A distinct human CD4+ T cell subset that secretes CXCL13 in rheumatoid synovitis

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2

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.

3

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.

4

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

5

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

6

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

7

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

8

(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

9

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

10

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

11

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

12

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

13

(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

14

4A). CXCL13 production differed between joints, and some synovial CD4+ T cells

showed low 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,

15

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

16

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.

17

DISCUSSION

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

18

proinflammatory cytokines, CD4+ T cells contribute at least partly to the chronic

inflammation of RA synovitis.

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

19

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

20

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

21

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

22

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.

23

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.

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

24

the integrity of the data and the accuracy of the data analysis.

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25

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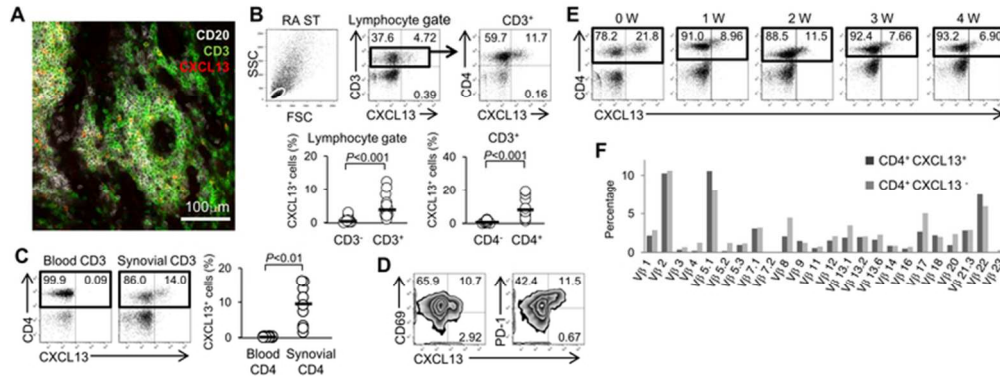
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Figure 1
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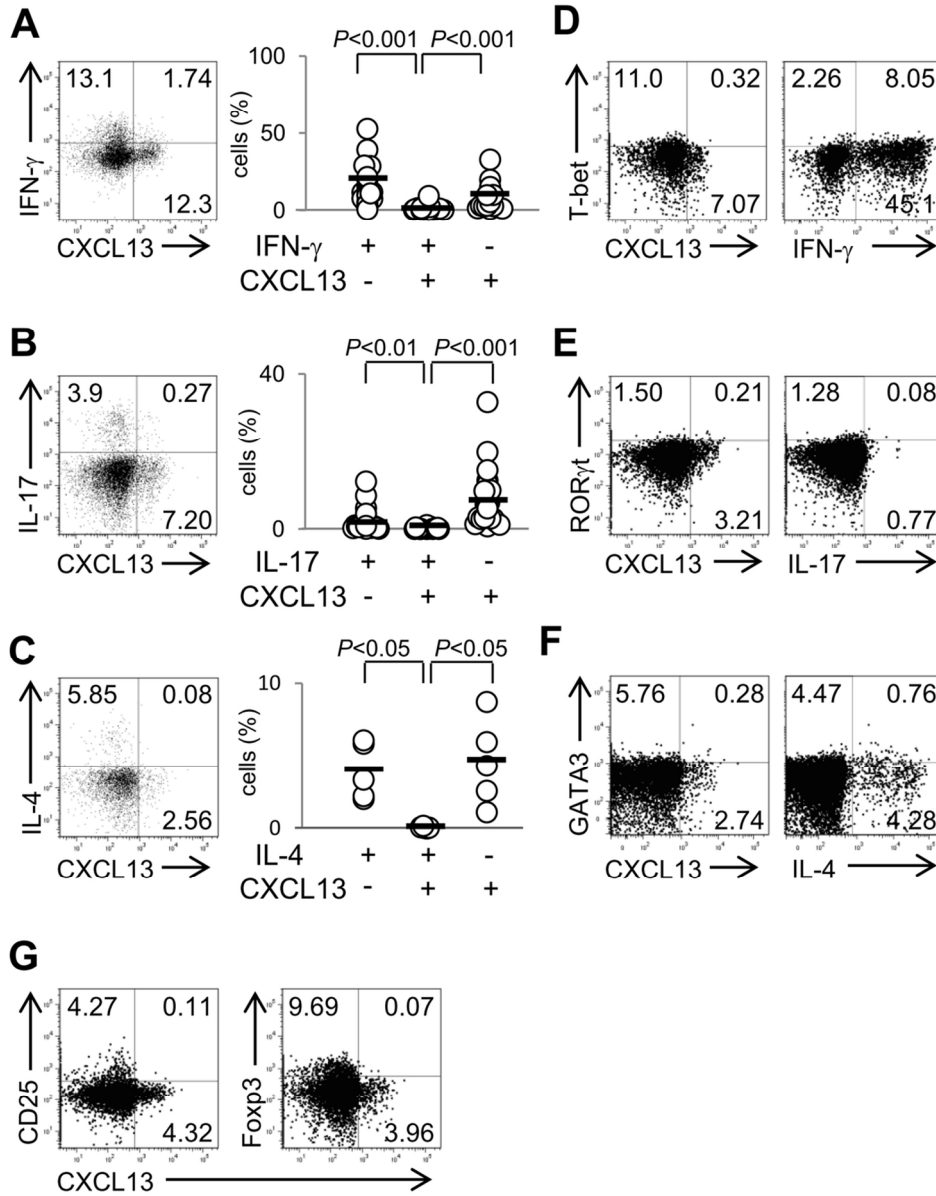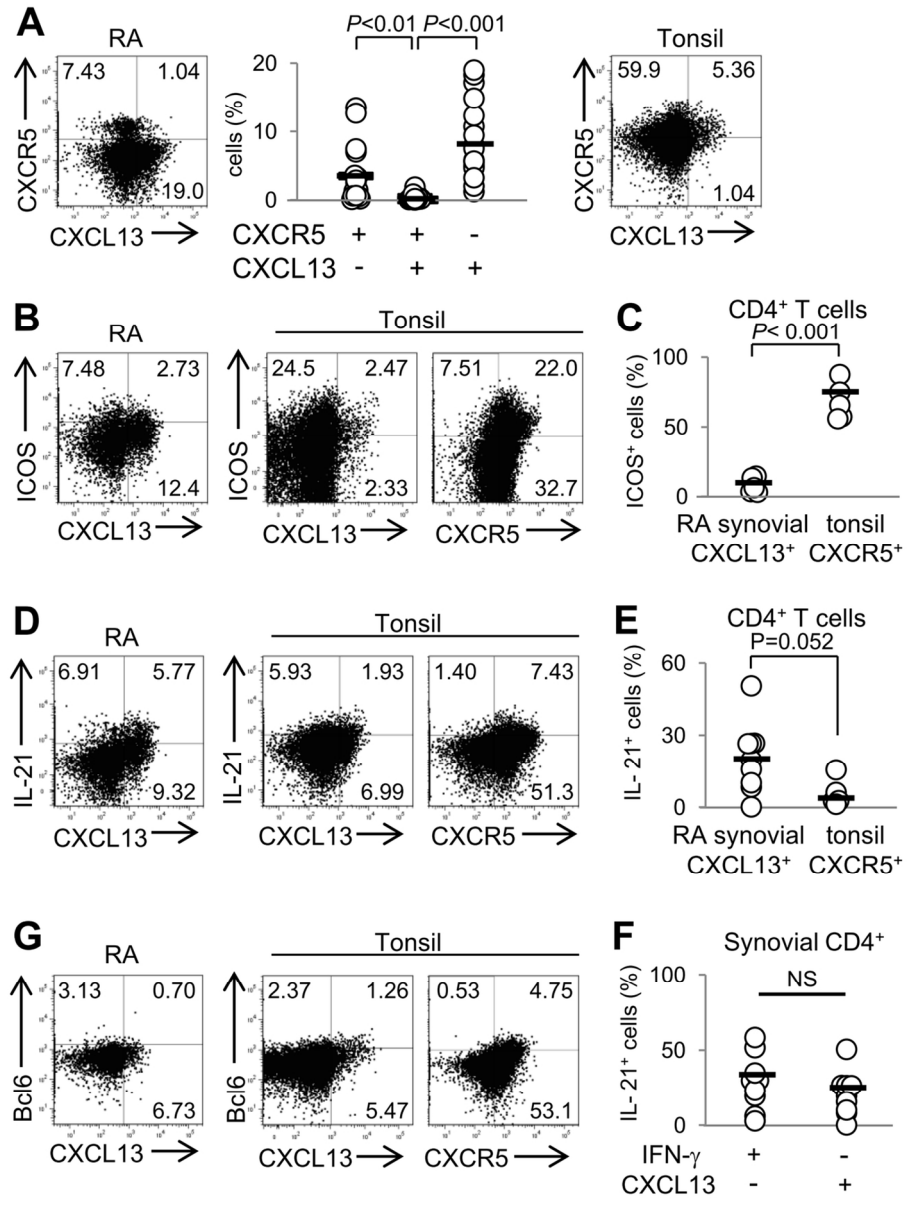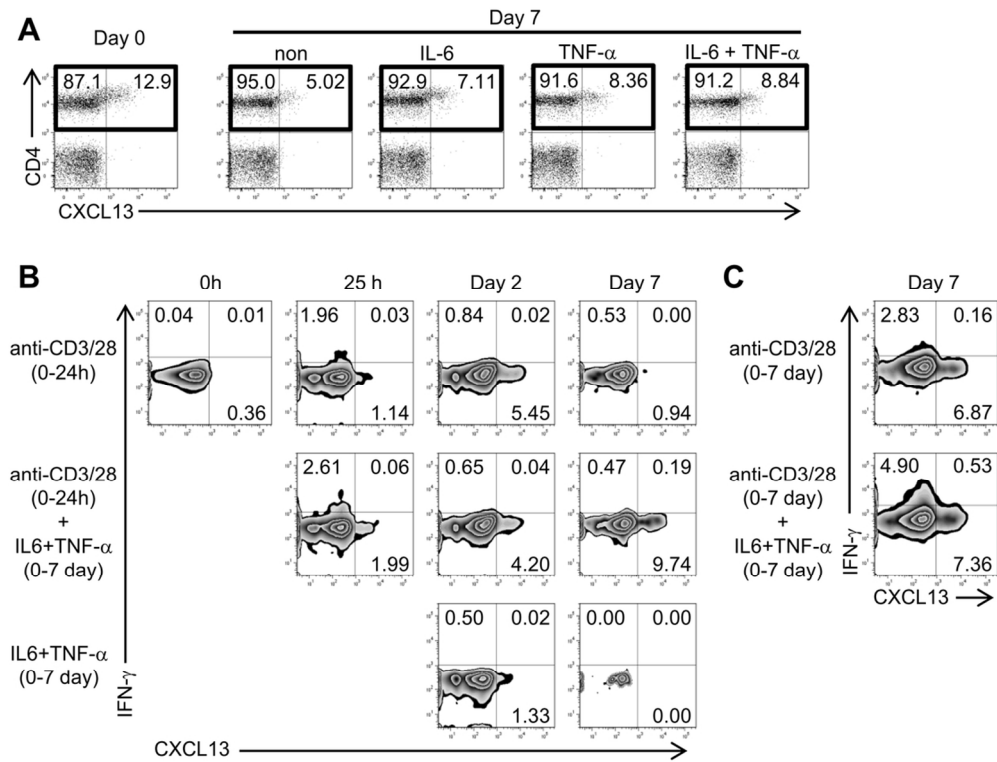
Figure 2
104x131mm (300 x 300 DPI)

