Th1 but not Th17 cells predominate in the joints of patients with rheumatoid arthritis

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ABSTRACT

Objectives: Recent animal studies have revealed critical roles of interleukin (IL)17, which is produced by a newly identified subset of helper T cells, Th17 cells, in the development of autoimmune diseases including arthritis. However, in human rheumatoid arthritis (RA), detailed characteristics and the prevalence of Th17 cells are unclear.

Methods: Peripheral blood mononuclear cells (PBMC) were obtained from 123 patients with RA and 28 healthy controls. Mononuclear cells were also prepared from synovial membrane or synovial fluid of 12 patients with RA. IL17 (IL17A) positive T cells were identified by a flow cytometer after ex vivo stimulation with phorbol myristate acetate and ionomycin. Disease activity was assessed with the 28-joint Disease Activity Score (DAS28).

Results: IL17 positive cells were detected in CD45RO+ CD4 T cells. Most IL17 positive T cells produced neither interferon (IFN)γ nor IL4, but tumour necrosis factor (TNF)α similar to murine Th17 cells. The frequency of Th17 cells was neither increased in RA nor correlated with DAS28. Unexpectedly, the frequency of Th17 cells was significantly decreased in the joints compared with PBMC of the same patients with RA, whereas Th1 cells were more abundant in the joints than in PBMC.

Conclusions: We could not obtain evidence that positively supports predominance of Th17 cells in RA. Further careful investigation is necessary before clinical application of IL17-targeting therapy.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder in which activated macrophages producing tumour necrosis factor (TNF)α play critical roles.[1] Disease association with certain major histocompatibility complex (MHC) class II haplotypes and histological similarity between rheumatoid synovium and skin delayed-type hypersensitivity (DTH) lesions, including infiltration of CD4 T cells,[2–4] suggest that the macrophages are activated by CD4 T cells, especially Th1 cells, although relative paucity of T cell-derived cytokines in RA joints has been reported.[5 6] The importance of Th1 responses in RA is also supported by predominance of Th1 responses in animal models of arthritis, in which increased disease susceptibility by defective interferon (IFN)γ/IFNγ receptor signalling has also been observed.[7–9]

Recently, a novel subset of helper T cells was identified that produces interleukin (IL)17 but not IFNγ or IL4. Development of IL17-producing T cells is counter-regulated by Th1 or Th2 cells, thereby they were recognised as a separate lineage of helper T cells, Th17 cells.[10 11] The importance of Th17 responses has been clearly demonstrated in murine models of autoimmune diseases including arthritis, which had formerly been regarded as Th1 diseases.[12–14] Their involvement in human inflammatory bowel disease was also recently shown.[15] In vitro studies demonstrated various disease-promoting effects of IL17 on RA. IL17 induced IL6 and IL8 production from epithelial cells, endothelial cells and fibroblasts.[16] IL17 stimulated macrophages to produce various cytokines including IL1 and TNFα.[17] IL17 was also shown to induce bone and cartilage destruction via osteoclastgenesis, induction of metalloproteinases and inhibition of proteoglycan synthesis.[18–20] In addition, IL17 was detected in the serum, synovial fluid and synovial membrane of patients with RA.[18 21–23] Expression of human IL17 mRNA was detected in CD45RO+ effector/memory CD4 T cells after activation.[16] However, it is unclear which helper T cell subset produces IL17 in RA. It was shown that Th1 or Th0 but not Th2 clones from patients with RA produced IL17, while IL17 production by Th1 and Th2 cells from patients with allergic dermatitis was also reported.[24 25] The prevalence of Th17 cells in RA is also unknown. In the present study, we identified and characterised T cells that produce IL17 in patients with RA. We further investigated their prevalence in peripheral blood and the joints.

PATIENTS AND METHODS

Patients

A total of 123 patients diagnosed as having RA (9 men and 114 women, aged 21–81 years, mean (SD) 59 (11) years) based on the 1987 criteria of the American College of Rheumatology[26] and 28 healthy controls (3 men and 25 women, age 20–84 years, mean 54 (16) years) were included. Disease duration of RA ranged from 6 months to 45 years (mean 13 (10) years). A total of 102 patients were taking disease-modifying antirheumatic drugs (DMARDs) (53, methotrexate (MTX); 21, bucillamine; 15, sulfasalazopyridine; 8, auranofin; 5, leflunomide, 4, mizoribine; 1, gold sodium thiomalate) either as monotherapy or in combination, 89 patients were taking prednisolone (PSL, 2.5–10 mg/day) and 11 patients were treated with anti-TNFα biologicals (6, infliximab; 5, eternacept). Disease activity was assessed with the 28-joint Disease Activity Score (DAS28).[27] The study protocol was approved by the Regional Committee of Ethics for Human Research at Faculty of Medicine of Kyushu University. All subjects signed an informed consent before participation in the study.

