Supplemental materials

Subsets of Human Blood CXCR5^{+} CD4^{+} T Cells

And Their Alteration in Autoimmune Disease

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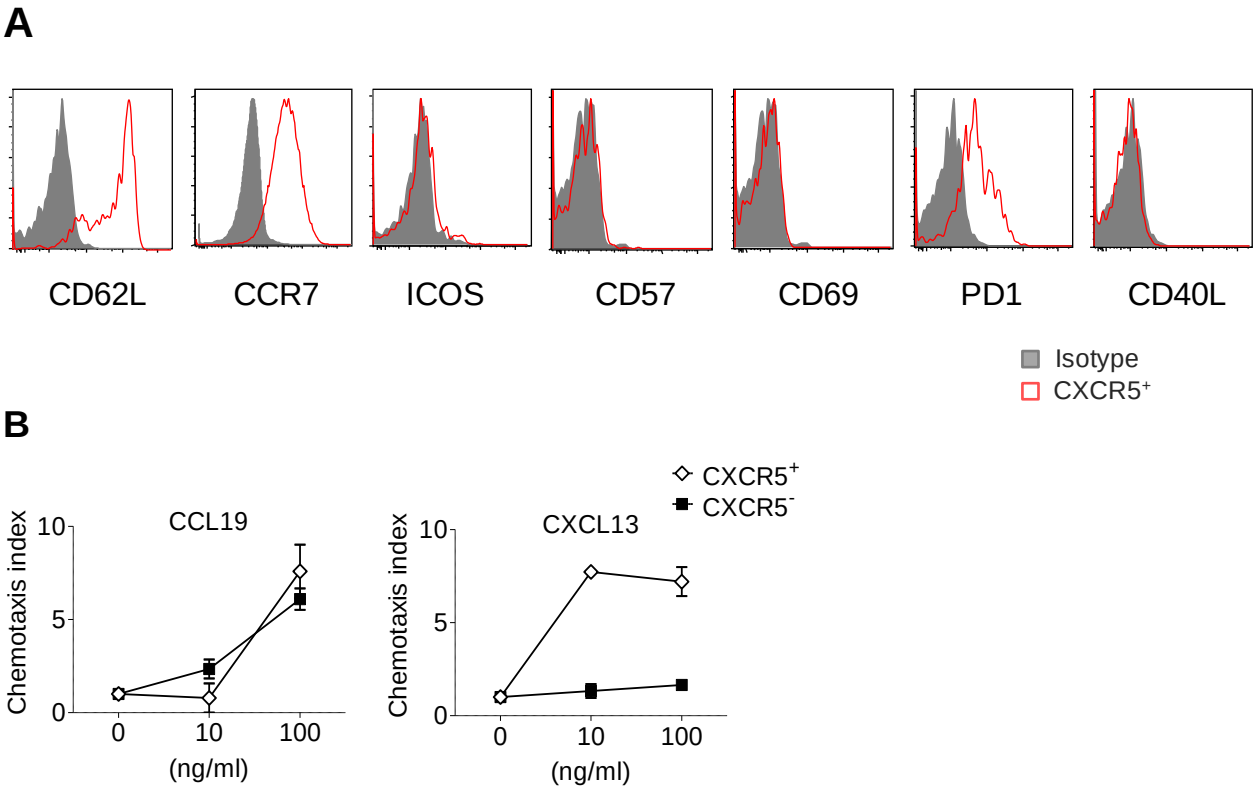
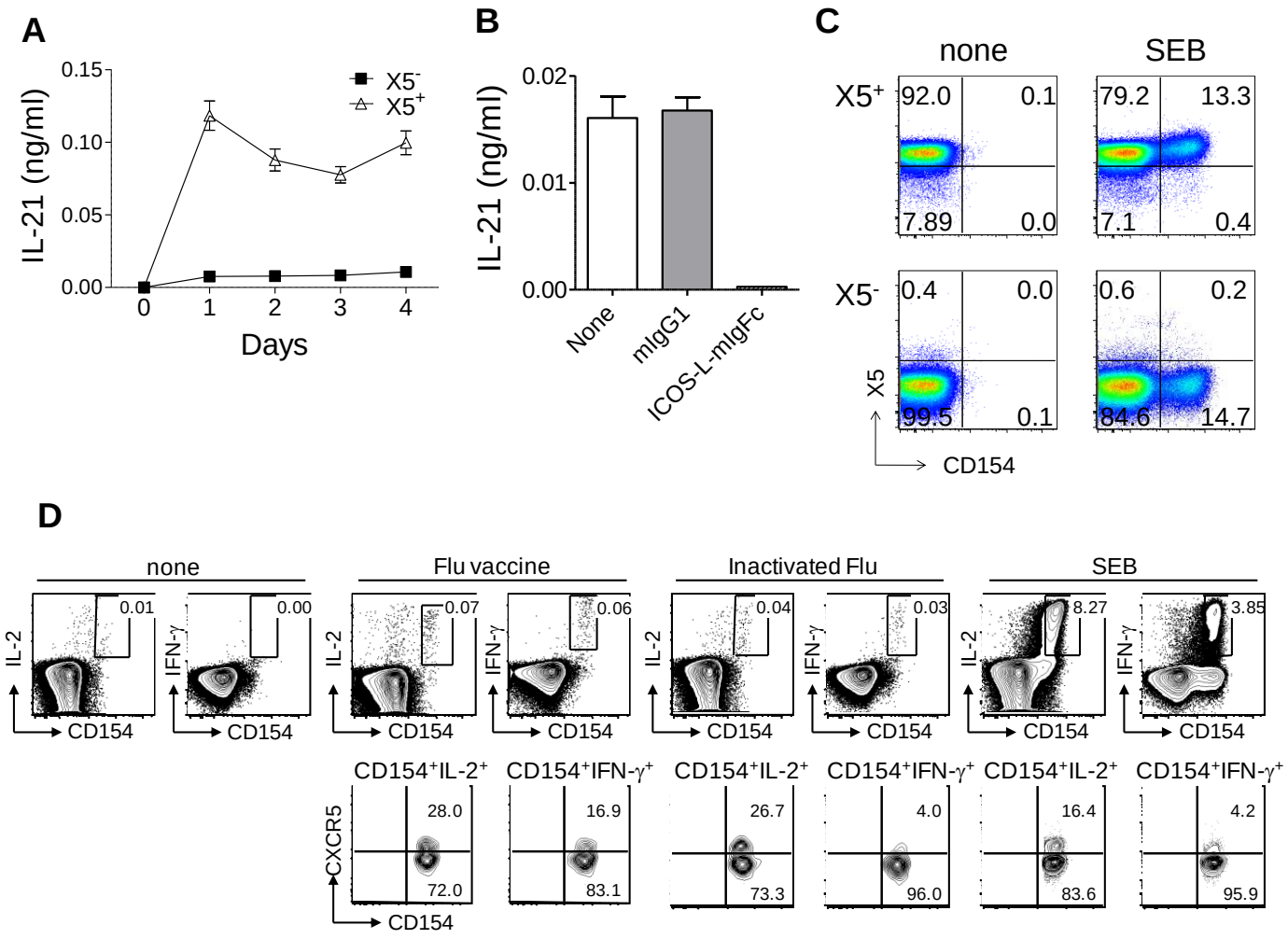
Supplemental Figure 1: Related to Figure 1



