A Distinct Human CD4+ T cell Subset That Secretes CXCL13 in Rheumatoid Synovium

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Objective. Human CD4+ T cells in synovitis of rheumatoid arthritis (RA) produce CXCL13, a chemokine crucial for the formation of germinal center. This study was undertaken to determine the relevance of this population to known subsets of helper T cells and to proinflammatory cytokines and how these cells are generated.

Methods. The expression of Th markers and CXCL13 in CD4+ T cells of RA synovitis and the involvement of proinflammatory cytokines in CXCL13 production were assessed. We also investigated whether CXCL13+CD4+ T cells could be newly induced.

Results. CXCL13+CD4+ T cells of RA synovitis were negative for interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-17, Foxp3, and CXCR5, and expressed low levels of ICOS, indicating that this population is a distinct human CD4 subset. T cell receptor (TCR) stimulation of CD4+ T cells from RA synovitis with low expression of CXCL13, promptly induced CXCL13 production, and addition of proinflammatory cytokines supported the long-term production of CXCL13, indicating that CXCL13-producing CD4+ T cells can be in a memory state ready to be reactivated upon TCR stimulation, and that proinflammatory cytokines are involved in persistent CXCL13 production. TCR stimulation of blood CD4+ T cells from healthy volunteers together with proinflammatory cytokines induced a population that produced CXCL13 but not IFN-γ.

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Synovial T cells recruited CXCR5+ cells in a CXCL13-dependent manner.

Conclusion. CXCL13-producing CD4+ T cells induced in RA synovitis may play a role in

the recruitment of CXCR5+ cells, such as B cells and circulating Tfh cells, and in ectopic

lymphoid neogenesis in inflammatory sites.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by proliferative synovitis with massive infiltration of lymphocytes, leading to destruction of cartilage, bone, and the joint structure (1). Immunogenetic studies have shown that genetic variations regarding T-cell-mediated immunity such as *HLA-DRB1* shared epitope, *PTPN22*, and *CTLA4* alleles are associated with RA (2-5), and T-cell-targeting treatments such as CTLA4-Ig are clinically effective (6). These findings strongly suggest a key role of T cells in the pathogenesis of RA. Animal models have shown that T helper 1 (Th1) cells and T helper 17 (Th17) cells play crucial roles in various autoimmune diseases (7-9). However, in rodent models of arthritis, deficiency in the interferon-γ (IFN-γ) receptor exacerbates the disease (10), and anti-IFN-γ treatment produces an insufficient clinical effect in RA patients (11). Although recent reports indicate a definite role of IL-17 in the pathogenesis of autoimmune arthritis in both animal models and human (9, 12, 13), the contribution of Th17 cells to the pathogenesis of human RA is still controversial. Increased Th17 cell number is not obvious in RA patients (14), and mast cells are considered the main source of human IL-17 rather than CD4+ T cells in RA synovial tissues (15, 16). Taken together, previous findings imply that a CD4+ T cell subset other than Th1 or Th17 cells may also contribute to the pathogenesis of RA.

One striking feature of RA is that synovitis is accompanied by ectopic lymphoid

structures (17, 18). The chemokine CXCL13 plays a crucial role in the formation of germinal center (GC) (19), and it has been reported that CXCL13 expression is elevated markedly in human RA synovitis (20). In mice, CXCL13 is produced mainly by stromal cells such as follicular dendritic cells (FDCs) in the secondary lymphoid organs (SLO)(21) but not by T cells such as follicular helper T (Tfh) cells in the GC (22). In 2008, Manzo et al. showed that CD4+ T cells of RA synovitis appear to produce CXCL13 spontaneously, and stimulation with anti-CD3/CD28 antibodies enhances ongoing CXCL13 production (23). Although these cells were shown to be negative for CXCR5 and BCL6, both hallmarks of Tfh cells, further relevance to known Th subsets such as Th1, Th17 or Tfh cell has been still unclear. Indeed, reports have shown that some subsets of human CD4+ T cells express CXCL13. Tonsillar CXCR5+ Tfh cells, which are positive for BCL6 as a nuclear marker of Tfh, express CXCL13 (24). Another study showed that human Th17 clones preferentially produce CXCL13 (25).

Here we show that CXCL13-producing CD4+ T cells in RA synovitis are a CD4 subset distinct from Th1, Th2, Th17, CXCR5+ Tfh, and Treg cells, and that proinflammatory cytokines and TCR stimulation are involved in long-term production of CXCL13 by this subset. Furthermore, T cell receptor (TCR) stimulation of blood CD4+ T cells from healthy volunteers together with proinflammatory cytokines

obviously induced a population of CD4+ T cells that produce CXCL13 but not IFN-γ.

PATIENTS AND METHODS

Preparation of specimens. Ethical approval for this study was granted by the ethics committee of Kyoto University Graduate School and Faculty of Medicine. Informed consent was obtained from all study participants. RA was diagnosed according to the American College of Rheumatology 1987 criteria (26). The characteristics of the RA patients are shown in Supplementary Table 1. Joint specimens were obtained during joint surgery or from an outpatient clinic. Tonsils were obtained from patients having a tonsillectomy because of tonsillar hypertrophy without acute inflammation. Synovial tissues and tonsils were minced and digested with 2.5 mg/ml collagenase (Roche) at 37°C for 1.5 h and then analyzed.

Peripheral blood mononuclear cells (PBMCs) or synovial fluid mononuclear cells (SFMCs) were collected using Lymphocyte Separation Solution 1.077 (Nacalai Tesque). CD4+ T cells were sorted with a CD4+ T Cell isolation kit (Miltenyi Biotec). The purity of sorted CD4+ T cells was more than 95%.

Flow cytometry. For intracellular staining of cytokines and chemokines, cells

were cultured for 5 h in complete RPMI 1640 (Life Technologies) supplemented with

10% FCS (Sigma-Aldrich), 55 μM mercaptoethanol (Life Technologies), 5 mM HEPES

(Life Technologies), and 100 unit/ml penicillin and streptomycin (Life Technologies)

containing 4 mM monensin (Sigma-Aldrich) in the presence or absence of phorbol

12-myristate 13-acetate (PMA) (Nacalai Tesque) and ionomycin (Nacalai Tesque).

Because stimulation with PMA and ionomycin reduced the production of CXCL13, we

optimized the concentrations of PMA and ionomycin to detect the production of CXCL13,

IFN-γ, and IL-17 simultaneously (see Supplementary Figure 1), and 0.3 ng/ml PMA and

0.5 μM ionomycin were used for stimulation unless otherwise mentioned. After staining

of surface molecules, cells were fixed with 4% paraformaldehyde, permeabilized with

0.5% saponin, and stained with anti-cytokine/chemokine antibody for 20 min at room

temperature. For intracellular staining of transcriptional factors, FOXP3 Fix/Perm

Buffer (BioLegend) and FOXP3 Perm Buffer (BioLegend) were used according to the

manufacturer's protocol after stimulation with PMA–ionomycin. The details of the

antibodies used in this study are shown in Supplementary Table 2. The data were

acquired with a FACSCanto II flow cytometer (BD Biosciences) and were analyzed with

FlowJo (Tree Star). Fixable Viability Dye eFluor 506 (eBioscience) was used to exclude

dead cells. The diversity of TCR-Vβ was analyzed with a TCR-Vβ Repertoire Kit

(Beckman Coulter).

Immunohistofluorescence. Frozen sections of RA synovial tissues were fixed with Fixation Buffer (BioLegend), permeabilized, stained with Permeabilization Wash Buffer (BioLegend) containing direct fluorescent antibodies, and mounted with Cell Staining Buffer (BioLegend). Images of stained sections were obtained with an LSM 710 confocal microscope (Carl Zeiss Japan) using a dry object lens of 20 magnifications at room temperature. ImageJ (NIH) was used to apply a gamma adjustment and Sigma filter.

Enzyme-linked immunosorbent assay (ELISA). The concentration of CXCL13 in the supernatant was measured by Human CXCL13/BLC/BCA-1 DuoSet (R&D systems) with a detection limit of 15 pg/ml.