Preparation of mononuclear cells

Synovial tissue was taken at the time of arthroplasty. Synovial fluid was aspirated when patients underwent symptomatic effusion. Synovial tissue samples were minced and incubated with 2 mg/ml collagenase (Wako, Osaka, Japan) for 90 min at 37°C. A single-cell suspension was layered on Ficoll-Paque separation media (GE Healthcare Bio-Science AB, Uppsala, Sweden) and centrifuged to obtain mononuclear cells. Peripheral blood mononuclear cells (PBMC) or synovial fluid mononuclear cells were similarly separated by Ficoll gradient centrifugation.

Flow cytometric analysis

Monoclonal antibodies (mAb) and reagents used for flow cytometric analysis were Alexa Fluor 488-conjugated anti-IL17 (IL17A) mAb (eBio64DEC17) (e-Bioscience, San Diego, California, USA) fluorescein isocyanate-conjugated anti-CD57 mAb (TB01) (Caltag Laboratories, Burlingame, California, USA), anti-CD45RO mAb (UCHL1), anti-IFNγ mAb (4S.B3) (e-Bioscience), phycoertythrin-conjugated anti-CD127 mAb (huIL7R-M21) (BD Bioscience, San Diego, California, USA), anti-CD28 mAb (15E8) (Caltag Laboratories), anti-IL17 mAb, anti-IFNγ mAb, anti-IL4 mAb (MP4-25D2), anti-TNFα mAb (MAb11) (e-Bioscience), allophycoerythrin-conjugated anti-CD4 mAb (S3.5) (Caltag Laboratories), PerCP-Cy5.5-cojugated CD3 mAb (SK7) (BD Bioscience), biotin-conjugated anti-CD4 mAb (Caltag Laboratories) and streptavidin-allophycoerythrin (e-Bioscience). Stained cells were run on a FACS Calibur flow cytometer (BD Bioscience), and the data were analysed using CELL Quest software (BD Bioscience).

Intracellular staining of cytokines

Mononuclear cells were stimulated with 50 ng/ml of phorbol myristate acetate (PMA) (Sigma-Aldrich, St Louis, Missouri, USA) and 750 ng/ml of Ionomycin (Sigma-Aldrich) for 6 h; 10 μl/ml of Brefeldin A (Sigma-Aldrich) was added to the culture for the last 5 h. After surface staining of the cells, intracellular staining was performed using BD Cytofix/Cytoperm solution and BD Perm/Wash solution following the manufacturer's instruction (BD Biosciences).

Statistical analysis

Statistical correlation was examined using Spearman rank correlation coefficient. For examining statistical comparison of unpaired and paired samples, Mann–Whitney U test and Wilcoxon signed rank test was used, respectively. p Values less than 0.05 were considered significant.

RESULTS

Identification of T cells capable of producing IL17 in PBMC of patients with RA

We identified IL17 (IL17A) positive cells in PBMC of patients with RA after stimulation with PMA and ionomycin. No staining for any cytokines was detected without stimulation in RA samples and healthy control samples (data not shown). IL17 positive cells were detected in CD3+ cells, and only in CD4+ T cells produced IL17 (fig 1A). IL17 production by CD45RO+ memory CD4 T cells was also observed by our analysis. We and others have found disease association of a subpopulation of memory phenotype CD4 T cells expressing CD57 and lacking CD28 with RA.[28 29] However, no CD57+ CD4 T cells were positive for IL17, whereas they all produced IFNγ (fig 1B).

In mice, IL17 is mainly produced by Th17 cells, which produce neither IFNγ nor IL4. Similar to murine Th17 cells, most IL17+ CD4 T cells did not produce IFNγ (fig 1C). There was also a small but substantial number of CD4 T cells positive for IL17 and IFNγ, as has been recently observed using human samples.[15] IL4 was not detected in IL17+ T cells, but TNFα was produced by nearly all IL17+ T cells.