Figure 3
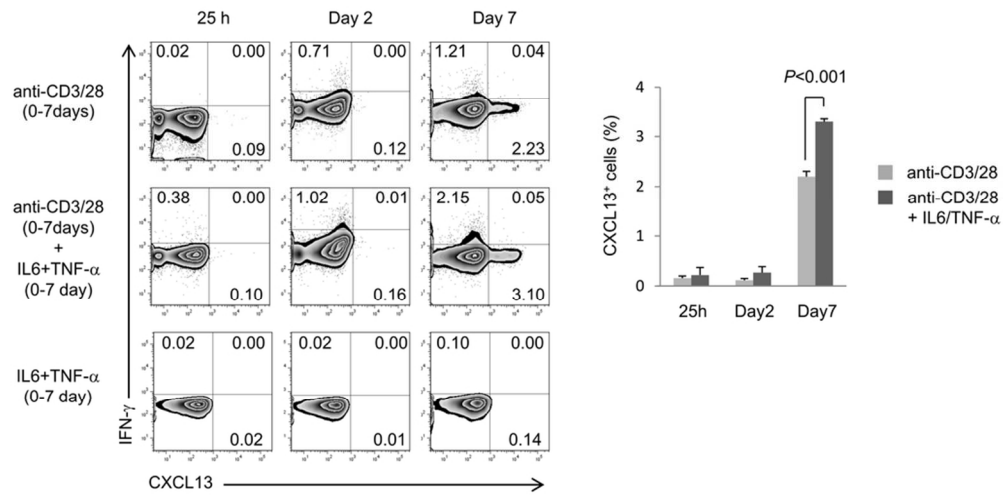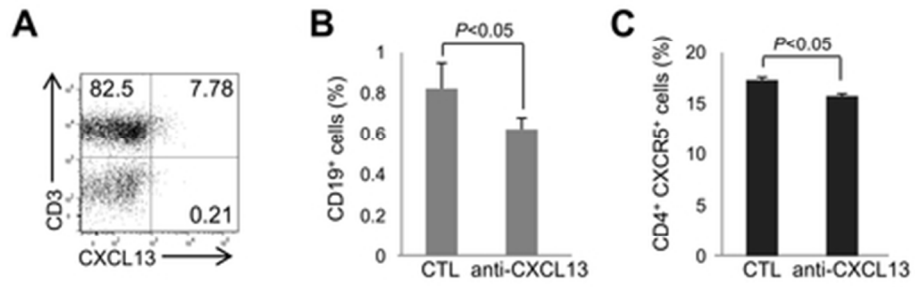111x149mm (300 x 300 DPI)

Figure 4
107x81mm (300 x 300 DPI)

Figure 5
87x43mm (300 x 300 DPI)

Figure 6
40x16mm (300 x 300 DPI)