Cell culture. Cells were cultured in 5% CO_2 in an incubator at 37°C. To analyze the maintenance of CXCL13 production, cells were cultured with complete RPMI 1640 medium in the presence or absence of recombinant human IL-6 (20 ng/ml; Wako) or recombinant human TNF-α (20 ng/ml; Miltenyi Biotec). To add TCR stimulation, cells were cultured with 5 µg/ml of plate-bound anti-CD3 antibody (eBioscience) and 10 µg/ml

of soluble anti-CD28 antibody (eBioscience). If necessary, cells were washed with complete RPMI 1640 medium and transferred to another well to stop TCR stimulation 24 h later. Proinflammatory cytokines were supplemented again after transfer. Cells were analyzed by flow cytometry after 5 h of culture with monensin.

Chemotaxis assay. Chemotaxis was assessed in a two-chamber Transwell cell migration system (5 μm pores, Corning). Conditioned medium of SFMCs cultured with complete RPMI 1640 medium at a concentration of 1×10^6 /ml for 72h at 37°C was collected and frozen until analysis. PBMCs from healthy volunteers (1×10^6) in RPMI 1640 containing 5% FCS were seeded in the upper well, and RPMI 1640 supplemented with 50% conditioned medium in the presence of 20 μg/ml anti-CXCL13 antibody (R&D systems) or control IgG1 (R&D systems) was added to bottom well as the chemoattractant. The cells were cultured for 4 h at 37°C and then collected and stained with anti-CD3, anti-CD4, anti-CD19, and anti-CXCR5 antibodies. The migrating cells were analyzed by flow cytometry.

Statistical analysis. The data were analyzed using Student's *t* test or Mann–Whitney U test as appropriate. A p value <0.05 was considered significant.

RESULTS

PD1+ effector memory CD4+ T cells of RA synovitis preferentially produce CXCL13. Immunohistofluorescence of RA synovial tissues showed the frequent presence of ectopic lymphoid structures formed by the accumulation of T cells and B cells and CXCL13 positive T cells (Figure 1A). To investigate the expression of CXCL13 quantitatively, we enzymatically digested RA synovial tissues and conducted flow cytometric analysis. About 5% of CD3+ T cells produced CXCL13 spontaneously, whereas a subpopulation of <2% of CD3– cells including larger cells than lymphocytes were positive for CXCL13 (Figure 1B and see Supplementary Figure 2). In both synovial tissues and synovial fluid from RA patients, CD4+ T cells produced similar amount of CXCL13 (See Supplementary Figure 3), and we treated both as RA synovial CD4+ T cells, unless otherwise mentioned. Spontaneous production of CXCL13 by RA synovial CD4+ T cells and negligible presence of CXCL13+CD4+ T cells in blood of RA patients were confirmed by flow cytometry, ELISA and qRT-PCR (Figure 1C and see Supplementary Figure 4). The CXCL13-producing CD4+ T cells were restricted to effector memory cells (See Supplementary Figure 5) as previously reported (23), and had a phenotype of CD69+ and programmed death 1 (PD-1)+ (Figure 1D). Interestingly,

we were able to confirm the production of CXCL13 by CD4+ T cells for more than 1 month without exogenous stimulation (Figure 1E), indicating the stability of this population. To investigate whether CXCL13-producing CD4+ T cells of RA synovitis express skewed TCR-Vβ repertoire, we analyzed the expression of TCR-Vβ in CD4+ T cells of RA synovitis. The diversity of TCR-Vβ was similar in CXCL13+ and CXCL13– CD4+ T cells (Figure 1F). These findings indicate that CXCL13-producing CD4+ T cells of RA synovitis are a stable PD1+ effector memory population of CD4+ alpha/beta T cells.

CXCL13-producing CD4+ T cells of RA synovitis are a distinct human CD4 subset. CD4+ T cells of RA synovitis produced CXCL13 but little IFN-γ or IL-17 without exogenous stimulation (see supplementary Figure 1 upper left). To clarify the relationship of synovial CXCL13+CD4+ T cells to Th1 and Th17 cells, synovial cells were stimulated with PMA and ionomycin, and then subjected to intracellular staining. In RA synovitis, there were significantly fewer IFN-γ/CXCL13 double-positive and IL-17/CXCL13 double-positive CD4+ T cells than single-positive cells producing each of cytokine/chemokine (Figures 2A and B). This indicates that CXCL13+CD4+ T cells formed a CD4+ T cell population distinct from T cells that produce IFN-γ or IL-17. T-bet was expressed in IFN-γ-producing Th1 cells but not in CXCL13+CD4+ T cells (Figure

2D). Although obvious detection and separation of RORγt in synovial CD4+ T cells was technically difficult (Figure 2E), these results collectively indicate that synovial CXCL13+CD4+ T cells are distinct from Th1 and Th17 cells. To investigate the relevance of these cells to Th2 and Treg cells, we also analyzed the expression of IL-4, GATA3, CD25, or Foxp3. CXCL13+CD4+ T cells of RA synovitis were negative for these markers (Figures 2C, F and G), indicating that they are not Th2 or Treg cells.

It has been reported that human CXCR5+ Tfh cells produce CXCL13 (24). Tfh cells relocate to the B cell zone by expressing CXCR5, leading to the formation of GC and establishment of long-lived memory B and plasma cells. Expression of ICOS, BCL6, PD-1, and IL-21 are also functionally crucial hallmarks of human Tfh cells (27-29). Especially, ICOS deficient humans have profound deficits in antibody production, class switch, GC formation, and the presence of circulating CXCR5+CD45RO+CD4+ T cells (27). Synovial CXCL13+CD4+ T cells are similar to Tfh cells in that both cell types express high levels of PD-1 (Figure 1D) (30) but produce little IFN-γ, IL-4, and IL-17 (Figures 2A−C) (24, 29). However, synovial CXCL13+CD4+ T cells were negative for CXCR5 expression (Figure 3A) and expressed less BCL6 (Figure 3G) as previously reported (23), and had lower expression of ICOS compared with tonsil CXCR5+ Tfh cells (Figures 3B and C). The expression pattern of CXCR5 and CXCL13 also differed

between CD4+ T cells from the tonsil and RA synovitis (Figure 3A). Although IL-21 positive cells are more frequent in RA synovial CXCL13+CD4+ T cells than in tonsil CXCR5+ Tfh cells (Figures 3D and E), RA synovial CXCL13+CD4+ T cells produced as much IL-21 as did synovial Th1 cells (Figure 3F), indicating that CXCL13+CD4+ T cells exert a significant level of B helper activity but have no specialized commitment to this activity compared to Th1 cells in the same environment. Taken together, these data indicate that CXCL13-producing CD4+ T cells are enriched in RA synovitis and comprise a CD4 subset distinct from Th1, Th2, Th17, Treg, and CXCR5+ Tfh cells. Their main role might be in the persistent production of CXCL13, which appears to promote ectopic formation of lymphoid structures in RA synovial tissues.

TCR stimulation reactivates CXCL13-producing CD4+ T cells in a memory state and proinflammatory cytokines support the long-term production of CXCL13. The obvious clinical results of anti-TNF (31) and anti-IL-6 receptor (32) treatment for RA support the role of TNF and IL-6 in RA pathogenesis. The presence of proinflammatory cytokines appeared to maintain the frequency of CXCL13-producing CD4+ T cells. Each of IL-6 and TNF-α had similarly contributed the maintenance of CXCL13 production and the combination of both cytokines enhanced the effect (Figure 4A). CXCL13 production differed between joints, and some synovial CD4+ T cells showed low

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spontaneous production of CXCL13 (Figure 1B). We hypothesized that a subset of cells

remains ready to produce CXCL13 upon TCR stimulation as memory cells. To verify this

hypothesis, we sorted whole CD4+ T cells expressing low CXCL13 from RA synovitis.

