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Effects of Infliximab Therapy on Gene Expression Levels of Tumor Necrosis Factor α, Tristetraprolin, T Cell Intracellular Antigen 1, and Hu Antigen R in Patients With Rheumatoid Arthritis

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Objective. **Tristetraprolin (TTP), T cell intracellular antigen 1 (TIA-1), and Hu antigen R (HuR) are adenine/uridine-rich element binding proteins (ABPs) that affect the production of tumor necrosis factor α (TNFα) by binding to TNF messenger RNA (mRNA). TTP promotes deadenylation, TIA-1 inhibits translation, and HuR stabilizes TNFα mRNA. The aims of this study were to understand the posttranscriptional control of TNFα production in patients with rheumatoid arthritis (RA), and to identify parameters that may predict the efficacy of anti-TNFα therapy.**

Methods. **Peripheral blood mononuclear cells from 38 patients with RA were obtained before therapy and 2 weeks and 54 weeks after administration of the first dose of infliximab, and from 20 healthy control subjects. TNFα, TTP, TIA-1, and HuR gene expression levels were analyzed by real-time polymerase chain reaction.**

Results. **At baseline, TTP and HuR gene expression levels, as well as the TTP:TNFα, TTP:HuR, and TIA-1:TNFα gene expression ratios were lower in patients with RA than in control subjects, while expression of TNFα, TIA-1, and TIA-1:HuR was higher in patients with RA. The TTP:HuR expression ratio decreased significantly after administration of infliximab. Positive correlations were observed between TNFα and TTP, TNFα and TIA-1, TIA-1 and HuR, and TNFα and HuR gene expression in both healthy control subjects and patients with RA. At baseline, the TIA-1:HuR ratio tended to be higher in patients who achieved 50% improvement according to the American College of Rheumatology criteria (ACR50) at week 54 than in those who did not achieve at least an ACR20 response.**

Conclusion. **Differences in ABP gene expression may affect TNFα gene expression. A higher TIA-1:HuR expression ratio might correlate with the response to infliximab therapy.**

Rheumatoid arthritis (RA) is a relatively common chronic systemic inflammatory disease, affecting nearly 1% of the world's population (1). Although the pathogenesis of RA is not fully understood, tumor necrosis factor α (TNFα) is one of the most important cytokines involved in the development of synovitis (2–4). TNFα is produced by activated macrophages, lymphocytes, and synovial cells and induces other proinflammatory agents, including interleukin-1α (IL-1α), IL-6, and IL-15. All of these cytokines are involved in synovial cell activation and proliferation, leading to pannus formation in the joints (5–8). They also enhance the synthesis and action of proteases such as metalloproteinases, eventually causing cartilage and bone destruction (5). Antagonists to these cytokines, such as infliximab, etanercept, adalimumab (TNFα antagonists), and tocilizumab (IL-6 antagonist), are effective in relieving these cytokine-induced symptoms of RA in individual patients (9–21). In fact, the beneficial effects of TNFα antago-

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nists confirm the central role of TNFα in the inflammatory process of RA. However, some patients do not respond to TNFα antagonists, and it is not currently possible to predict the efficacy of these drugs prior to therapy. Thus, a better understanding of the mechanisms that control TNFα production is needed to develop new therapies and to envision the responses of individual patients to anti-TNFα therapy.

Production of TNFα is regulated both transcriptionally and posttranscriptionally. Degradation of messenger RNA (mRNA) regulated by adenine/uridine-rich elements (AREs), which are present in the 3′-untranslated region of transcripts encoding inflammatory cytokines such as TNFα, is a paradigm for posttranscriptional regulation (22–24). ARE-binding proteins (ABPs) that can affect the production of cytokines and other inflammatory molecules have been identified (25). Of these, tristetraprolin (TTP), T cell intracellular antigen 1 (TIA-1), and Hu antigen R (HuR) are the most studied to date (26–42).

TTP is a widely expressed protein with 2 zinc finger domains that act as active RNA binding sites. TTP is an immediate early response gene expressed in fibroblasts and other cells upon induction by a variety of stimuli (29–32). TTP binds to AREs of TNFα mRNA and promotes mRNA degradation, thereby reducing the production of TNFα (33). TTP-knockout mice display an inflammatory phenotype characterized by inflammatory arthritis, dermatitis, cachexia, autoimmunity, and myeloid hyperplasia; this phenotype can be prevented by administration of anti-TNFα antibodies (34).

TIA-1 contains 3 RNA recognition motifs (RRMs) that confer high-affinity binding to uridine-rich motifs (35). Recent studies have shown that upon binding to AREs, TIA-1 works not as a transcript destabilizer but as a translational silencer (28). Mild arthritis develops in TIA-1–knockout mice, and severe arthritis develops in TIA-1/TTP–double-knockout mice (36).

The other ABP, HuR, is a member of the embryonic lethal abnormal vision RNA-binding proteins and is ubiquitously expressed in proliferating cells (27). HuR has 3 RRMs that bind to ARE at the poly A tail of various mRNAs, and it participates in the regulation of ARE-mediated mRNA stabilization (37,38). Overexpression of HuR stabilizes mRNA-containing TNFα AREs, implicating TNFα AREs as a target for HuR (39). Although HuR gene–knockout mice have been not reported and our knowledge on HuR function is limited, HuR is assumed to accelerate the posttranscriptional production of TNFα by stabilizing its mRNA (41,42).

Recently, we reported that the TTP gene is overexpressed in synovial tissue from patients with RA compared with that from patients with osteoarthritis (OA) (40). Interestingly, when TTP and TNFα gene expression was compared, synovial tissue from patients with elevated serum C-reactive protein (CRP) levels tended to have a low TTP:TNFα gene expression ratio. Thus, appropriate expression of the TTP gene may be important in reducing the severity of RA. This prompted us to speculate that the magnitude and balance of expression of these ABP genes are of importance in determining the severity of RA. Inadequate expression of these genes may result in more severe disease or refractory responses to therapies including anti-TNFα agents. However, although measurement of gene expression in the joint synovium is informative, it would be impossible to obtain clinical samples at the desired time points for adequate monitoring of the disease activity or drug efficacy. It is also almost impossible to obtain samples from healthy control subjects.

The aims of this study were to understand the posttranscriptional control of TNFα production in RA and to identify parameters that could predict the efficacy of anti-TNFα therapy. For this purpose, we measured gene expression of TNFα, TTP, HuR, and TIA-1 in peripheral blood mononuclear cells (PBMCs) from patients with RA. The samples were obtained at baseline and 2 weeks and 54 weeks after administration of the first dose of the anti-TNFα monoclonal antibody, infliximab, and were compared among each other and with those obtained from healthy control subjects.

PATIENTS AND METHODS

Patients. Thirty-eight patients with RA (15 men and 23 women, mean ± SD age 53.0 ± 11.5 years) and 20 healthy control subjects (14 men and 6 women, mean ± SD age 31.9 ± 8.40 years) were included in this study. All patients fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 criteria for the classification of RA (43) and had active arthritis in spite of oral methotrexate therapy (at least 6 mg/week for more than 6 weeks). The characteristics of the participants are listed in Table 1. Written informed consent was obtained from all patients, and the study was approved by the appropriate ethics committee.