Frequency of IL17 positive T cells in RA PBMC

We examined whether the frequency of CD4 T cells capable of producing IL17 was increased in RA. PBMC samples were obtained from 123 patients with RA and 28 healthy controls. The mean percentages of IFNγ+ IL17– cells (Th1), IFNγ+ IL17+ cells and IFNγ– IL17+ cells (Th17) in RA were 16.6 (7.5), 0.27 (0.28) and 1.58 (0.95), respectively. There was no significant difference in the frequency of IFNγ+ IL17– cells, IFNγ+ IL17+ cells or IFNγ– IL17+ cells between RA and controls.

We next looked for any correlation between the frequency of IL17 positive cells and disease activity of RA assessed by DAS28. A total of 69 patients with RA whose clinical variables necessary for the scoring were available were included for the analysis. As shown in fig 2B, the frequency of IFNγ+ cells or IL17+ cells did not correlate with the DAS28. Since there was a significant correlation in the frequency between IFNγ+ cells and IL17+ cells ($r = 0.48$, $p<0.01$), likely because both cytokines were produced by CD45RO+ cells, we also analysed correlation of the ratio of IFNγ+ cells to IL17+ cells with DAS28. However, no significant correlation was detected between the DAS28 and IFNγ+/IL17+ ratio (fig 2B, right). There was also no correlation between the frequency of IL17+ cells or IFNγ+/IL17+ ratio and the DAS28 C-reactive protein (CRP) score, or each component of the scoring including number of tender or swollen joints, visual analogue scale (VAS), erythorcyte sedimentation rate (ESR) and CRP (data not shown).

We also performed correlation or association analysis for background variables of RA. Patients' age or disease duration had no correlation with the frequency of IL17 positive T cells. Sex, seropositivity for rheumatoid factor, history of joint surgery or the presence of bone erosion had no significant association. Interestingly, patients taking PSL or MTX had a higher frequency of IL17+ cells (table 1). An even stronger association was found with the IFNγ+/IL17+ ratio, likely due to a slightly decreased frequency of IFNγ+ cells in these patients. There was no difference in disease activity or other variables of RA between patients taking PSL or MTX and those not taking them (data not shown). A statistically significant association was still detected between MTX use and the IFNγ/IL17 ratio in patients with PSL (MTX+, 9.49 (6.81); MTX–, 12.61 (7.22); $p = 0.0033$). Although there were only 11 patients treated with anti-TNFα reagents, and thereby the number was not statistically significant, such treatment seemed to have no effect on the frequency of IL17+ cells.

Paucity of T cells capable of producing IL17 in RA joints

Although we did not obtain any evidence that supports the importance of IL17-producing T cells in RA by analysing PBMC, it was possible that pathogenic T cells were enriched in the joints. Therefore, we examined the frequency of IL17 positive T cells in the joints of RA, either from synovial membrane or synovial fluid. As depicted in fig 3A, most of the CD4 T cells in the joints were CD45RO+ memory cells. By contrast, only part of CD4 T cells was CD45RO+ in PBMC, indicating accumulation of effector/memory T cells in the joints. There were