We stimulated the cells with anti-CD3/CD28 antibodies and proinflammatory cytokines,

and then analyzed the expression of CXCL13 and IFN-γ (Figure 4B). CD3/CD28

stimulation of the cells for the first 24 h promptly induced the production of both IFN-γ

and CXCL13, but IFN-γ production stopped within 48 h even in the presence of both

IL-6 and TNF-α. By contrast, CXCL13 production increased gradually for 2 days

(Figure 4B, top and middle panels). These results indicate that CXCL13-producing cells

can be in a memory state ready to produce CXCL13 upon TCR stimulation and that

production of CXCL13 is more persistent than that of IFN-γ. CXCL13 production was

maintained for 7 days by addition of proinflammatory cytokines without further TCR

stimulation (Figure 4B, middle), indicating that proinflammatory cytokines play an

important role in supporting the long-term production of CXCL13. The summaries of

the time course for the production of CXCL13 and IFN-γ in three RA patients are shown

in Supplementary Figure 6. Proinflammatory cytokines alone were unable to induce

significant expression of CXCL13 (Figure 4B, bottom). Conversely, continuous TCR

stimulation maintained production of both CXCL13 and IFN-γ regardless of the

presence or absence of IL-6 and TNF-α (Figure 4C). The low expression of IFN-γ in RA

(33) suggests that the persistent production of CXCL13 in RA synovitis may be

attributed to the effect of proinflammatory cytokines. Lower production of CXCL13 by

synovial CD4+ T cells from RA patients with ESR less than 30 mm/h or with low disease

activities (DAS28 <3.2) (see Supplementary Figure 7) supports these findings, while

treatment with DMARDs or biologics had no correlation with CXCL13 production.

TCR stimulation together with proinflammatory cytokines induces CXCL13-producing CD4+ T cells. We investigated whether CXCL13-producing CD4+ T

cells can be induced from blood CD4+ T cells. To our surprise, TCR stimulation of blood

CD4+ T cells from healthy volunteers induced a small but obvious population of CD4+ T

cells that produced CXCL13 but not IFN-γ on day 7 (Figure 5). Addition of

proinflammatory cytokines to TCR stimulation significantly induced more

CXCL13-producing CD4+ T cells than did TCR stimulation alone (Figure 5). The

absence of prompt production of CXCL13 during the first 2 days suggest that

CXCL13+CD4+ cells were newly formed during 7 days. Thus, proinflammatory

cytokines together with TCR signaling are involved in this unique mode of CXCL13

production and in the formation of this CD4 subset.

CXCL13-producing CD4+ T cells contribute to the recruitment of CXCR5+ cells.

Given the low expression of Th cytokines (Figures 2A–C) and Tfh functional markers (Figures 3A and B), by producing CXCL13, CXCL13-producing CD4+ T cells of RA may play a role mainly in recruiting CXCR5+ cells such as B cells and Tfh cells to sites of inflammation. To examine this possibility, we investigated whether CXCR5+ cells could migrate to the conditioned medium in a CXCL13-dependent manner. Because of limited cell number of patient samples, we used the conditioned medium of SFMCs instead of sorted CD4+ T cells after the confirmation that T cells were the main source of CXCL13 in the sample (Figure 6A). Conditioned medium recruited B cells and CXCR5+CD4+ T cells (Figures 6B and C) in a CXCL13 dependent manner.   CXCL13-producing CD4+ T cells may modify the local chemokine environment coordinately with stromal cells to recruit CXCR5+ naïve B cells and CXCR5+ blood CD4+ T cells containing circulating Tfh cells (34), which contribute to the generation of ectopic tertiary lymphoid organs (TLO) in RA synovial tissues, leading to the production of autoantibodies in TLO (18, 35) and persistent proliferating synovitis. Lower production of CXCL13 by synovial CD4+ T cells from RA patients negative for rheumatoid factor (see Supplementary Figure 7) may support this.

DISCUSSION

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Accumulating clinical reports indicate that proinflammatory cytokines play a crucial role in the pathogenesis of RA (31, 32). However, it has been unclear how CD4+ T cells contribute to the persistent inflammation of synovitis, how proinflammatory cytokines affect CD4+ T cells of RA, and the position of CXCL13-producing CD4+ T cells within Th subsets. In this study, we demonstrated that CXCL13-producing CD4+ T cells of RA synovitis form a distinct CD4 subset and that proinflammatory cytokines are involved in both the persistent CXCL13 production and the generation of this subset. We designate the T cell subset as inflammatory CXCL13-producing helper T (iTh13) cells.

CD4 subsets such as Th1, Th2, and Th17 cells produce cytokines promptly upon TCR stimulation. We demonstrated that the CXCX13-producing CD4 subset also can be in a memory state ready to produce CXCL13 upon TCR stimulation and that proinflammatory cytokines support their long-term production of CXCL13 (Figure 4B). By contrast, the amount of IFN-γ is relatively small (33), and mast cells rather than CD4+ T cells are a main source of IL-17 in RA synovitis (15). Taken together, these findings suggest that, by producing CXCL13 persistently with the support of proinflammatory cytokines, CD4+ T cells contribute at least partly to the chronic inflammation of RA synovitis.

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CXCL13 is expressed in ectopic lymphoid structures including islets of NOD

mice, the aorta of ApoE deficient mice, and synovial tissues of RA patients (23, 36, 37),

and the ectopic expression of CXCL13 is sufficient to generate ectopic lymphoid

structures (38). While medial smooth muscle cells produce CXCL13 in ApoE deficient

mice (37), histologic analysis previously showed that both stromal and T cells produce

CXCL13 (23). In our study, flow cytometric analysis of single cell suspension of RA

synovial tissues also showed that both cells produce CXCL13 and that PD1+ effector

memory CD4+ T cells of RA synovitis preferentially produce CXCL13 (Figure 1). As a

distinct CD4 subset, iTh13 cells may play a role coordinately with stromal cells in the

modification of the local chemokine environment to recruit CXCR5+ cells such as B cells

and circulating Tfh cells to the sites of inflammation, followed by ectopic lymphoid

neogenesis (see Supplementary Figure 8). In other inflammatory conditions such as

infection, antigens and proinflammatory cytokines may also induce iTh13 cells at the

sites of inflammation where they may modify the local chemokine environment to

induce TLO by producing CXCL13. After clearance of the infection, disappearance of

proinflammatory cytokines and antigens should lead to the cessation of CXCL13

production by iTh13 cells and to the disappearance of TLO. Thus, iTh13 cells and

CXCL13 may play a crucial role in the generation of TLO at sites of inflammation. In

RA synovial tissues, existing proinflammatory cytokines could support persistent

CXCL13 production and ectopic lymphoid structures.

Because both Tfh and iTh13 cells have similarity in expression of CXCL13,

IL-21 and PD-1, their differentiation and function need to be further determined. Tfh

and iTh13 cells might share their function or the process of development to some extent

as well as Th1 and Tfh cells share the process of differentiation in the early phase (39).

However, expression of CXCR5 is crucial for the localization of T cells in the germinal

center of SLO. Recently, CXCR5+Foxp3+ follicular regulatory T (Tfr) cells were shown

to localize in the germinal center of SLO and to inhibit humoral immunity more

effectively compared to conventional CXCR5– Treg cells. Therefore, iTh13 cells, which

are negative for CXCR5, are considered to localize and exert B cell helper activity in

SLO less effectively than CXCR5+ Tfh cells.

Various studies demonstrate that B cells contribute to the production of

autoantibodies in the ectopic lymphoid structure of RA synovial tissues (35, 40).

Presumably, infiltrating CXCR5+ Tfh cells support the differentiation of B cells. It

remains to be determined whether iTh13 cells can also affect synovial cells such as

dendritic cells, macrophages, and fibroblast-like synoviocytes via CXCL13 or other

molecules specific for iTh13. At minimum, the persistent recruitment of CXCR5+ cells

by RA iTh13 cells should contribute to the maintenance or enlargement of ectopic

lymphoid structures and surrounding synovial tissues as a TLO.

In conclusion, our data, together with previous findings, indicate that iTh13

cells, a distinct CD4 subset, with the support of proinflammatory cytokines persistently

recruit CXCR5+ cells into sites of inflammation, leading to the maintenance of TLO and

chronic inflammation. Further investigation of iTh13 cells, including their roles in

chronic inflammatory diseases, their regulatory or specific genes, and the mechanisms

underlying their differentiation, may lead to alternative treatments for chronic

inflammatory diseases including RA and new insights into human immunity.

