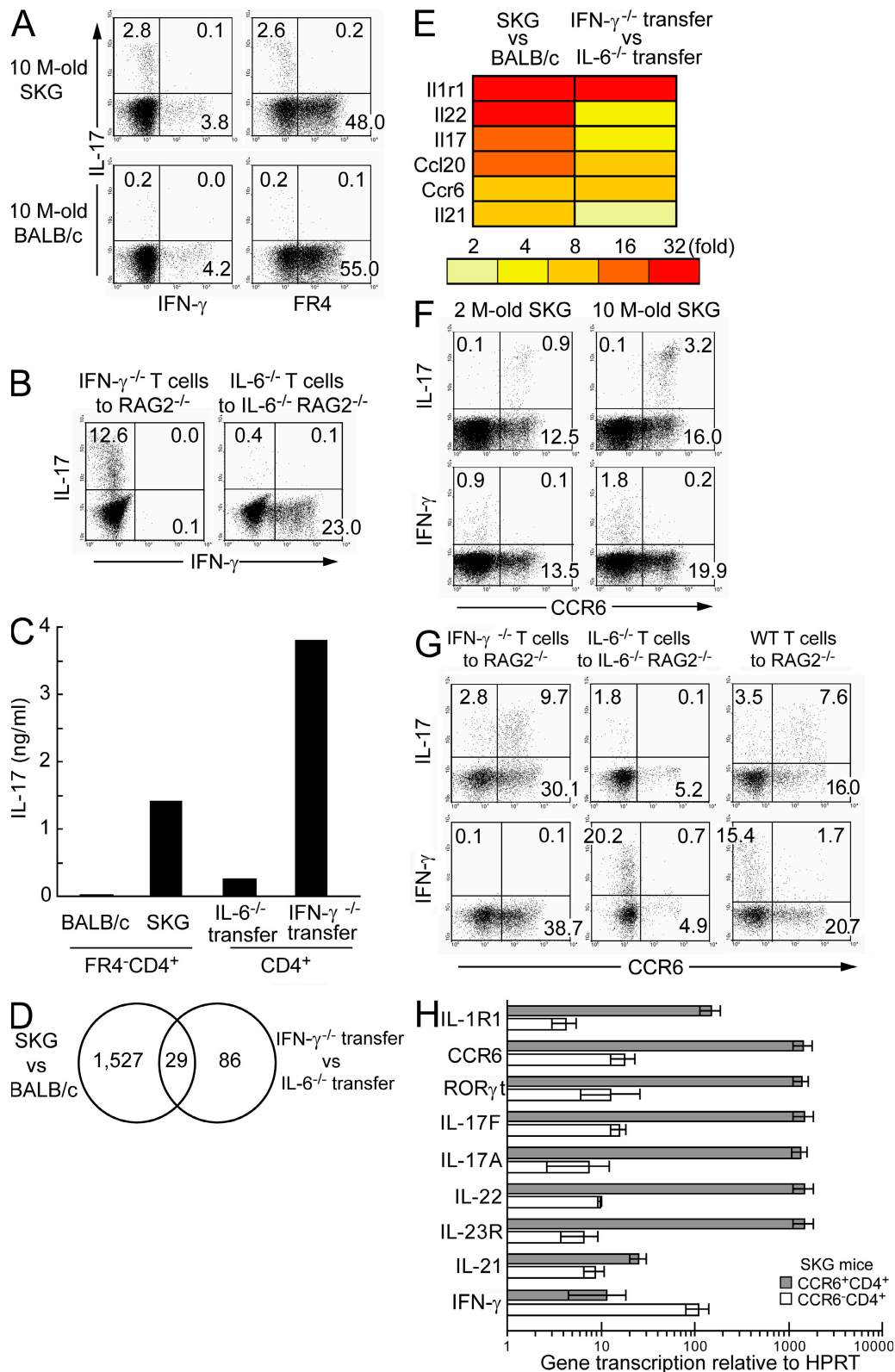


Figure 1. Gene microarray analysis of Th17 cells and their predominant expression of CCR6. (A) LN CD4⁺ T cells from 10-mo-old BALB/c or SKG mice were stained for intracellular IL-17 and IFN- γ , or these cytokines and cell-surface FR4. (B) CD4⁺ T cells from IFN- γ ^{-/-} or IL-6^{-/-} mice were transferred to RAG2^{-/-} mice or IL-6^{-/-}RAG2^{-/-} mice, respectively. Intracellular IL-17 and IFN- γ in recipient splenic CD4⁺ T cells were stained on day 7. (C) Purified FR4⁺CD4⁺ T cells from BALB/c or SKG mice or CD4⁺ T cells after homeostatic proliferation, as shown in A and B, were stimulated with PMA/ionomycin for 3 h,