Infliximab therapy and assessment of efficacy. Patients were treated with 3 mg/kg of infliximab at weeks 0, 2, 6, and 14, and every 8 weeks thereafter. Infliximab efficacy was evaluated using the ACR preliminary criteria for improvement in RA (44), 54 weeks after the initiation of infliximab therapy. Patients who achieved 50% improvement (an ACR50 response) at week 54 (n = 14) were included in the responder group, while those with less than 20% improvement at week 54 (n = 9) were classified as nonresponders.

Table 1. Characteristics of the patients with RA and healthy controls*

Characteristic	RA patients	Healthy controls
Age, years	53.0 ± 11.5 (25–69)	31.9 ± 8.40 (20–52)
No. men/no. women	15/23	14/6
Disease duration, years	8.68 ± 5.74 (1.67–24)	–
C-reactive protein, mg/dl	3.21 ± 1.93 (0.31–7.77)	–
ESR, mm/hour	59.5 ± 22.1 (15–104)	–
Rheumatoid factor, IU/ml	227 ± 362 (5–1,790)	–
Methotrexate, mg/week	7.67 ± 1.31 (6–12)	–
Prednisolone, mg/day	7.03 ± 4.00 (0–17.5)	–

* Except where indicated otherwise, values are the mean ± SD (range). RA = rheumatoid arthritis; ESR = erythrocyte sedimentation rate.

Samples and complementary DNA (cDNA) synthesis. Peripheral blood was obtained from healthy control subjects and from patients with RA, before (week 0) and 2 weeks and 54 weeks after they received the first dose of infliximab. PBMCs were isolated from heparinized peripheral blood using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden), following the protocol recommended by the manufacturer. Cells were spun down to pellets, and total RNA was extracted from the cell pellets using Isogen (Nippongene, Tokyo, Japan). Complementary DNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD), following the instructions provided by the manufacturer.

Quantification of gene expression by real-time polymerase chain reaction. The cDNA samples were amplified with specific primers and fluorescence-labeled probes for the target genes. Amplified product genes were monitored on an ABI 7700 Sequence Detection system (Applied Biosystems, Tokyo, Japan). qPCR MasterMix was purchased from Eurogentec (Seraing, Belgium). The final magnesium concentration was 5 n*M*, the final primer concentration was 200 n*M* for each 5′ and 3′ primer, and the final probe concentration was 100 n*M*. Primers and fluorescent probes for TNFα, TTP, TIA-1, HuR, and GAPDH were purchased from Applied Biosystems. Thermal cycler conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Serial dilutions of a standard sample were included in every assay, and standard curves for the genes of interest and the GAPDH gene were generated. All measurements were performed in triplicate. The level of gene expression was calculated from the standard curve and was expressed relative to GAPDH gene expression.

Statistical analysis. The Wilcoxon rank test for paired samples was used to compare the gene expression levels among samples obtained at week 0, week 2, and week 54. The Mann-Whitney U rank test was used to compare the expression levels of genes in patients with RA and healthy control subjects. Pearson's correlation coefficient was calculated to assess the correlations between the expression of 2 genes. All data are expressed as the mean ± SD. *P* values less than 0.05 were considered significant. Statistical analyses were performed using StatView version 5.0 software (SAS Institute, Cary, NC).

RESULTS

Expression levels of TNFα and ABP genes. At week 0 (baseline), TNFα gene expression in PBMCs was higher in patients with RA than in healthy control subjects (for patients with RA, mean ± SD 2.80 ± 2.48; for control subjects, 0.88 ± 0.46 [$P < 0.0001$]). In contrast, expression levels of the TTP gene were lower in patients with RA than in control subjects (1.20 ± 0.95 and 2.60 ± 1.54, respectively [$P < 0.0001$]). Expression levels of the TIA-1 gene were higher and those of the HuR gene were lower in patients with RA than in control subjects (for TIA-1, 3.34 ± 1.79 and 1.79 ± 0.39, respectively [$P < 0.0005$]; for HuR, 1.79 ± 0.83 and 2.15 ± 0.59, respectively [$P = 0.018$]) (Figure 1). When the expression levels of 2 genes in a given sample were compared, the TTP:TNFα, TTP:HuR, and TIA-1:TNFα ratios were significantly lower in patients with RA than in control subjects (for TTP:TNFα, 0.55 ± 0.43 and 3.09 ± 1.17, respectively [$P < 0.0001$]; for TTP:HuR, 0.90 ± 1.09 and 1.19 ± 0.53, respectively [$P < 0.005$]; for TIA-1:TNFα, 1.80 ± 1.42 and 2.40 ± 0.87, respectively [$P = 0.014$]), while the TIA-1:HuR gene expression ratio was significantly higher in PBMCs from patients with RA than in those from control subjects (1.85 ± 0.52 and 0.85 ± 0.14, respectively [$P < 0.0001$]) (Figure 1). Among these comparisons, the difference in the TTP:TNFα ratio appeared to be most prominent, and this significant difference may imply that TTP is important as a negative regulator of inflammation in RA.

TNFα and ABP gene expression levels before and after infliximab therapy. We compared the gene expression levels of TNFα, TTP, TIA-1, and HuR in PBMC samples obtained at baseline and 2 weeks and 54 weeks after administration of the first dose of infliximab. No significant differences were noticed between baseline and week 2 samples (for TNFα, 2.80 ± 2.48 at week 0

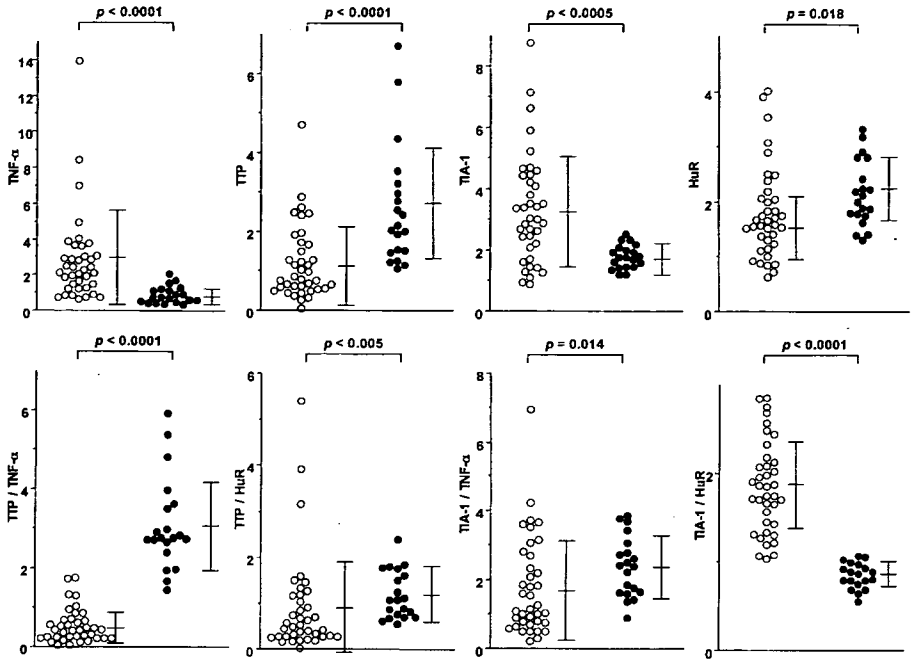

Figure 1. Expression levels of the tumor necrosis factor α (TNFα) gene and 3 adenine/uridine-rich element binding protein (tristetraprolin [TTP], T cell intracellular antigen 1 [TIA-1], and Hu antigen R [HuR]) genes in peripheral blood mononuclear cells from 38 patients with active rheumatoid arthritis at week 0 (open circles) and from 20 healthy control subjects (solid circles). Bars show the mean ± SD. *P* values were calculated by Mann-Whitney U test.