Figure legends

Figure 1. Spontaneous production of CXCL13 by CD4+ T cells in RA synovitis. **A,**

Immunohistofluorescence of ectopic lymphoid structures in RA synovial tissue stained

for CD20 (white), CD3 (green), and CXCL13 (red). **B,** Flow cytometric analysis of

spontaneous CXCL13 production by single cells from RA synovial tissue without

PMA–ionomycin stimulation (n=14). **C,** Spontaneous CXCL13 production by blood and

synovial CD4+ T cells from RA patients (n=10; ST:2, SFMC:8). **D,** Expression of CD69

and PD-1 by CD4+ T cells of RA synovitis. **E,** RA SFMCs were cultured with complete

RPMI and CXCL13 production was analyzed once a week. **F,** The diversity of TCR-Vβ expressed by CXCL13+ or CXCL13– CD4+ T cells of RA synovitis Numbers in quadrants show percentages of the indicated populations in **B** and **D**, and percentages within CD4+ T cells in **C** and **E**. Bars represent the mean. Representative data from three or more independent experiments are shown in **D**, **E** and **F**. ST = synovial tissue.

Figure 2. CXCL13-producing CD4+ T cells of RA synovitis are distinct from Th1, Th17, Th2 and Treg cells. **A–G** Flow cytometric analysis of synovial cells stimulated with PMA–ionomycin for 5 h. Representative dot plots gated on CD3+CD4+ cells of three or more independent experiments are shown. Summaries of the populations indicated are shown in **A** (ST: n=18, SFMC: n=6), **B** (ST: n=18, SFMC: n=6) and **C** (ST: n=2, SFMC: n=3). Numbers show percentages.

Figure 3. Comparison of CXCL13+CD4+ T cells of RA synovitis with tonsil CXCR5+ Tfh cells. Flow cytometric analysis of unsorted cells without exogenous stimulation (**A–C**) or with PMA–ionomycin stimulation (**D–G**). Summaries of the populations indicated are shown in **A** (ST: n=15, SFMC: n=2), **C** (ST: n=3, SFMC: n=2, tonsil: n=5), and **E** (ST: n=1, SFMC: n=8, tonsil: n=5) **F,** Expression of IL-21 in Th1 cells and CXCL13+CD4+ T cells

of RA synovitis (ST: n=1, SFMC: n=8). NS = not significant. Numbers show percentages.

Figure 4. Proinflammatory cytokines and TCR stimulation regulate CXCL13-producing helper T cells. **A,** RA SFMCs were cultured in the presence or absence of IL-6 and/or TNF-α for 7 days and then analyzed. Numbers show percentages within CD4+ T cells. **B** and **C,** Sorted whole CD4+ T cells with low spontaneous production of CXCL13 from RA synovitis were cultured in the presence or absence of IL-6 and TNF-α. TCR stimulation was added for the first 24 h (**B**), or for 7 days (**C**). Numbers show percentages. Representative data of three or more independent experiments are shown. Cells were analyzed without PMA-ionomycin stimulation.

Figure 5. Induction of CXCL13-producing CD4+ T cells from blood CD4+ T cells. Sorted blood CD4+ T cells of healthy volunteers were stimulated with anti-CD3/CD28 antibodies for 7 days in the presence or absence of IL-6 and TNF-α. Cells were analyzed without PMA-ionomycin. Representative dot plots and graphical summary of triplicates. Numbers show percentage. Data are shown as mean ± SEM.

Figure 6. Specific recruitment of CXCR5+ cells. **A**, Flow cytometric analysis of SFMCs

used in this analysis. Numbers show percentages. **B** and **C,** SFMCs were cultured in complete RPMI for 72 h and conditioned medium was harvested. The concentration of CXCL13 in the conditioned medium measured by ELISA was 1.4 ng/ml. PBMCs of healthy volunteers were loaded in the upper well and cultured for 4 h. Cells migrating into medium containing 50% conditioned medium with the indicated antibody were analyzed by flow cytometry. Percentage of migrating CD19+ cells among loaded cells (**B**) and percentage of migrating CXCR5+CD4+ cells among migrating CD4+ cells (**C**) are shown. Data are shown as mean ± SEM of quadruplicates.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Dr. Yoshitomi 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.