in the negative selection of highly self-reactive T cells that include potentially arthritogenic CD4⁺ T cells (8). Our previous report showed that SKG CD4⁺ T cells spontaneously differentiated to arthritogenic Th17 cells, which were nonredundant in mediating SKG arthritis (6). The study also showed that not only self-reactive CD4⁺ T cells in SKG mice but also naive T cells in normal mice were able to differentiate to Th17 cells when they were subjected to homeostatic proliferation in a T cell-deficient environment (6). In both systems, IL-6 deficiency inhibited Th17 cell differentiation, whereas IFN- γ deficiency augmented it (6). Use of these two *in vivo* Th17 cell induction systems allowed us to determine the genes commonly up-regulated in Th17 cells. We show in this report that Th17 cells predominantly express CC chemokine receptor (CCR) 6 and produce its ligand, CCL20. Inflamed synovial cells in both SKG arthritis and human RA also produce CCL20, facilitating the migration of arthritogenic Th17 cells to inflamed joints. Thus, CCR6 is an important functional marker for Th17 cells and contributes to their preferential migration to a particular inflammation site.

RESULTS AND DISCUSSION

Gene microarray analysis of Th17 cells

Th17 cells increase with age in SKG mice, constituting ~3–10% of LN CD4⁺ T cells at 10 mo of age compared with ~0.2–0.7% in age-matched BALB/c mice (Fig. 1 A). Notably, they scarcely express the folate receptor 4 (FR4), which is highly expressed by CD25⁺CD4⁺ natural regulatory T cells and central memory-like CD4⁺ T cells but not by effector or effector memory-like CD4⁺ T cells (Fig. 1 A) (11–12). Th17 cells can therefore be enriched by sorting FR4⁻CD4⁺ T cells from SKG mice. A large number of Th17 cells also developed spontaneously when CD4⁺ T cells from IFN- γ -deficient (IFN- γ ^{-/-}) BALB/c mice were transferred to T cell-deficient RAG2-deficient (RAG2^{-/-}) mice and subjected to homeostatic proliferation, whereas they failed to develop in a similar transfer of IL-6-deficient (IL-6^{-/-}) CD4⁺ T cells to IL-6^{-/-} RAG2^{-/-} mice (Fig. 1 B) (6). The numbers of Th17 cells assessed by cytofluorometric analysis were well correlated with the amounts of the IL-17 protein secreted after *in vitro* stimulation with PMA/ionomycin (Fig. 1 C).

To explore the functional molecules specifically expressed by Th17 cells, we conducted gene microarray analysis between 10-mo-old SKG FR4⁻CD4⁺ cells and age-matched

BALB/c FR4⁻CD4⁺ cells, and between IFN- γ ^{-/-}CD4⁺ cells and IL-6^{-/-}CD4⁺ T cells transferred to RAG2^{-/-} mice, as described in the previous paragraph. The analysis revealed that 1,556 and 115 genes were up-regulated in 10-mo-old SKG FR4⁻CD4⁺ and IFN- γ ^{-/-}CD4⁺ T cells after homeostatic proliferation, respectively, with 29 genes shared by the two groups of genes (Fig. 1 D). The 29 genes included those encoding cytokines, chemokines, and their receptors, such as IL-1R1, IL-17, IL-22, IL-21, CCR6, and CCL20 (Fig. 1 E; the rest of the genes are shown in Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20071397>).