and 2.84 ± 1.99 at week 2; for TTP, 1.20 ± 0.95 at week 0 and 1.17 ± 1.32 at week 2; for TIA-1, 3.34 ± 1.79 at week 0 and 3.88 ± 1.79 at week 2; for HuR, 1.79 ± 0.83 at week 0 and 2.06 ± 0.91 at week 2) (Figure 2). However, the TTP:HuR gene expression ratio decreased 2 weeks after initiation of infliximab therapy (0.90 ± 1.09 at week 0 and 0.71 ± 0.88 at week 2; $P = 0.015$), while no significant changes were noted in the TTP:TNFα, TIA-1:TNFα, and TIA-1:HuR ratios (for TTP:TNFα, 0.55 ± 0.43 at week 0 and 0.50 ± 0.39 at week 2; for TIA-1:TNFα, 1.80 ± 1.42 at week 0 and 1.94 ± 1.19 at week 2; for TIA-1:HuR, 1.85 ± 0.52 at week 0 and 1.88 ± 0.34 at week 2) (Figure 2).

At week 54, the TNFα gene expression level and the TIA-1:HuR gene expression ratio increased from those observed at week 0 (for TNFα, 2.80 ± 2.48 at week 0 and 5.05 ± 3.89 at week 54 [$P = 0.015$]; for TIA-1:HuR, 1.85 ± 0.52 at week 0 and 2.35 ± 0.71 at week 54 [$P = 0.010$]). TTP gene expression increased from that at week 2 (1.17 ± 1.32 at week 2 and 1.61 ± 0.94 at week 54; $P = 0.0065$). In contrast, the TIA-1:TNFα gene expression ratio decreased, from 1.94 ± 1.19 at week 2 to 1.22 ± 0.81 at week 54 ($P = 0.026$). Fluctuations in TIA-1 gene expression and the TTP:TNFα and TTP:HuR gene expression ratios differed greatly among individual patients (Figure 2).

Relationship between TNFα and ABP gene expression levels in patients with RA. We next examined the correlation between the gene expression levels in PBMC samples from healthy control subjects and those in samples from patients with RA at week 0, week 2, and week 54. We anticipated that although posttranscriptional regulation of TNFα production would be adequately executed in healthy individuals, some disturbance might be present in patients with active RA. These disturbances may be partially responsible for the higher disease activity in these patients to whom infliximab is prescribed. In particular, we were interested in investigating whether the correlation between TNFα and the ABPs that have been shown to suppress TNFα production (TTP and TIA-1) would be altered. In addition, we were interested in determining whether a disturbance in posttranscriptional regulation of TNFα production, if it does exist, would be affected by infliximab therapy.

In the control samples, gene expression of TNFα correlated with the expression levels of all genes examined (for TTP and TNFα, r = 0.64 and $P = 0.0017$; for

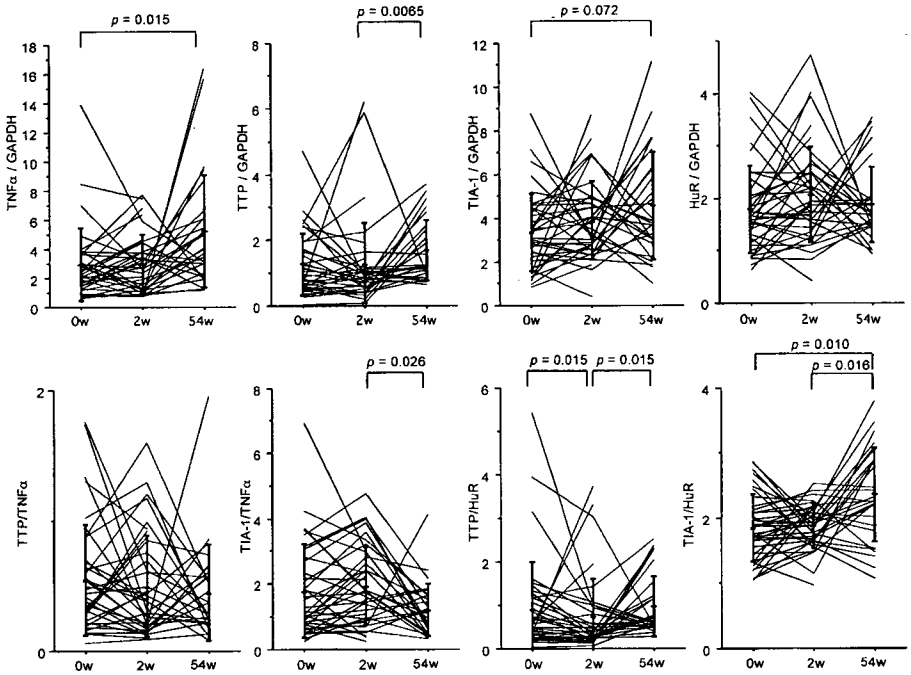

Figure 2. TNFα and adenine/uridine-rich element binding protein gene expression levels in peripheral blood mononuclear cells from patients with rheumatoid arthritis, before (0w) and 2 weeks and 54 weeks after administration of the first dose of infliximab. Bars show the mean ± SD. *P* values were calculated by Wilcoxon's rank sum test. See Figure 1 for definitions.

TTP and HuR, r = 0.64 and $P = 0.0017$; for TIA-1 and TNFα, r = 0.62 and $P = 0.0030$; for TIA-1 and HuR, r = 0.73 and $P = 0.0001$; for TNFα and HuR, r = 0.60 and $P = 0.0041$; for TTP and TIA-1, r = 0.59 and $P = 0.0049$) (Figure 3A). In samples obtained from patients with RA at week 0, the correlations between TTP and TNFα and between TIA-1 and HuR were significant (for TTP and TNFα, r = 0.40 and $P = 0.016$; for TIA-1 and HuR, r = 0.87 and $P < 0.0001$) (Figure 3B), while the correlation between TTP and HuR was not significant. Similar correlations were noted at week 2 and at week 54 (for TTP and TNFα, r = 0.34 and $P = 0.039$ at week 2 and r = 0.39 and $P = 0.042$ at week 54; for TIA-1 and HuR, r = 0.94 and $P < 0.0001$ at week 2 and r = 0.84 and $P < 0.0001$ at week 54) (Figures 3C and D).