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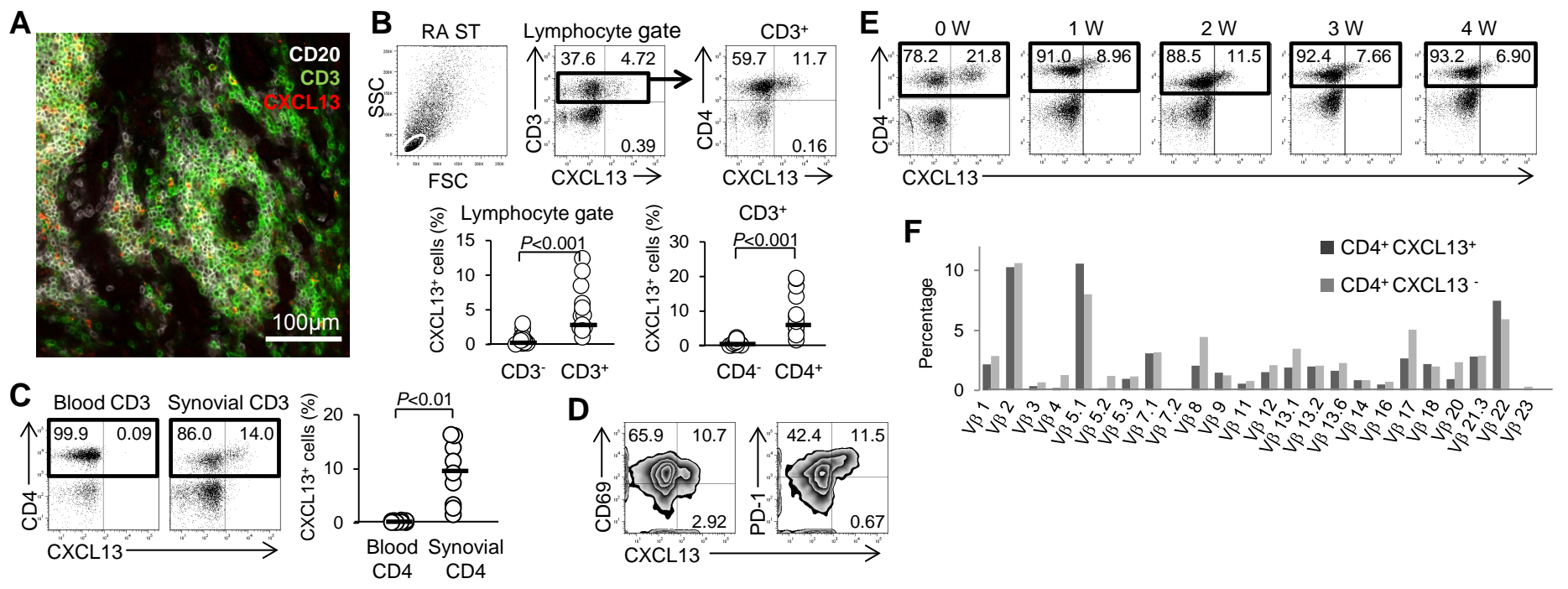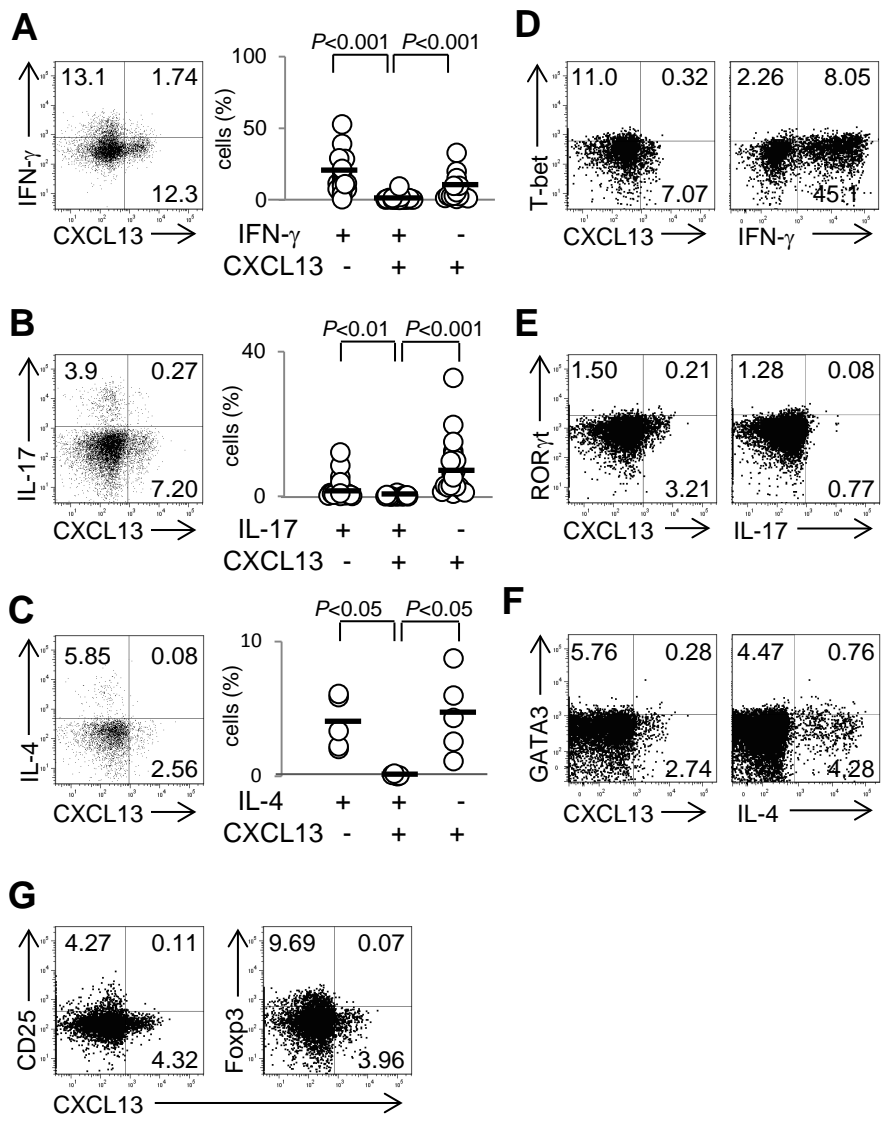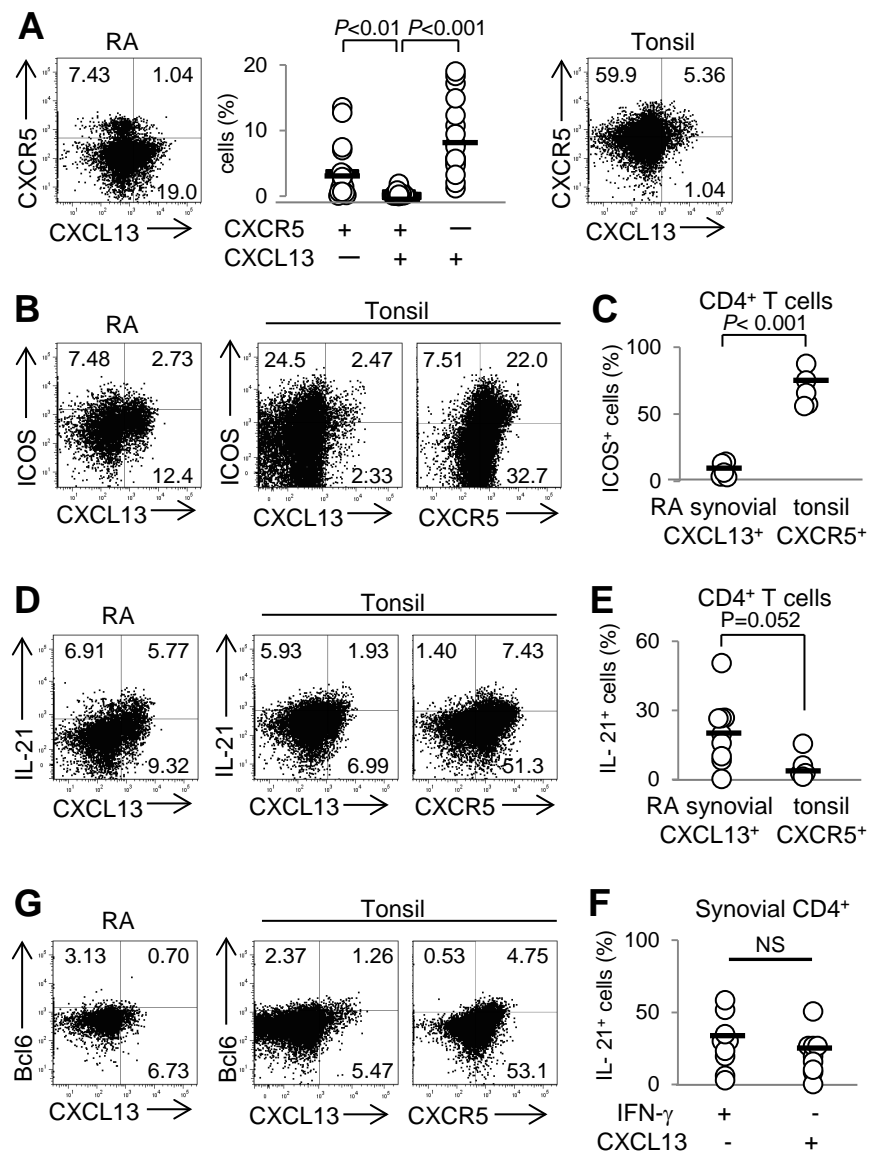
Fig1