Expression of CCR6 by Th17 cells

With our interest in Th17 cell-specific cell-surface molecules, we analyzed CCR6 expressed by Th17 cells in young (2-mo-old) or aged (10-mo-old) SKG mice, or BALB/c Th17 cells induced via homeostatic proliferation (Fig. 1 B). The majority of Th17 cells in SKG mice expressed CCR6 whether the mice were young or aged, whereas SKG Th1 cells did not (Fig. 1 F). After cell transfer to RAG2^{-/-} mice, the majority of IL-17⁺CD4⁺ T cells that had differentiated from IFN- γ ^{-/-} or WT T cells were CCR6⁺, whereas IFN- γ ⁺CD4⁺ T cells derived from WT CD4⁺ T cells were CCR6⁻ (Fig. 1 G). Furthermore, IL-6^{-/-}CD4⁺ T cells not only failed to give rise to IL-17⁺CCR6⁺ cells but also preferentially differentiated to IFN- γ ⁺CCR6⁻ cells (Fig. 1 G).

It was also noted that CCR6⁺ cells, either IL-17⁺ or IL-17⁻, increased in number among CD4⁺ T cells in aged SKG mice compared with aged BALB/c or young SKG mice (Fig. 1 F and Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20071397>). Both IL-17⁺CCR6⁺ and IL-17⁻CCR6⁺ populations similarly increased in RAG2^{-/-} mice transferred with IFN- γ ^{-/-} cells and, to a lesser extent, in those transferred with WT T cells, but not in IL-6^{-/-} cell-transferred RAG2^{-/-} mice (Fig. 1 G and Fig. S2 B). In addition, CCR6⁻CD4⁺ T cells in BALB/c mice gave rise to IL-17⁺CCR6⁺CD4⁺ T cells via homeostatic proliferation when transferred to RAG2^{-/-} mice (Fig. S2 C).

We also examined a possible correlation between CCR6 expression and transcription of the other genes depicted by the microarray analysis in Fig. 1 E or those genes reported to be functional in Th17 cells (e.g., IL-17, IL-17F, IL-21, IL-22, IL-1R1, IL-23R, and ROR γ t; Fig. 1 H) (7, 13, 14). When CCR6⁺ or CCR6⁻CD4⁺ T cells purified from aged SKG mice

and the amounts of IL-17 in culture supernatants were measured by ELISA. (D and E) Total RNA extracted from activated cells, as shown in C, was subjected to gene microarray for SKG versus BALB/c FR4-CD4⁺ T cells or IFN- γ ^{-/-} versus IL-6^{-/-}CD4⁺ T cells after homeostatic proliferation. The expression of each gene was averaged from three independent experiments and analyzed by GeneSpring software. The Venn diagram shown in D represents a group of up-regulated genes in SKG FR4-CD4⁺ T cells and IFN- γ ^{-/-}CD4⁺ T cells after homeostatic proliferation. Genes for cytokines, chemokines, or their receptors commonly up-regulated in the two groups of genes are shown in E. Graded colors represent relative expression levels. (F) LN CD4⁺ T cells from 2- or 10-mo-old SKG mice were stained for CCR6 and intracellular IL-17 or IFN- γ . (G) CD4⁺ T cells from IFN- γ ^{-/-}, IL-6^{-/-}, or WT mice were stained for CCR6 and intracellular IL-17 or IFN- γ after homeostatic proliferation, as shown in B. (H) Total RNA extracted from purified SKG CCR6⁺ or CCR6⁻CD4⁺ T cells stimulated with PMA/ionomycin for 3 h was subjected to quantitative RT-PCR for the indicated genes and normalized for hypoxanthine-guanine phosphoribosyltransferase (HPRT) messenger RNA expression, as previously described (reference 7). Data are shown as the mean \pm SD of three independent experiments. Results in A–C, F, and G represent three to five independent experiments. The percentage of cells in each quadrant is shown in A, B, F, and G.

were activated in vitro with PMA/ionomycin and expression levels of individual genes were analyzed by quantitative RT-PCR, CCR6⁺CD4⁺ SKG T cells indeed transcribed most of the genes much more actively than CCR6⁻CD4⁺ T cells, in contrast with much less transcription of the IFN- γ gene. As both aged SKG CD4⁺ T cells and BALB/c IFN- γ ^{-/-}CD4⁺ T cells highly expressed the *Il1r1* gene after homeostatic proliferation (Fig. 1 E), IL-17⁺CD4⁺ T cells in aged SKG mice expressed the IL-1R1 protein higher than IL-17⁻CD4⁺ T cells by cytofluorometric analysis (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20071397>).