Interestingly, the significant relationships for gene expression between TIA-1 and TNFα, TNFα and HuR, and TTP and TIA-1 were not observed in samples obtained from patients with RA at week 0. However, significant relationships between TIA-1 and TNFα and between TNFα and HuR gene expression were observed in week 2 and week 54 samples (for TIA-1 and TNFα, r = 0.18 and $P = 0.27$ at week 0, r = 0.38 and $P = 0.022$ at week 2, and r = 0.51 and $P = 0.009$ at week 54; for TNFα and HuR, r = 0.10 and $P = 0.54$ at week 0, r = 0.45 and $P = 0.007$ at week 2, and r = 0.72 and $P = 0.0002$ at week 54 (Figures 3B, C, and D). These observations suggest that regulatory mechanisms that control the expression of these molecules are disturbed in patients with active RA and are somewhat restored after the initiation of anti-TNFα therapy.

Relationship between TNFα and ABP gene expression and efficacy of infliximab therapy. Our working hypothesis was that differences in the regulation of ABP production might lead to differences in the severity of RA and the efficacy of TNFα-blocking agents. Thus, we anticipated that we might observe some differences in the expression of these molecules between patients whose disease responded to infliximab and infliximab nonresponders.

At the time of this study, 27 patients with RA had received at least 9 courses of infliximab therapy (week 54). At week 54, 18 patients (66.7%) had achieved at least an ACR20 response; 14 patients (51.9%) had achieved an ACR50 response, and 8 patients (29.6%) had achieved an ACR70 response. The 14 patients who achieved an ACR50 response at week 54 were included in the responder group, while the 9 patients who did not

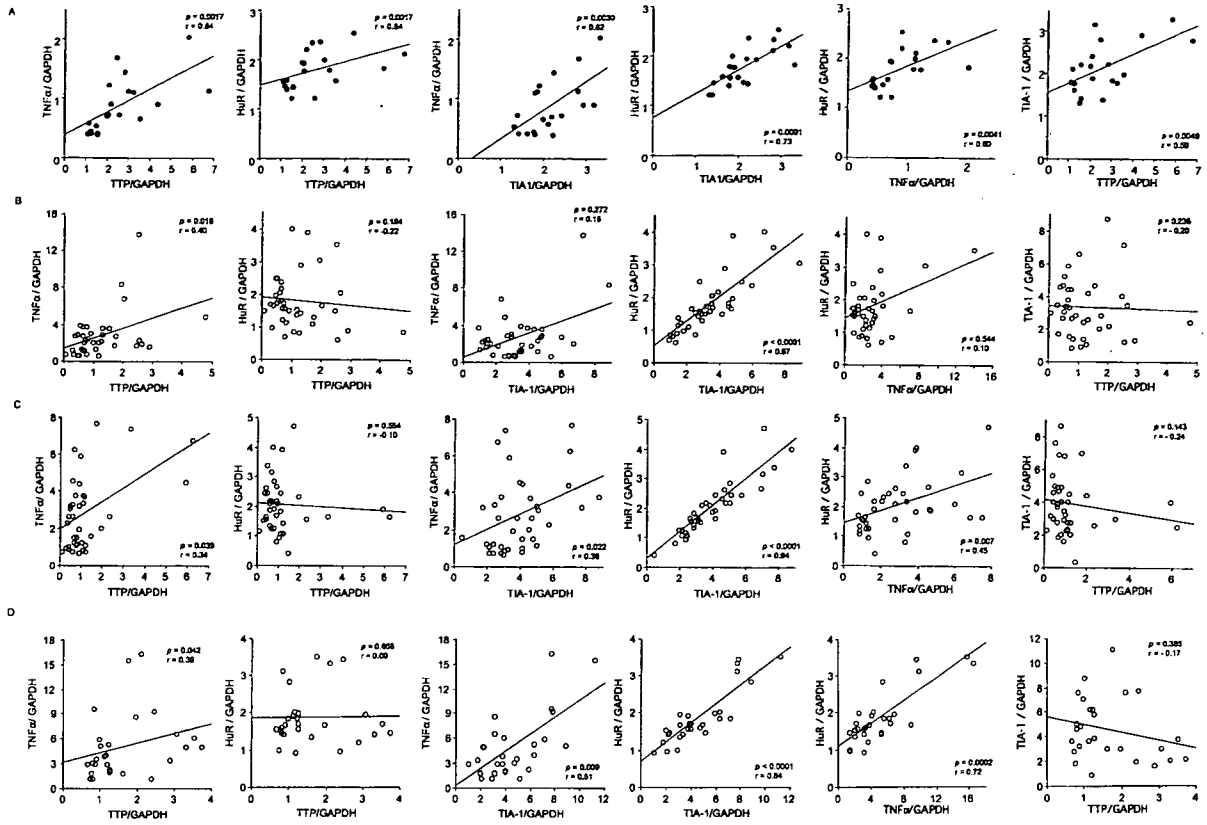

Figure 3. Relationships between expression levels of tumor necrosis factor α (TNFα) and adenine/uridine-rich element binding protein genes in peripheral blood mononuclear cells obtained from patients with rheumatoid arthritis (RA; open circles [n = 38]) and from healthy control subjects (solid circles [n = 20]). **A,** Healthy control subjects. **B,** Patients with RA, before initiation of infliximab therapy. **C,** Patients with RA, 2 weeks after administration of the first dose of infliximab. **D,** Patients with RA, 54 weeks after initiation of infliximab therapy. *P* values were calculated using Pearson's correlation coefficient. See Figure 1 for other definitions.

achieve at least an ACR20 response at week 54 were included in the nonresponder group. No significant differences in the expression of TNFα and ABP genes were observed between these 2 groups (for TNFα, 3.27 ± 3.53 in responders and 2.18 ± 1.25 in nonresponders; for TTP, 1.20 ± 1.31 in responders and 1.09 ± 0.50 in nonresponders; for TIA-1, 3.55 ± 1.64 in responders and 2.86 ± 1.01 in responders; for HuR, 1.72 ± 0.68 in responders and 1.86 ± 0.92 in nonresponders) (Figure 4). The TIA-1:HuR gene expression ratio at week 0 tended to be higher in the responder group than in the nonresponder group (2.051 ± 0.471 and 1.626 ± 0.365, respectively [P = 0.059 by Mann-Whitney U test]). No statistically significant differences were observed between responders and nonresponders for other clinical parameters measured at week 0 or week 2, including serum CRP levels, the erythrocyte sedimentation rate, and other ACR-defined improvement parameters, and the matrix metalloproteinase 3 level (data not shown).