Fig2

A
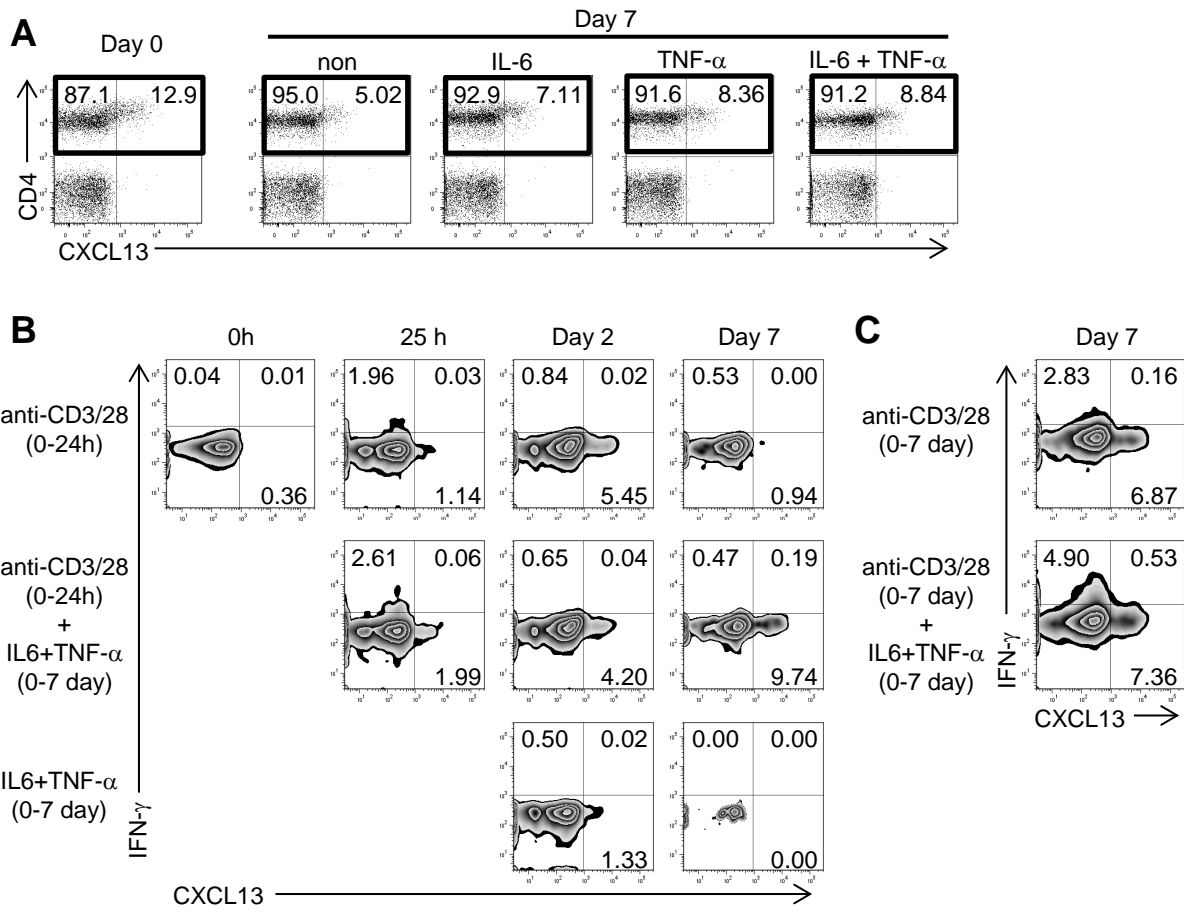
RA

CXCR5 → CXCL13

7.43 | 1.04
19.0

cells (%)

P<0.01 *P*<0.001

| CXCR5 | + | + | − |
| CXCL13 | − | + | + |

Tonsil

CXCR5 → CXCL13

59.9 | 5.36
1.04

B

RA

ICOS → CXCL13

7.48 | 2.73
12.4

Tonsil

ICOS → CXCL13

24.5 | 2.47
2.33

ICOS → CXCR5

7.51 | 22.0
32.7

C

CD4+ T cells

P< 0.001

ICOS+ cells (%)

RA synovial CXCL13+   tonsil CXCR5+

D

RA

IL-21 → CXCL13

6.91 | 5.77
9.32

Tonsil

IL-21 → CXCL13

5.93 | 1.93
6.99

IL-21 → CXCR5

1.40 | 7.43
51.3

E

CD4+ T cells

P=0.052

IL-21+ cells (%)

RA synovial CXCL13+   tonsil CXCR5+

G

RA

Bcl6 → CXCL13

3.13 | 0.70
6.73

Tonsil

Bcl6 → CXCL13

2.37 | 1.26
5.47

Bcl6 → CXCR5

0.53 | 4.75
53.1

F

Synovial CD4+

NS

IL-21+ cells (%)

| IFN-γ | + | − |
| CXCL13 | − | + |

Fig3

Fig4

Fig5

A

CD3 ↑ | 82.5 | 7.78
CXCL13 → | | 0.21

B

P<0.05

CD19⁺ cells (%)

CTL   anti-CXCL13

C

P<0.05

CD4⁺ CXCR5⁺ cells (%)

CTL   anti-CXCL13

D
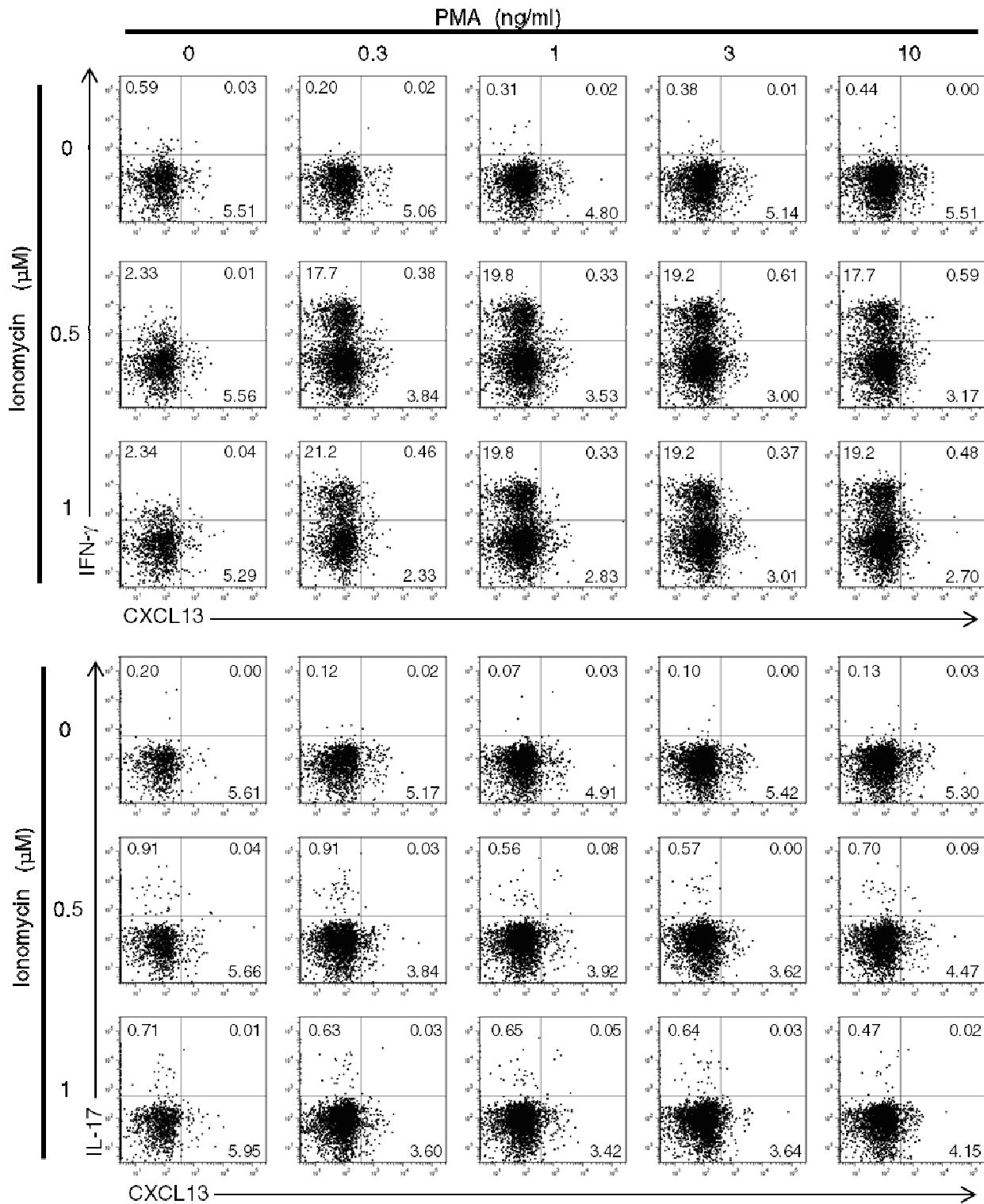
P<0.05

CD4⁺ CXCR5⁻ cells (%)

CTL   anti-CXCL13
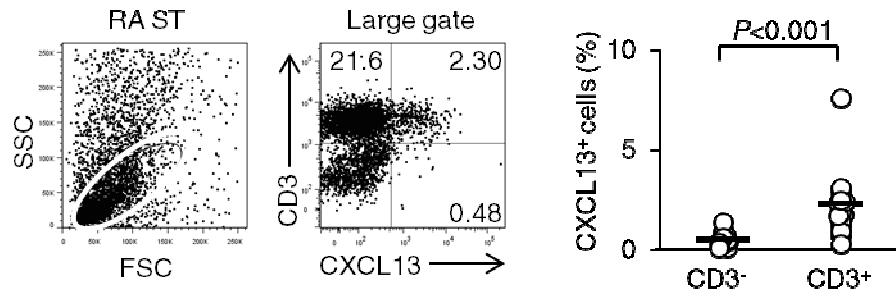
Fig6

Supplementary Figure 1. Optimization of the concentration of PMA and ionomycin to detect IFN-γ, IL-17, and CXCL13. CD4+ T cells from rheumatoid synovitis were stimulated with PMA and ionomycin at the indicated concentrations for 5 h and then analyzed by flow cytometry. Numbers in the quadrants represent the percentages of the indicated populations.