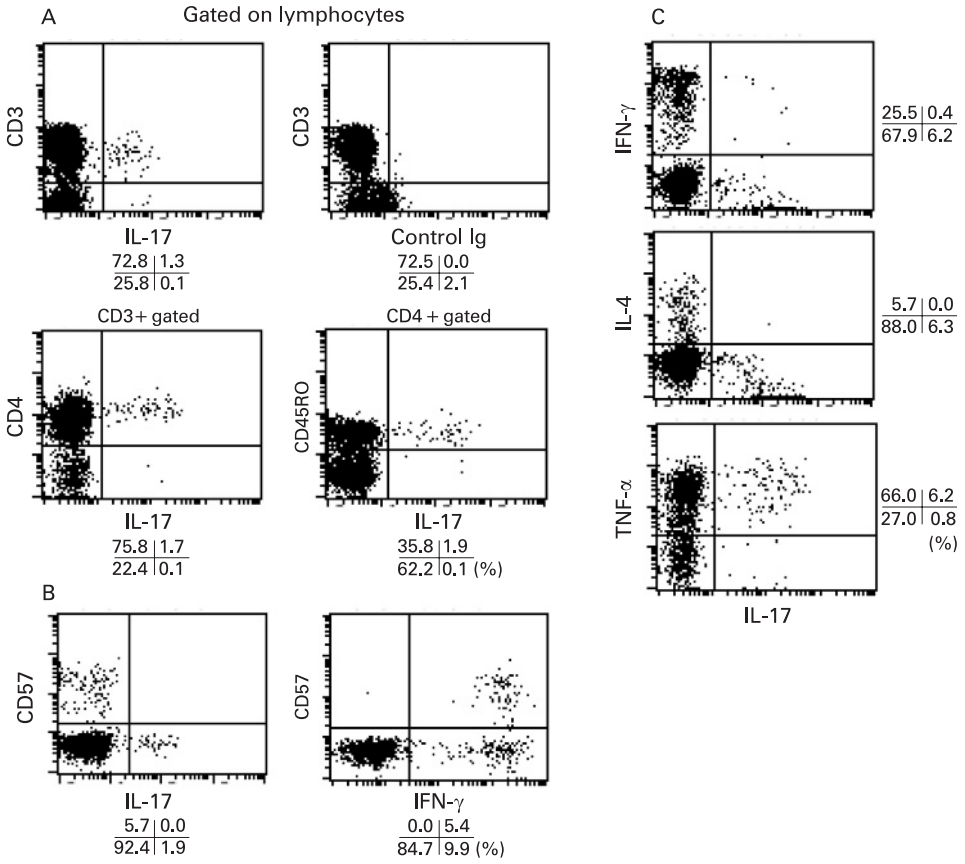

Figure 1 Characterisation of peripheral blood mononuclear cells (PBMC) capable of producing interleukin (IL)17 in rheumatoid arthritis (RA). Cytokine production of PBMC was examined by intracellular staining after stimulation with phorbol myristate acetate (PMA) and ionomycin in the presence of Brefeldin A. A. IL17 positive cells were shown after setting on different gates as indicated in the figure. Isotype-matched mouse IgG1 monoclonal antibody (mAb) was used as a negative control for IL17 staining. B. IL17 or interferon (IFN)γ production of CD57+ cells was examined. Figures are shown after gated on CD4+ cells. C. Production of various cytokines by IL17 positive cells was examined. Figures are shown after being gated on CD4+ cells. Representative data are shown in these figures.

consistently more IFNγ positive CD4 T cells in the joints than in PBMC from the same patients. The majority of IFNγ+ cells were also positive for TNFα (data not shown), Unexpectedly, IL17+ CD4 T cells, irrespective of whether IFNγ positive or negative, were scarcely found in the joints. IL17+ cells were not detected in CD4 negative lymphocytes, either (data not shown).

We analysed 12 paired samples for comparing the frequency of IFNγ and/or IL17 positive T cells between PBMC and the