DISCUSSION

In this study, we investigated the role of ABPs in the pathogenesis of RA, as well as any association between gene expression and the efficacy of anti-TNFα therapy, by monitoring PBMC samples obtained before and after infliximab therapy. A similar study using synovial tissue would have been preferable but is quite impractical. We anticipated that the gene expression levels of TNFα and the ABPs in PBMCs would reflect the inflammation status of patients with RA, and we focused on TTP, TIA-1, and HuR, which are clinically important ABPs. Results of previous studies suggested that TTP and TIA-1 are antiinflammatory factors, while HuR is considered an inflammation-accelerating factor

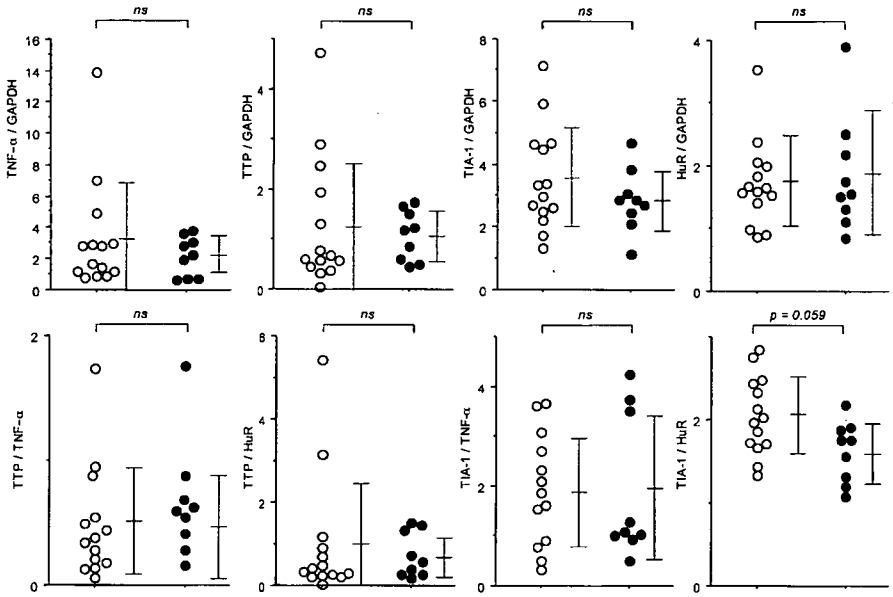

Figure 4. Relationships between expression levels of TNFα and adenine/uridine-rich element binding protein genes in peripheral blood mononuclear cells, and efficacy of infliximab therapy. Open circles represent the 14 rheumatoid arthritis patients who achieved 50% improvement according to the American College of Rheumatology response criteria (ACR50) at week 54. Solid circles represent the 9 patients who had not achieved at least an ACR20 response at week 54. Bars show the mean ± SD. *P* values were calculated by Mann-Whitney U test. NS = not significant (see Figure 1 for other definitions).

(28,33,34,36,39,41,42). We speculated that the balance or imbalance in the production of these factors may induce differences in TNFα production, and hence, RA activity.

The aim of anti-TNFα therapy is to neutralize TNFα in the circulation and suppress the harmful effects of this cytokine in vivo. In doing so, the physiologic mechanisms that control TNFα gene transcription may be attenuated in the short term, resulting in increased TNFα production. If this is the case, differences in the posttranscriptional regulation of TNFα production may affect the disease activity or efficacy of TNFα-blocking agents in individual patients with RA. Therefore, examining the gene expression of TNFα and ABP may provide not only a better understanding of the pathogenesis of RA but also a clue to the factors that affect and allow us to predict the efficacy of TNFα-blocking drugs.

Our results showed that prior to the start of infliximab therapy, the TNFα gene was overexpressed and the TTP gene was underexpressed in patients with RA compared with healthy control subjects. Furthermore, the TTP:TNFα and TTP:HuR ratios were significantly lower in patients with RA than in healthy control subjects. Because TTP is a destabilizer of TNFα mRNA, and various stimuli including TNFα itself promote TTP production (33), our results imply that the negative feedback mechanism of TTP production is not sufficient to counter the excessive TNFα production that occurs during active RA. TIA-1 gene expression and the TIA-1:HuR expression ratio were higher in patients with RA than in control subjects, while the TIA-1:TNFα ratio was lower. Because TIA-1 is a translational silencer of TNFα, it is conceivable that TIA-1 is produced as another negative feedback mechanism against TNFα overproduction to compensate for the TTP decrement in patients with active RA, although such compensation is still not sufficient to mitigate the symptoms of RA activity. Interestingly, although the scatter in expression of the TIA-1 gene was small among healthy control subjects, it was quite large among patients with RA. This may reflect the interindividual differences in the regulation of TIA-1 expression, which become evident when a person acquires an inflammatory disorder mediated by TNFα.

Considering the crucial role of excessive TNFα production in the RA inflammatory process, abnormal regulation at the posttranscriptional level may be one of the factors that promote this excessive TNFα production, and thus, more severe arthritis. We previously reported that TTP gene expression is significantly higher in RA synovial tissue compared with that in OA synovial

tissue (40). One of the explanations for this discrepancy is that in the previous study, RA synovial samples were obtained from patients who had undergone surgery, and disease activity greatly varied among these patients. In contrast, all of the PBMC samples used in the present study were obtained from patients with active arthritis and were obtained just prior to initiation of anti-TNFα therapy. Another interpretation is that TTP production may be higher at sites of active inflammation (e.g., synovial tissue in patients with RA) than in PBMCs.

To investigate the effect of infliximab on the production of TNFα posttranscriptional regulatory factors, we compared the gene expression levels of TNFα and the 3 ABPs before and 2 weeks and 54 weeks after administration of the first dose of infliximab. There were no significant changes in gene expression, but the TTP:HuR ratio significantly decreased after infliximab therapy. This change may have resulted from the decrease in TTP gene expression and the increase in HuR gene expression in response to infliximab-induced TNFα removal. The results hinted at this situation but were not statistically significant. In addition, the TIA-1:TNFα ratio tended to increase at week 2. This may also have resulted from a subtle reduction in TNFα gene expression and an increase in TIA-1 gene expression. When gene expression at week 0 and week 54 were compared, TNFα gene expression and the TIA-1:HuR ratio were significantly higher in week 54 samples than in week 0 samples. Inhibition of the function of the TNFα protein by infliximab may have led to a relative increment in expression of the TNFα gene and the TIA-1 gene.

Interestingly, parameters investigated in this study tended to fluctuate toward the reverse direction when changes between week 0 and week 2 and those between week 2 and week 54 were compared. In addition, there seemed to be a large interindividual difference in the fluctuation of these parameters, especially TTP, TIA-1, HuR, the TIA-1:TNFα ratio, and the TIA-1:HuR ratio. These results imply that the impact of infliximab on the posttranscriptional regulation of TNFα production varies among individual patients with RA. These variations may affect the long-term efficacy of anti-TNFα drugs. Recently, it has been discussed whether infliximab therapy could be postponed in some patients without causing a flare in disease activity (45). Gene expression levels of TNFα and ABPs that are involved in the posttranscriptional regulation of TNFα production are possible candidates for parameters that could be used to predict whether infliximab may be withdrawn without a severe flare in disease activity.

A significant positive relationship was observed between the gene expression of TNFα and that of TTP, in PBMC samples from both patients with RA and healthy control subjects. This result is consistent with results of a previous study showing induction of TTP protein biosynthesis by TNFα production in macrophages as a part of a negative feedback mechanism (33). The correlation between TNFα and TTP was most prominent in healthy subjects, which may imply that the balance of TNFα and TTP gene expression is inappropriately regulated in at least some patients with RA. The positive relationship of TNFα and TIA-1, in both patients with RA and healthy control subjects, implies that a negative feedback mechanism between TNFα and TIA-1 may also exist. In addition, a strong positive relationship was observed between the gene expression of TIA-1 and that of HuR in both patients with RA and healthy control subjects, implying that either a common mechanism controlling the expression of TIA-1 and HuR is present or that one of these proteins controls the expression of the other. Interestingly, we also observed a strong positive relationship between the expression of TIA-1 and that of HuR genes in synovial tissue obtained from patients with RA after surgery (46).