Supplementary Figure 2. Flow cytometric analysis of synovial tissues including large cells. The CXCL13 production by single cells of RA synovial tissue without PMA–ionomycin stimulation was analyzed (n=14). A representative dot plot gated on cells including large cells is shown. Numbers in the quadrants represent the percentages of the indicated populations. Bars represent the mean.
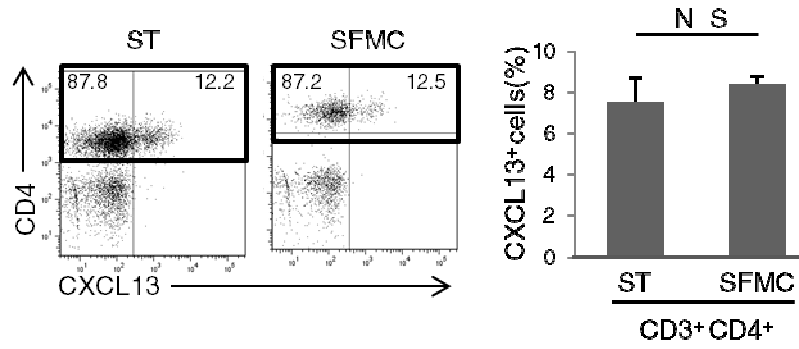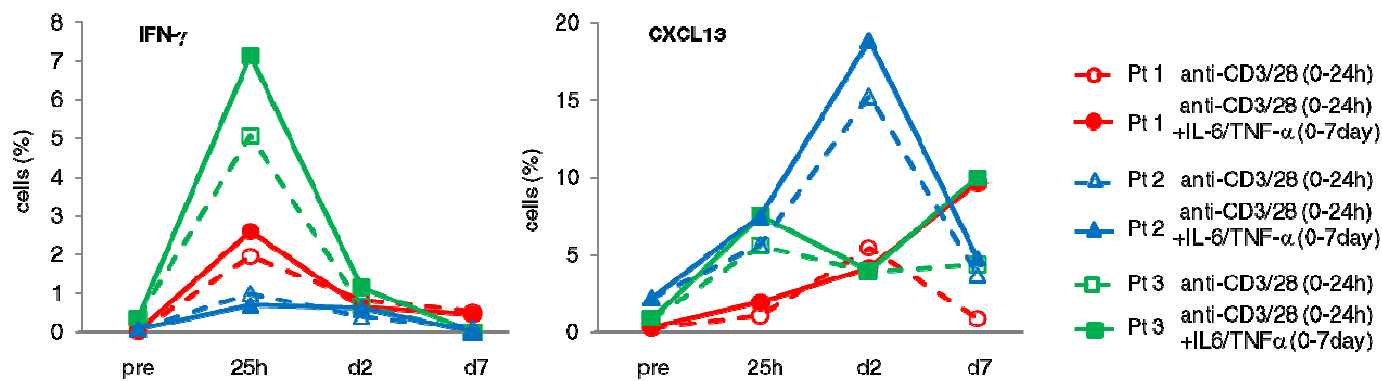
Supplementary Figure 3. Spontaneous CXCL13 production in synovial tissue (ST) and synovial fluid mononuclear cells (SFMC) of RA. CD4^{+} T cells from ST or SFMC of RA were analyzed by flow cytometry without PMA–ionomycin stimulation (n=3). Numbers indicate percentage of indicated populations in CD4^{+} T cells. NS: not significant.
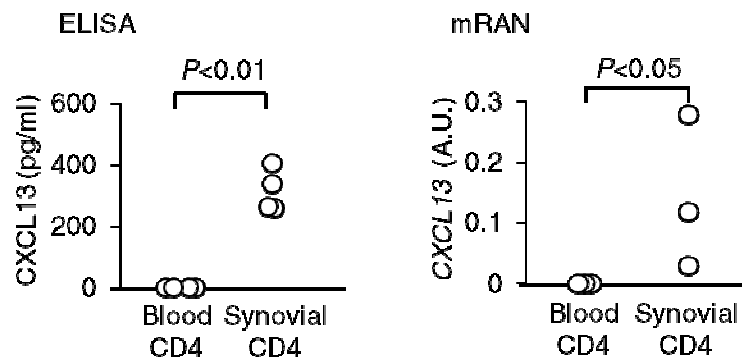
Supplementary Figure 4. Spontaneous CXCL13 production by blood and synovial CD4+ T cells from RA patients. CD4+ T cells were sorted with magnetic beads and then cultured with complete RPMI 1640 medium at a concentration of 1×10^6 /ml for 36 h at 37°C. The concentration of CXCL13 in the supernatant or synovial fluid was measured by ELISA (left; ST: n=1, SF: n=3). The expression of mRNA in sorted CD4+ T cells was quantified by qPCR using TaqMan Gene Expression Assay (Applied Biosystems); *CXCL13* (Hs00757930_m1) and *GAPDH* (Hs02758991_g1). The expression of *CXCL13* was normalized to the expression of *GAPDH* (right; ST: n=3)
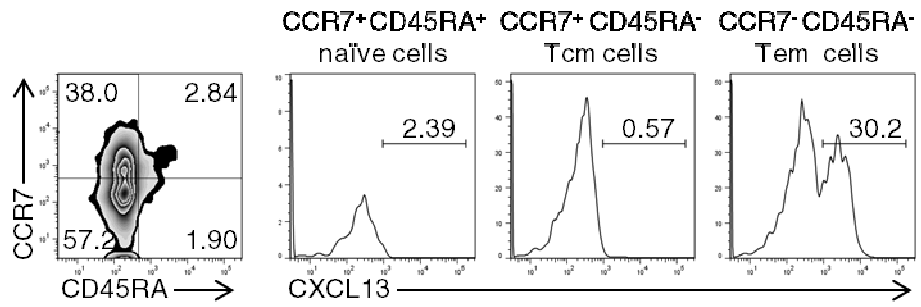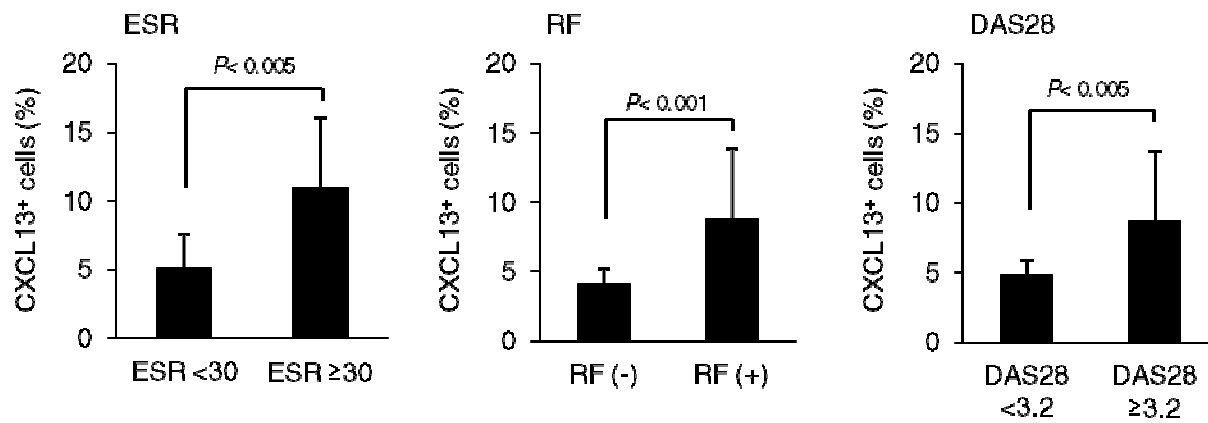
Supplementary Figure 5. CXCL13+CD4+ T cells of RA synovitis are restricted to effector memory helper T cells. Spontaneous CXCL13 production by CCR7+CD45RA+ naïve cells, CCR7+CD45RA− central memory (Tcm) cells, and CCR7−CD45RA− effector memory (Tem) CD4+ T cells is shown. Numbers in quadrants and histograms show percentages of the indicated populations. Representative data from three independent experiments are shown.

Supplementary Figure 6. The summaries of the time course for the production of CXCL13 and IFN-γ in three RA patients. Sorted whole CD4+ T cells with low spontaneous production of CXCL13 from RA synovitis were cultures as in Figure 6B. Closed symbols with solid line and Open symbols with dashed line indicate cells cultured with and without proinflammatory cytokines, respectively.