Previous studies with mice have shown that CCR6 is mainly expressed on memory T cells, some natural CD25⁺CD4⁺ regulatory T cells, B cells, some DCs, and Langerhans cells, and suggested that CCR6 may be required for the trafficking of these cells via CCL20 (15–20). The present results show that, in addition to these cell populations, Th17 cells also express CCR6 and that CCR6 expression can distinguish Th17 cells from Th1 cells. The condition that facilitates in vivo Th17 cell differentiation might also promote differentiation of naive CD4⁺ T cell to CCR6⁺IL-17⁻IFN- γ ⁻ cells,

which are FR4 high and, therefore, phenotypically similar to natural CD25⁺CD4⁺ regulatory T cells and central memory-like T cells, as recently shown (12), and in accord with the results by others (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20071397>).

Induction of CCR6 in Th17 cells in vitro

ROR γ t crucially controls Th17 cell differentiation from naive CD4⁺ T cells (7). To test whether forced expression of ROR γ t directs the differentiation of CD4⁺ T cells to CCR6⁺ Th17 cells, ROR γ t was transduced in CCR6⁻CD4⁺ T cells by a bicistronic retroviral vector expressing ROR γ t and GFP (7). Notably, GFP-high (i.e., ROR γ t-high) cells expressed CCR6 as well as IL-17, whereas GFP-low cells did not (Fig. 2, A and B). Th17 cells can also be induced in vitro from naive CD4⁺ T cells by TCR stimulation in the presence of IL-6 and TGF- β , as reported by others (21–23). Interestingly, this in vitro induction of a large number of Th17 cells did not accompany CCR6 expression (Fig. 2 C). Yet, further addition of IL-1, based on the high expression of IL-1R by IL-17⁺ cells (Fig. 1 E and Fig. S3), elicited CCR6 expression in a