Recently, it was shown in HuR-transgenic mice that HuR and TIA-1 act in concert to suppress TNFα production, suggesting that HuR may be an inflammation suppressor in vivo (26), contrary to previous studies in which HuR was reported to be a proinflammatory factor (39,41,42). Thus, it seems that ABPs do not function independently of each other, and the precise roles of these ABPs in vivo are still to be elucidated. In contrast to what is observed in healthy control subjects, positive correlations between TTP and HuR gene expression and between TTP and TIA-1 were not present in RA samples obtained before and 2 weeks after initiation of infliximab therapy. It is possible that an imbalance in gene expression is one of the causes of excessive TNFα production, which in turn leads to higher disease activity in patients with RA.

Currently, it is not clear why the efficacy of TNFα-blocking agents differs greatly among patients with RA. We wanted to find some clues that may help answer this question. The TIA-1:HuR gene expression ratio tended to be higher in the responder group, but the difference between responders and nonresponders was not statistically significant. It may be possible that patients with lower gene expression of HuR and higher expression of TIA-1 have decreased TNFα mRNA stability and translation, and hence, lower TNFα production. In these patients, infliximab might be more effective in neutralizing circulating TNFα than in patients

with a lower TIA-1:HuR expression ratio. Measurement of such gene expression in patients prior to infliximab therapy might be a useful predictor of the potential efficacy of infliximab. However, we were unable to draw a definitive conclusion in this study, and additional studies with a larger number of samples should be performed to confirm our observations and speculations. In a recent study, it was shown that failure to suppress serum CRP at week 2 of therapy identified the majority of patients who were nonresponders by week 12 (47). However, in our series of patients, we did not find a significant relationship between a reduction in the CRP level at week 2 and the efficacy of infliximab at week 54.

Our study had several limitations. Although we included all of our patients who were receiving infliximab therapy, the cohort number was not large enough for strong statistical analyses. Protein analyses would also be useful to accurately determine the ABP levels actually present in the cells. Other ABPs that were not studied here may also have important roles in the pathogenesis of RA. Furthermore, the ABPs studied here can also affect the mRNA of other inflammatory molecules such as cyclooxygenase 2 (48–50); these interactions may have impacted our findings and therefore should be considered in any interpretation of the data.

In conclusion, by analyzing the gene expression levels of TNFα and ABP in PBMCs, we observed a relationship between the expression of TTP, TIA-1, and HuR that might have an impact on TNFα gene expression and thereby protein production. Our results also implied that the TIA-1:HuR gene expression ratio before infliximab therapy may predict the efficacy of treatment. Further studies are necessary to enhance our understanding of RA pathogenesis and to identify possible targets of therapy as well as parameters that predict the efficacy of pharmaceutical agents.

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AUTHOR CONTRIBUTIONS

Dr. Sumida had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Sugihara, Tsutsumi, E. Suzuki, Sumida.

Acquisition of data. Sugihara, Tsutsumi, E. Suzuki, T. Suzuki, Ogishima, Hayashi, Chino, Ishii, Mamura, Goto, Matsumoto, Ito, Sumida.

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

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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 RORγt, 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-1β, 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.

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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 RORγt 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.
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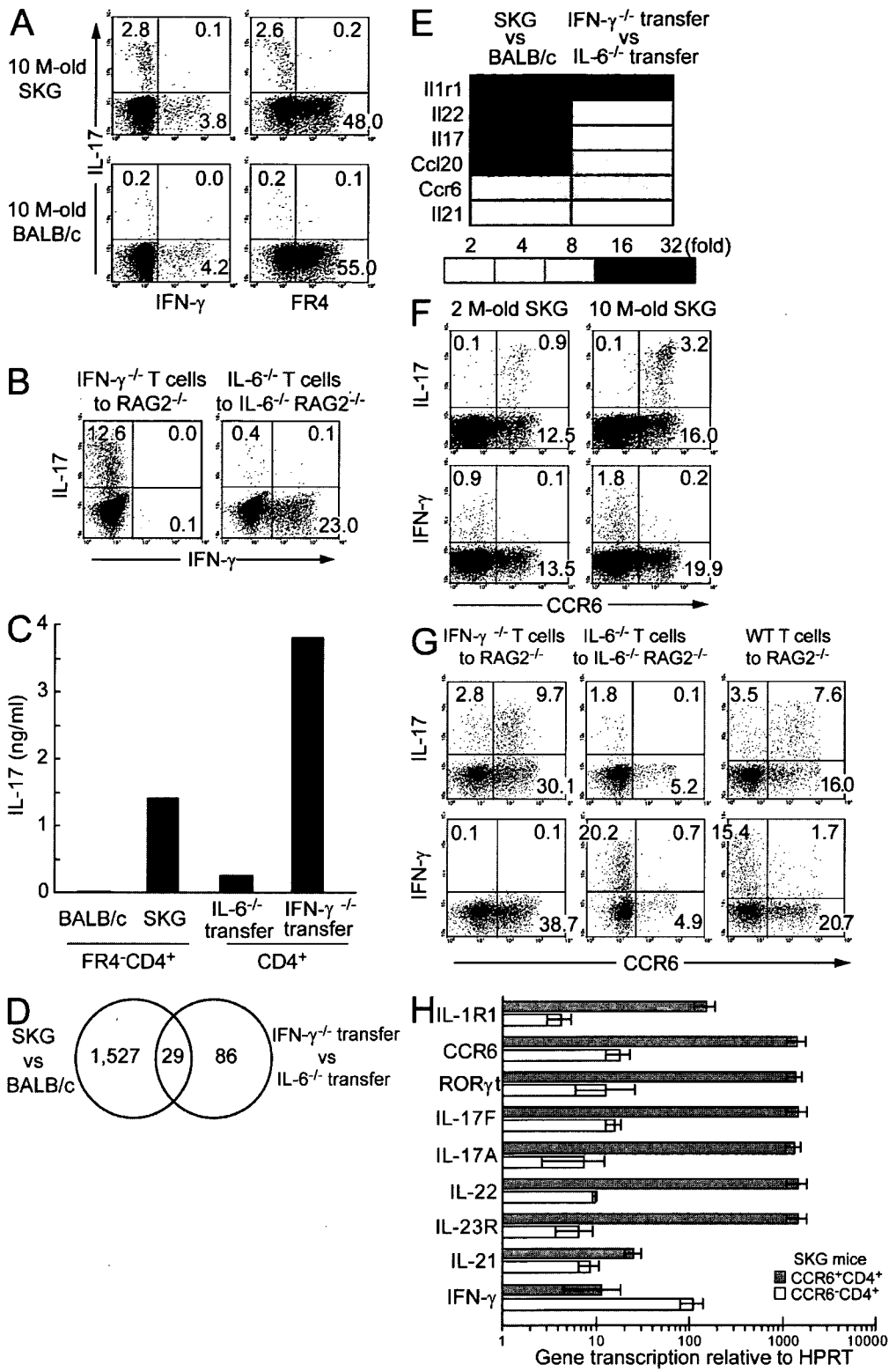