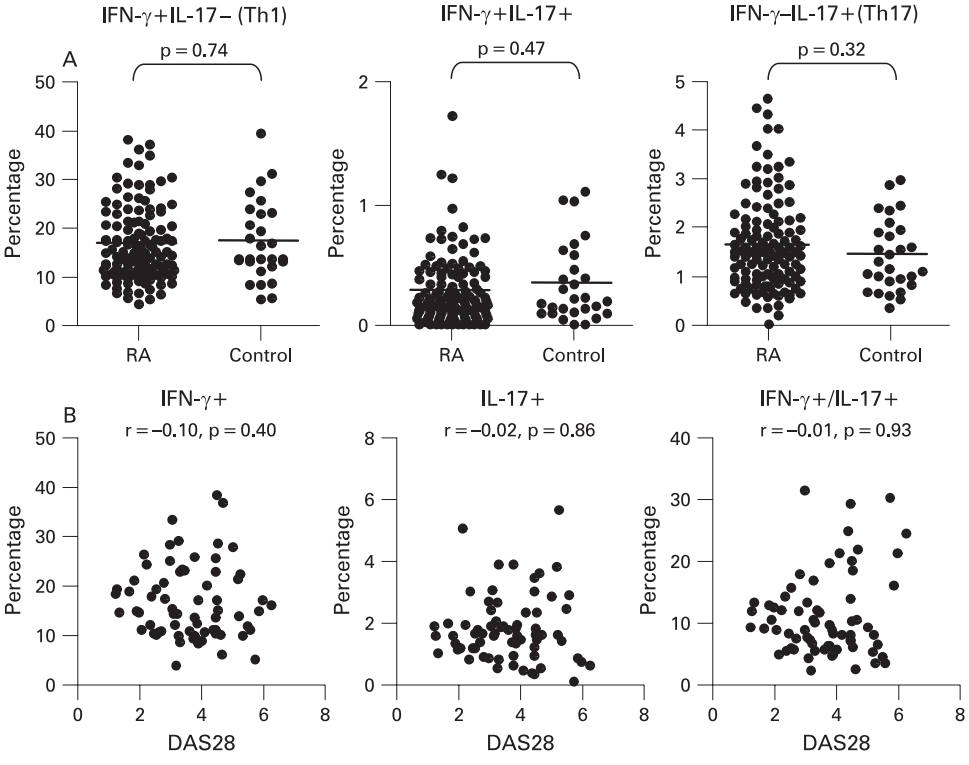

Figure 2 Association of the frequency of interleukin (IL)17 positive T cells in peripheral blood mononuclear cells (PBMC) with rheumatoid arthritis (RA). A. The frequency of interferon (IFNγ)+ IL17– (left), IFNγ+ IL17+ (middle), or IFNγ– IL17+ (right) cells in PBMC was compared between RA and healthy control samples (labelled as Control). The results of statistical analysis are indicated in each panel. B. Correlation between 28-joint Disease Activity Score (DAS28) and frequency of IFNγ+ (left) or IL17+ (middle) cells or the ratio of IFNγ+ cells to IL17+ cells (right) is shown. IFNγ+ cells consists of IFNγ+ IL17– cells and IFNγ+ IL17+ cells, while IL17+ cells consists of IFNγ+ IL17+ cells and IFNγ– IL17+ cells. The results of statistical analysis are indicated in each panel. There was also no correlation between DAS28 and the frequency of IFNγ+ IL17–, IFNγ+ IL17+, or IFNγ– IL17+ cells (not shown).

Table 1 Association of medication with the frequency of interleukin (IL)17+ or interferon (IFN)γ + CD4 T cells in peripheral blood mononuclear cells (PBMC)

	IFNγ + (%)	IL17 + (%)	IFNγ/IL17 (ratio)
PSL+ (n = 89)	17.01 (7.97)	1.98 (1.20)	11.00 (7.15)
PSL– (n = 34)	16.12 (6.84)	1.46 (0.89)	14.35 (8.56)
	p = 0.80	p = 0.028*	p = 0.018*
DMARD+ (n = 102)	16.67 (7.71)	1.85 (1.15)	11.27 (6.58)
DMARD– (n = 21)	17.65 (7.53)	1.85 (1.16)	14.83 (11.24)
	p = 0.40	p = 0.83	p = 0.40
MTX+ (n = 53)	15.90 (8.17)	2.14 (1.34)	9.65 (6.51)
MTX– (n = 70)	17.48 (7.14)	1.60 (0.86)	13.65 (8.00)
	p = 0.07	p = 0.038*	p<0.001*
anti-TNF+ (n = 11)	17.73 (6.08)	2.18 (1.41)	10.33 (4.34)
anti-TNF– (n = 112)	16.75 (7.82)	1.82 (1.12)	12.03 (7.89)
	p = 0.42	p = 0.41	p = 0.94

Values are mean (SD) unless otherwise stated.
*Statistically significant.
DMARD, disease-modifying anti-rheumatic drug; MTX, methotrexate; PSL, prednisolone; TNF, tumour necrosis factor.

joints (Fig 3B). The frequency of IFNγ+ IL17– cells were significantly increased in the joints whereas frequency of IL17+ T cells, especially those which were negative for IFNγ (Th17 cells), was significantly lower in the joints compared with PBMC. There was no difference in the profile of IFNγ and IL17 production between CD4 T cells from synovial membrane and those from synovial fluid. We did not detect positive staining for IFNγ or IL17 in mononuclear cells from the joint without in vitro stimulation (data not shown).