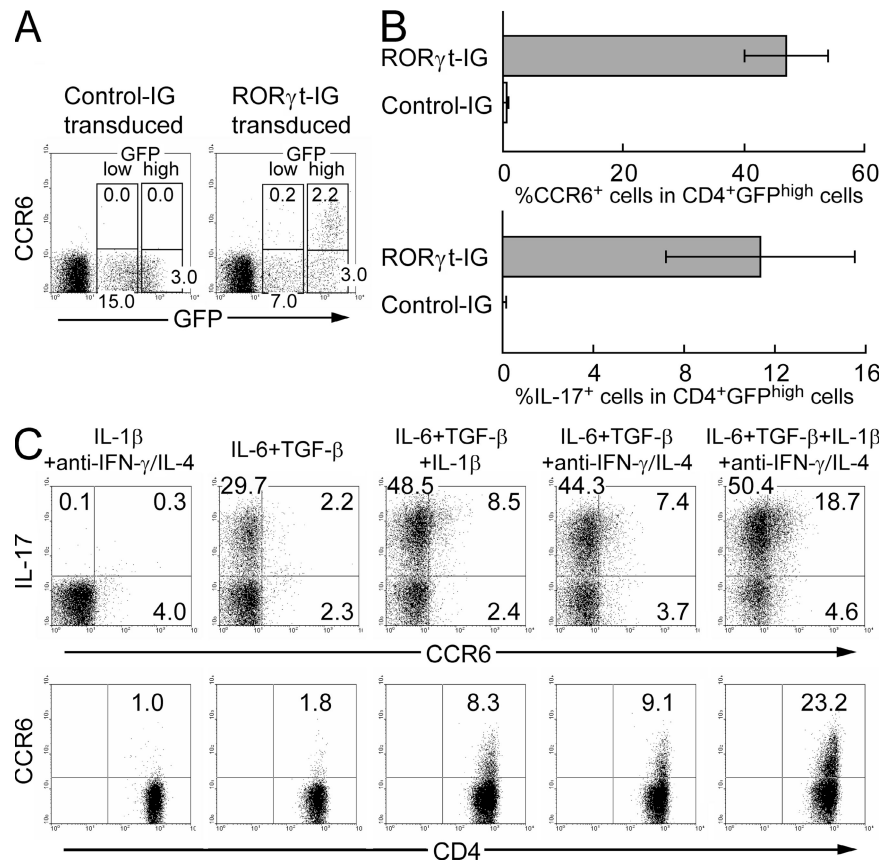


Figure 2. Induction of CCR6 on Th17 cells in vitro. (A) Retroviral vector, pMxs-ROR γ t-IRES-GFP (ROR γ t-IG), or control pMxs-IRES-GFP (Control-IG), was transduced into BALB/c CCR6⁻CD4⁺ T cells, as previously described (reference 7), and the cells expressing GFP were stained for CCR6 on day 4 after transduction. (B) The mean percentages \pm SD of CCR6⁺ or IL-17⁺ cells among CD4⁺GFP^{high} cells (as gated in A) in three independent experiments are shown. (C) CD4⁺CD25⁻CCR6⁻ T cells were in vitro driven to differentiate to Th17 cells in the indicated conditions, as previously described (reference 21). CCR6 and intracellular IL-17 gated on CD4⁺ T cells were stained on day 5. Results in A and C represent three independent experiments, and the percentage of cells in each quadrant is shown.