Figure 1. Gene microarray analysis of Th17 cells and their predominant expression of CCR6. (A) LN CD4+ T cells from 10-mo-old BALB/c or SKG mice were stained for intracellular IL-17 and IFN-γ, or these cytokines and cell-surface FR4. (B) CD4+ T cells from IFN-$\gamma^{-/-}$ or IL-$6^{-/-}$ mice were 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,

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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-$\gamma^{-/-}$) 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-$\gamma^{-/-}CD4^+$ 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-$\gamma^{-/-}CD4^+$ 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-$\gamma^{-/-}$ 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-$\gamma^{-/-}$ 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 RORγt; 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-$\gamma^{-/-}$ 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-$\gamma^{-/-}CD4^+$ 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-$\gamma^{-/-}$, 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 ± 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.

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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

RORγt crucially controls Th17 cell differentiation from naive CD4$^+$ T cells (7). To test whether forced expression of RORγt directs the differentiation of CD4$^+$ T cells to CCR6$^+$ Th17 cells, RORγt was transduced in CCR6$^-$CD4$^+$ T cells by a bicistronic retroviral vector expressing RORγt and GFP (7). Notably, GFP-high (i.e., RORγt-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

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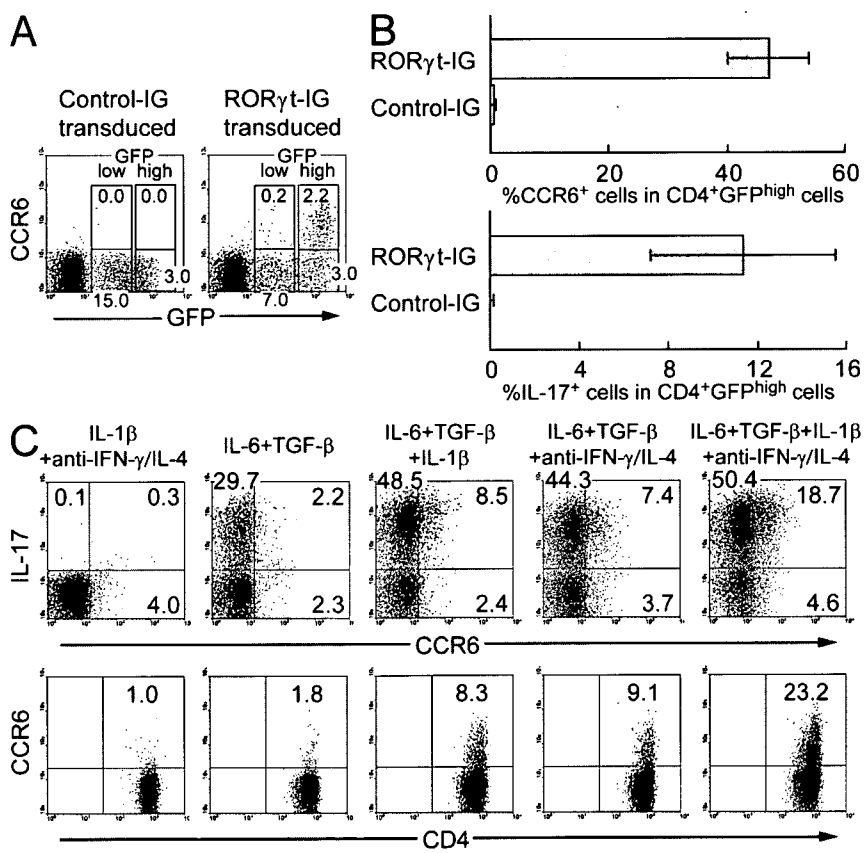

Figure 2. Induction of CCR6 on Th17 cells in vitro. (A) Retroviral vector, pMxs-RORγt-IRES-GFP (RORγt-IG), or control pMxs-IRES-GFP (Control-IG), was transduced into BALB/c CCR6$^-$CD4$^+$ T cells, as previously described (reference 7), and the cells expressing GFP were stained for CCR6 on day 4 after transduction. (B) The mean percentages ± 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.