Supplementary Figure 7. Relevance between RA clinical parameters and iTH13 cells. The frequencies of CXCL13+CD4+ T cells among CD4+ T cells in patient with low ESR (<30; ST: n=10, SF: n=2), high ESR (≥30; ST: n=10, SF: n=2), negative rheumatoid factor (RF) (ST: n=2, SF: n=2), positive RF (ST: n=18, SF: n=2), low disease activity (DAS28<3.2; ST: n=4), and moderate or high disease activity (DAS28≥3.2; ST: n=16, SFMC: n=4)
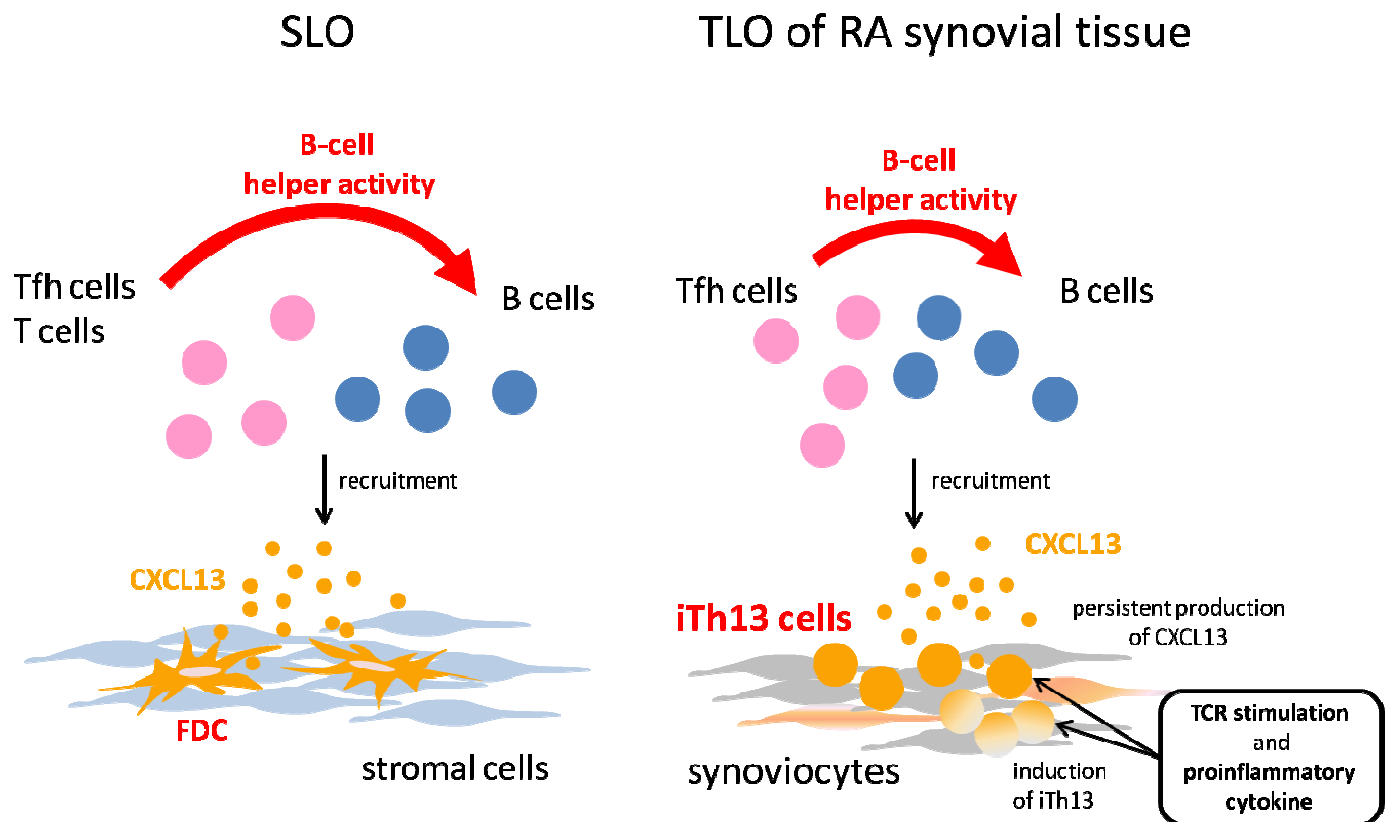
Supplementary Figure 8. Proposed role of iTh13 cell in ectopic formation of TLO at sites of inflammation.
Left: Follicular dendritic cells (FDCs) recruit CXCR5^{+} B cells and CXCR5^{+} Tfh cells to form the GC in the secondary lymphoid organs (SLO). B helper activity of Tfh cells promotes class switch, and establishment of long-lived memory B or plasma cells. (16, 17, 23-25).

Right: In the peripheral sites of inflammation such as RA synovial tissues, TCR stimulation and proinflammatory cytokines induce iTh13 cells (Figure 3D) and maintained the persistent production of CXCL13 (Figure 3, B and C). iTh13 cells modify local chemokine environment coordinately with stromal cells of synoviocytes to form TLO by recruiting CXCR5^{+} B cells and CXCR5^{+} Tfh cells (Figure 3E). Presence of TCR stimulation and proinflammatory cytokines may lead to the maintenance of TLO, to the generation of autoantibodies (15, 30), and to the enlargement of TLO and surrounding tissues.

Supplementary Table 1. Clinical details of patients with RA

Number of patients	24
Sex, male/female	7/17
Age	65.1 ± 10.5
Disease duration (y)	13.5 ± 9.24
Positive ACPA, n (%)	22 (91.7)
ESR (mm)	37.9 ± 37.0
C-reactive protein (mg/l)	1.59 ± 1.61
DAS28(ESR)	4.48 ± 1.27
Drug use, n (%)	22 (91.7%)
DMARDs	19 (79.2%)
Steroid	13 (51.2%)
Biologics	3 (12.5%)

SD = standard deviation; ACPA = anti-citrullinated protein antibody; ESR = erythrocyte sedimentation ratio; MMP3 = metalloproteinase-3; DAS28 = 28-joint Disease Activity Score; DMARDs = Disease Modifying Anti-rheumatic Drugs.

Supplementary Table 2. Anti-human antibodies used in this study

Antigen	Manufacturer	Clone	Format	Application
CD3ε	BioLegend	UCTH1	Brilliant Violet	Flow cytometry
CD3ε	BioLegend	UCTH1	PE	Immunohistofluorescence
CD4	BioLegend	RPA-T4	PerCP-Cy5.5	Flow cytometry
CD25	BioLegend	BC96	FITC	Flow cytometry
PD-1	BioLegend	EH12.2.H7	APC-Cy7	Flow cytometry
IL-4	BioLegend	8D4-8	PE	Flow cytometry
IL-17	BioLegend	BL168	PE-Cy7	Flow cytometry
RORγt	BioLegend	AFKJS-9	PE	Flow cytometry
CD19	BD Bioscience	HIB19	FITC	Flow cytometry
CD45RA	BD Bioscience	H100	FITC	Flow cytometry
T-bet	BD Bioscience	O4-46	PerCP-Cy5.5	Flow cytometry
GATA3	BD Bioscience	L50-823	PE-Cy7	Flow cytometry
Bcl-6	BD Bioscience	K112-91	PE	Flow cytometry
IFN-γ	BD Bioscience	B27	FITC	Flow cytometry
CD4	eBio Science	RPA-T4	APC-eFluor780	Flow cytometry
CD20	eBioscience	L26	Alexa488	Immunohistofluorescence
CD69	eBioscience	FN50	PE	Flow cytometry
CCR7	eBioscience	3D12	PE	Flow cytometry
ICOS	eBioscience	ISA-3	PE-Cy7	Flow cytometry
IL-21	eBioscience	3A3-N2	PE	Flow cytometry
Foxp3	eBioscience	236A/E7	PE	Flow cytometry
CXCR5	R&D Systems	51505.111	PE	Flow cytometry
CXCL13	R&D Systems	53610	APC	Flow cytometry / Immunohistofluorescence