DISCUSSION

In the present study, we identified and characterised T cells capable of producing IL17 in human RA. Similar to murine Th17 cells, most IL17 positive CD4 T cells did not produce IFNγ or IL4 but did produce TNFα. Therefore, these cells are likely the counterparts of murine Th17 cells. Consistent with the recent studies on the human IL17-producing T cells,[15] we also detected a relatively small but substantial percentage of cells producing IL17 and IFNγ. It is notable that IL17 was originally considered as a Th1 cytokine. It was reported that IL17 was produced by Th1 or Th0 cell clones, but not by Th2 cell clones, from patients with RA.[25] It is unclear if such CD4 T cells producing IFNγ and IL17 are a special subset detected in humans, or are also present in mice. It is also unknown whether these double positive T cells represent another subset of helper T cells that require differential signals for differentiation, because differentiation of murine naïve CD4 T cells to Th17 cells is inhibited by the presence of IFNγ.[10 11] In fact, differential requirement for cytokine signals, especially for transforming growth factor (TGF)β, in the development of Th17 between human and mice was recently demonstrated.[30 31] Further investigation is needed to clarify these issues.

Although CD57+ CD28– CD4 T cells have been reported to be associated with RA,[28 29] we did not detect IL17 production by

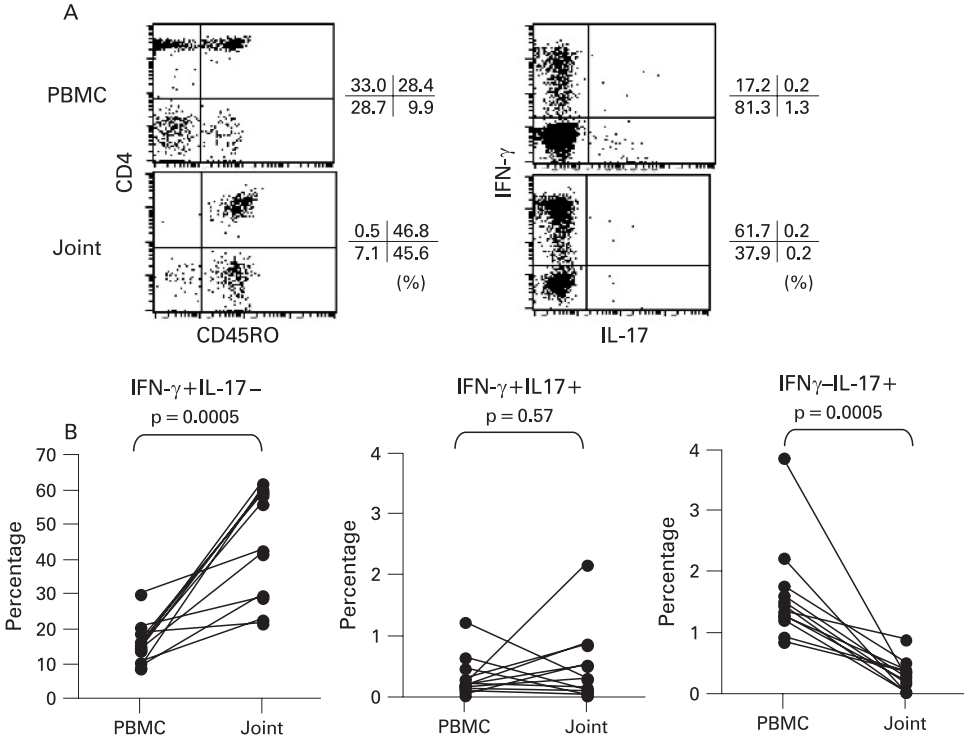

Figure 3 Analysis of T cells capable of producing interleukin (IL)17 in rheumatoid arthritis (RA) joints. A. Expression of CD4 and CD45RO (left panels) on freshly isolated peripheral blood mononuclear cells (PBMC) (upper panels) or in the joint (lower panels). Expression of IL17 and interferon (IFN)γ in these CD4 T cells after stimulation with phorbol myristate acetate (PMA) and ionomycin was analysed (right panels). All figures are shown after being gated on CD4+ cells. B. The frequency of IFNγ+ IL17– (left), IFNγ+ IL17+ (middle), or IFNγ– IL17+ (right) cells in PBMC or the joints (synovial membrane, n = 4; synovial fluid, n = 8) of the same patient. The results of statistical analysis are indicated in each panel.

these T cells. Instead, nearly all of them produced IFNγ (fig 1B). Consistent with our results, a high level of IFNγ production as well as a lack of IL17 production by anti-CD3 mAb-stimulated CD28- CD4 T cells was reported.[32] Therefore it seems that this RA-associated T cell subset is highly polarised toward the Th1 phenotype. We found the frequency of IL17 positive T cells in PBMC was neither increased in RA nor correlated with disease activity of RA. Interestingly, we found the frequency of IL17+ CD4 T cells was slightly increased in patients with RA taking PSL or MTX. However, it is unclear whether this correlation was the result of the treatment or an influence of other unidentified factors. Analysis of samples before and after the start of treatment with these drugs is needed.