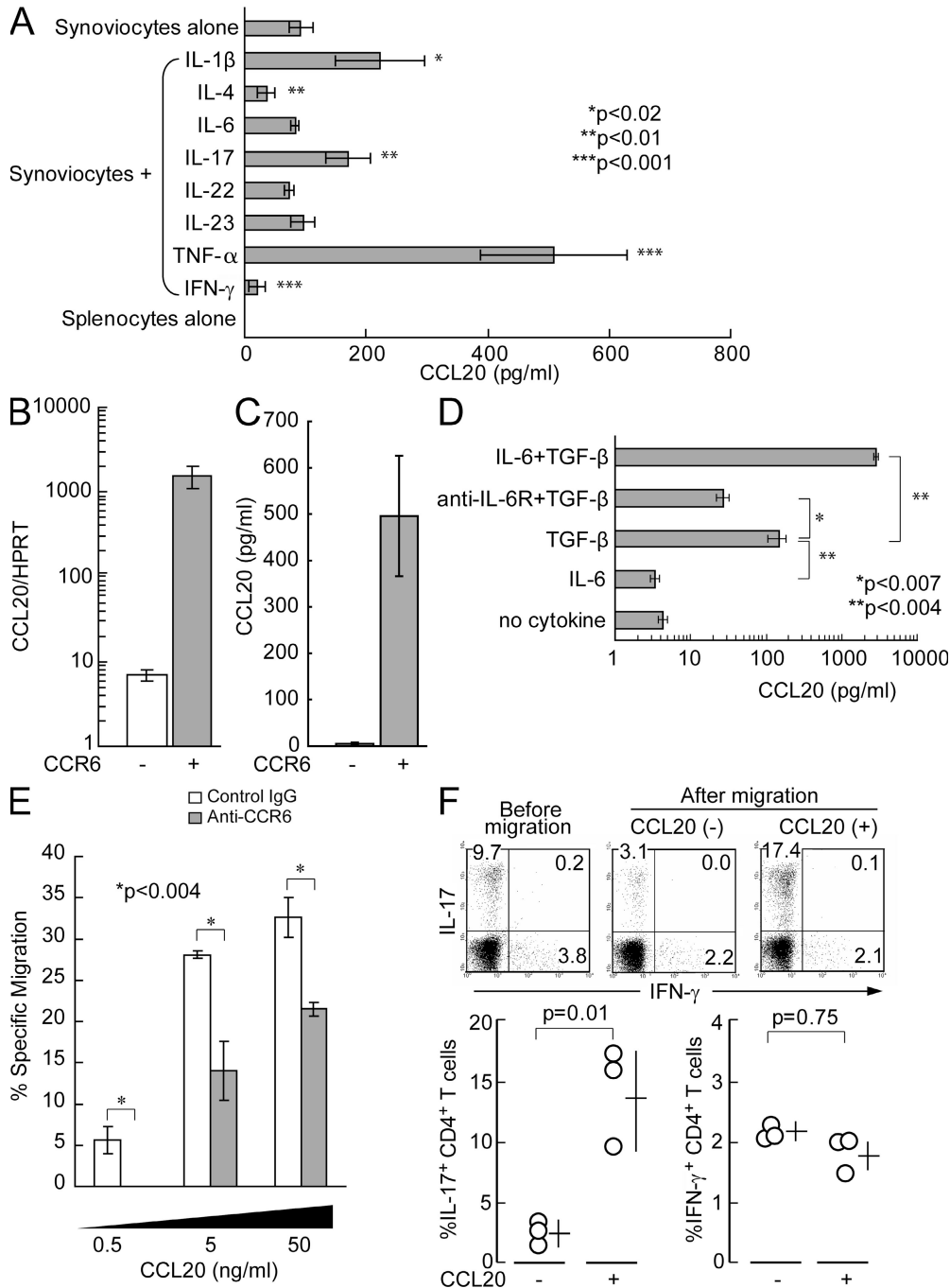


Figure 3. Cross-regulation of synoviocyte CCL20 production by cytokines and preferential migration of Th17 cells in response to CCL20. (A) 2.5×10^4 adherent synoviocytes, prepared as previously described (reference 10), were cultured with 10 ng/ml of the indicated cytokine for 24 h. The amounts of CCL20 in culture supernatants were measured by ELISA. Data are shown as the mean \pm 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 \pm 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 μ g/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 \pm 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-17⁺ or IFN- γ ⁺ 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 \pm SD. Results in A, D, and E represent three independent experiments.

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 ROR γ t at an amount sufficient for directing IL-17 production in vitro (7), a higher amount of ROR γ t 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-1 β , 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,

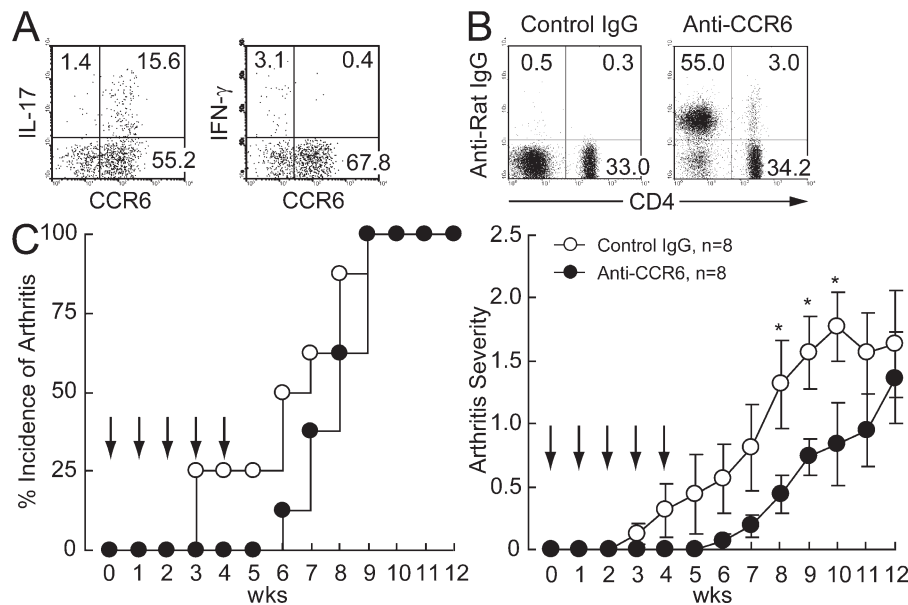


Figure 4. Contribution of CCR6/CCL20 to trafficking of Th17 cells in SKG arthritis. (A) CD4⁺ T cells infiltrating arthritic joints were prepared from collagenase-digested ankle joints of SKG mice, and stained for CCR6 and intracellular IL-17 or IFN- γ . (B) SKG mice received an i.v. injection of 100 μ g 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 10⁶ SKG CD4⁺ T cells, and then injections of 100 μ g 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 \pm 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.