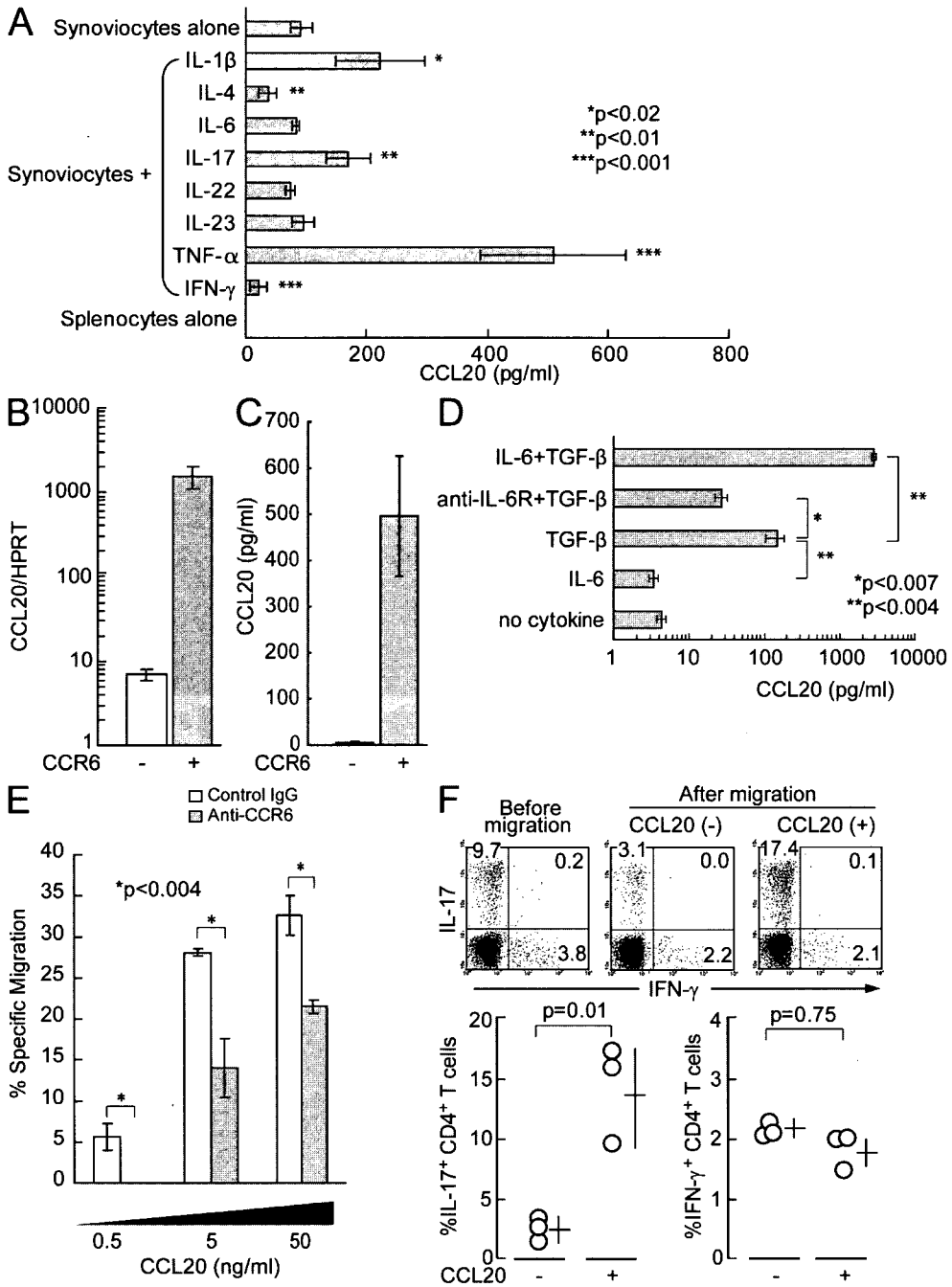

Figure 3. Cross-regulation of synoviocyte CCL20 production by cytokines and preferential migration of Th17 cells in response to CCL20. (A) 2.5 × 10^4 adherent synoviocytes, prepared as previously described (reference 10), were cultured with 10 ng/ml of the indicated cytokine for 24 h. The amounts of CCL20 in culture supernatants were measured by ELISA. Data are shown as the mean ± 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.

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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,

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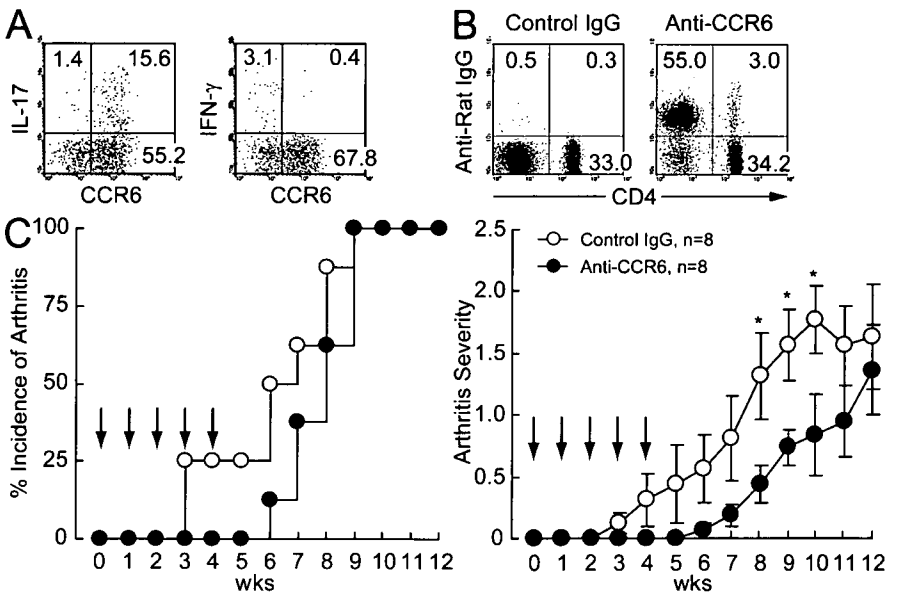

Figure 4. Contribution of CCR6/CCL20 to trafficking of Th17 cells in SKG arthritis. (A) CD4$^+$ T cells infiltrating arthritic joints were prepared from collagenase-digested ankle joints of SKG mice, and stained for CCR6 and intracellular IL-17 or IFN-γ. (B) SKG mice received an i.v. injection of 100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

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

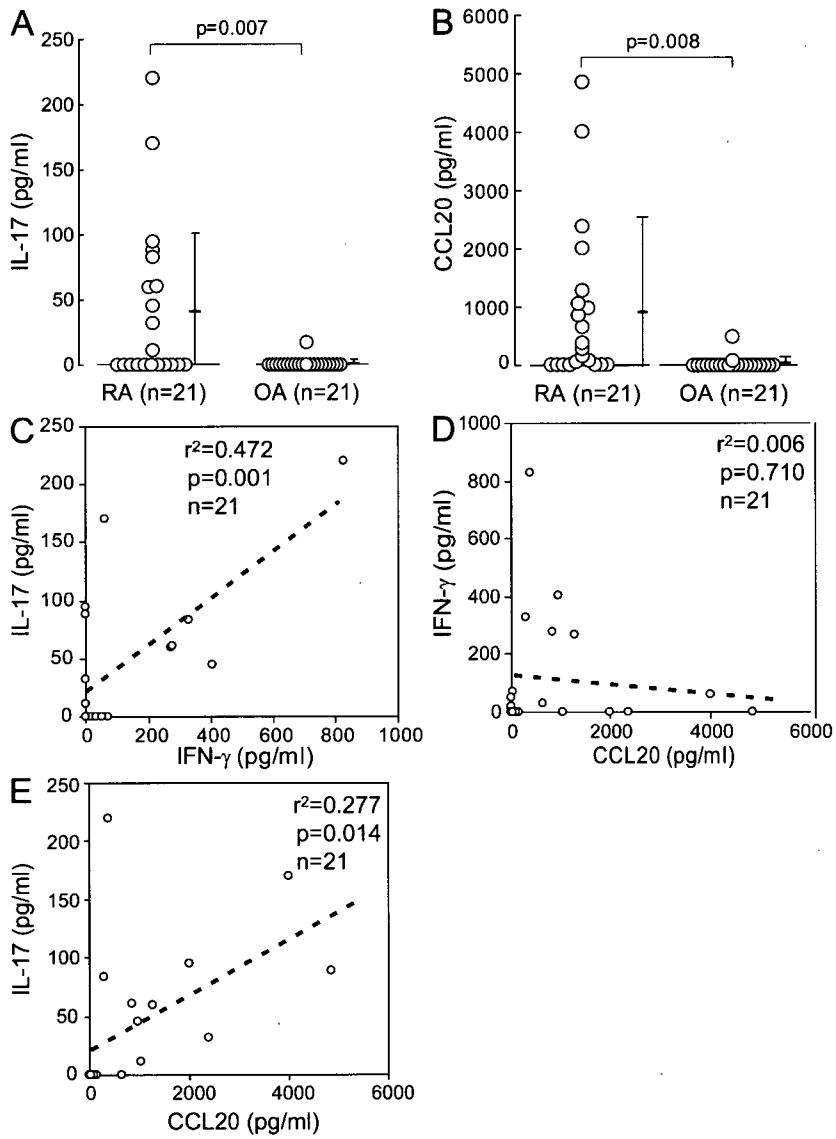

Figure 5. Production of IL-17 and CCL20 in RA joints. (A and B) The concentrations of IL-17 and CCL20 in synovial fluid from RA or OA patients were measured by ELISA. Vertical bars represent the means ± SD. (C–E) Scatter plots show the correlation between IL-17 and IFN-γ (C), CCL20 and IFN-γ (D), or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

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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).

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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.

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