Recent studies emphasise an importance of IL17 in the pathogenesis of RA. However, as mentioned above, there was no association between the frequency of T cells capable of producing IL17 in PBMC and RA. In addition, we unexpectedly found a paucity of IL17 positive CD4 T cells, especially Th17 cells, in the joints of patients with RA. This was unlikely to be due to cellular damage caused by collagenase treatment or other preparation process from the synovial membrane or synovial fluid, because IFNγ was vigorously produced by the same samples. Consistent with our results, it was shown by immunohistochemical analysis that less than 1% of T cells in RA synovium positively stained for IL17.[21] We also confirmed higher levels of IFNγ and lower levels of IL17 production in the culture supernatants of synovial T cells stimulated with anti-CD3 mAb (data not shown). It should be taken into consideration that we identified T cells that had differentiated and were capable of producing IL17, because we analysed IL17 production from T cells stimulated ex vivo with PMA and ionomycin. Therefore, it is possible that, although there are many T cells that potentially produce IFNγ, there are more T cells producing IL17 in situ. To circumvent these problems, we stained the cells directly ex vivo or after incubation for 5 h with Brefeldin A alone, but we did not detect positive staining for either IL17 or IFNγ by these methods. It is also possible that a small number of Th17 cells play an important effector role, as high levels of IL17 production was shown in the joint of patients with RA.[18 21 22] However, it is of note that in most of these studies emphasising the importance of IL17 in RA, osteoarthritis (OA) samples were used as controls. Therefore, their results do not necessarily mean an enrichment of IL17-producing cells among CD4 T cells, because RA joints usually contain much higher numbers of total CD4 T cells than OA joints. Similarly, although it is generally accepted that the concentration of IFNγ is not high in the synovial fluid of RA, it is still higher than in OA.[33]

The importance of Th17 cells in autoimmune diseases has been clearly shown in mice.[14] However, in most cases, disease is induced by immunisation with Freund complete adjuvant containing *Mycobacterium tuberculosis*, which is a potent inducer of Th17 responses.[34] This might have led to an overestimation of the role of Th17 responses in disease pathogenesis. In fact, it was demonstrated in murine collagen induced arthritis (CIA) that immunisation with Freund incomplete adjuvant induced IL4-dependent arthritis.[35] Another example is murine proteoglycan-induced arthritis (PGIA). IFNγ deficient mice were resistant for the induction of PGIA,[36] while they were more susceptible for the induction of CIA.[7] Thus, the nature of arthritogenic T cell response depends on disease models, and it is possible that human RA is not necessarily a Th17-predominated disease. Another likely explanation for the lack of association between Th17 and RA in this study is that predominance of Th17 response is limited in early stage of RA. In fact, there is a report showing increased IL17 production only at very early stage of RA.[23] Unfortunately, most of the patients included in this study have relatively long disease duration, and there were only seven patients within 1 year after onset, three of whom were taking neither PSL nor DMARDs. None of these patients with early RA were included in the analysis of T cells in the joints, but their PBMC did not show an apparent increase of IL17+ cells (mean (SD) percentage 1.6 (1.3)). The long disease duration also makes it difficult to analyse the association between ongoing bone destruction and Th17, as many of the patients had already reached an advanced stage of joint destruction. Thus, we could not conclude whether Th17 is involved in joint destruction or not from the data of the present study. It is important to further examine the involvement of IL17-producing T cells in early RA. Nevertheless, the present study at least provides a suggestion that targeting IL17 may not be effective in established stages of RA.

Involvement of IL17 has not only been demonstrated in autoimmune inflammation but also in host defence against various pathogens including *Klebsiella pneumoniae*, *Candida albicans* and *Mycobacterium tuberculosis*.[37–39] We have also found IL17 plays a critical role in host defence against *Escherichia coli*.[40] Because blocking IL17 in humans might also induce an immunocompromised state, it is necessary to carefully investigate the involvement of IL17 in the pathogenesis of RA before clinical application of IL17-targeting therapy.

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Competing interests: None.

Ethics approval: The study protocol was approved by Regional Committee of Ethics for Human Research at Faculty of Medicine of Kyushu University. All subjects signed informed consent before participation in the study.

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