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

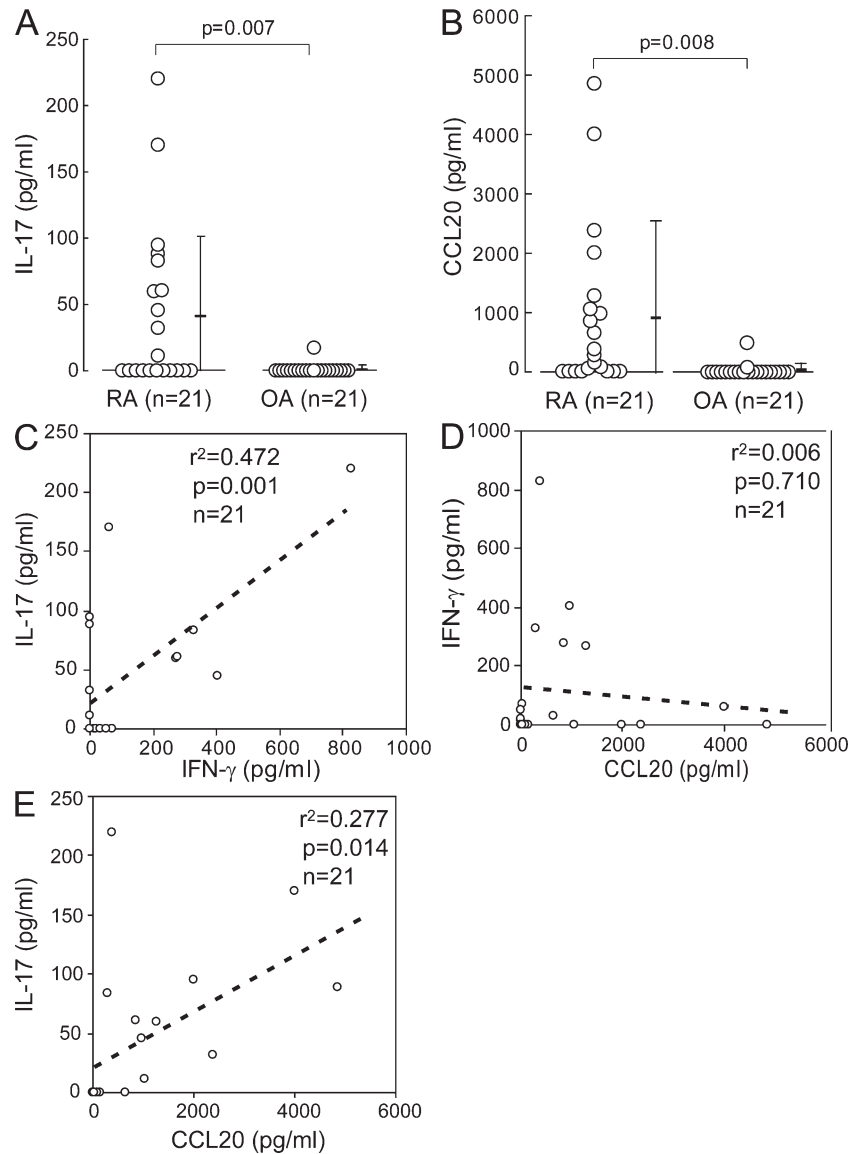


Figure 5. Production of IL-17 and CCL20 in RA joints. (A and B) The concentrations of IL-17 and CCL20 in synovial fluid from RA or OA patients were measured by ELISA. Vertical bars represent the means \pm SD. (C–E) Scatter plots show the correlation between IL-17 and IFN- γ (C), CCL20 and IFN- γ (D), 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 $P = 0.003$ ($n = 13$).

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-1 β , 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-TCR $\alpha\beta$ (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 μ M 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- μ m pore size Transwell system (Costar). 10⁶ 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).

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

- Harris, E.D. 1997. Rheumatoid Arthritis. W.B. Saunders Co., Philadelphia. 417 pp.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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 ROR γ directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell.* 126:1121–1133.
- 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.
- 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- α , IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J. Clin. Invest.* 114:582–588.
- 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.
- 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.
- 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.
- 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.
- 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.
- 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 α . *J. Immunol.* 162:186–194.
- Charbonnier, A.S., N. Kohrgruber, E. Kriehuber, G. Stingl, A. Rot, and D. Maurer. 1999. Macrophage inflammatory protein 3 α is involved in the constitutive trafficking of epidermal Langerhans cells. *J. Exp. Med.* 190:1755–1768.
- 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 3 α is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J. Exp. Med.* 192:705–718.
- 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)-3 α , MIP-3 β , and secondary lymphoid organ chemokine. *J. Exp. Med.* 191:1381–1394.
- 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.
- 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.
- Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGF β in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* 24:179–189.
- Betelli, 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.
- 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(H)17 lineage. *Nature.* 441:231–234.
- 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.
- Matsui, T., T. Akahoshi, R. Namai, A. Hashimoto, Y. Kurihara, M. Rana, A. Nishimura, H. Endo, H. Kitasato, S. Kawai, et al. 2001. Selective

- 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.