(A) Blood CXCR5 (CXCR5)$^{+}$ CD4^{+} T cells do not express activation markers. The expression of each molecule was analyzed by FACS. Gated to CD3^{+}CD4^{+}CXCR5^{+} T cells.

(B) Blood CXCR5^{+} CD4^{+} T cells migrate in response to CCL19 and CXCL13. Migration of CD4^{+} T cells was assayed by using Boyden chamber (Neuro Probe). CXCL13 or CCL19 (10 and 100 ng/ml; both from R&D Systems) was added to the lower chamber (27 μl), while CD4^{+} T cells (8.5 x 10^{5} cells/ml) were added to the upper chamber (50 μl) separated with polycarbonate filter with 5 μm pores. The chamber was incubated 2 h at 37°C, and the filters were stained with Diff-Quik solutions (Dade Behring) after the upper side was cleaned. Migrating cells were counted four fields at x 200 magnification per well. The data were expressed as chemotaxis index calculated with this formula: chemotaxis index = the number of migrating cells exposed to the chemokines / the number of cells in background. n=3, Mean ± s.d. Representative data of three experiments.

Supplemental Figure 2: Related to Figure 2



(A) Kinetics of IL-21 secretion by blood CXCR5$^+$CD4$^+$ T cells. Isolated CXCR5$^+$ and CXCR5$^-$ CD4$^+$ T cells were cultured with autologous naïve B cells in the presence of SEB. The secreted IL-21 levels were analyzed at the indicated time points. n=3, Mean ± s.d. Representative data of three independent experiments.

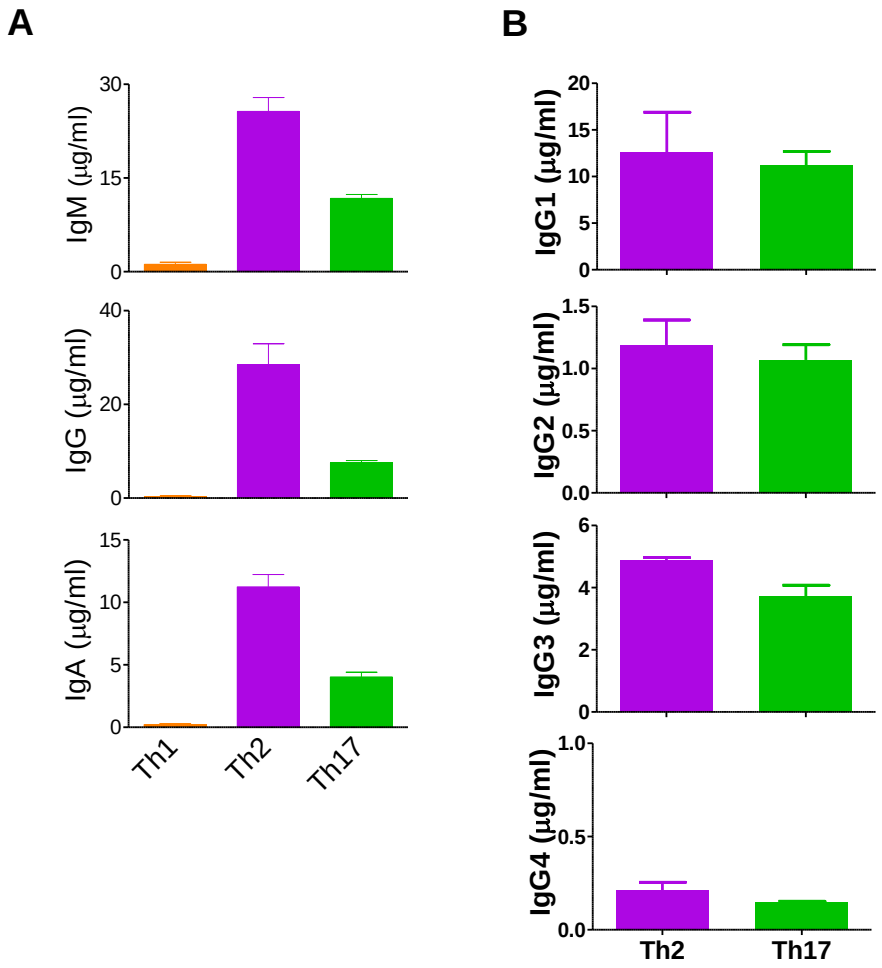
(B) Blocking ICOS inhibits IL-21 secretion by CXCR5$^+$ CD4$^+$ T cells. Blood CXCR5$^+$ CD4$^+$ T cells were cultured with SEB-pulsed naïve B cells in the presence of either control mIgG1 or ICOS-L-mIgFc. The levels of IL-21 in cultures were measured at day 2. n=3, Mean ± s.d. Representative data of three independent experiments.

(C) CXCR5$^-$ CD4$^+$ T cells do not upregulate CXCR5 expression during 6 h stimulation with SEB. Isolated CXCR5$^+$ and CXCR5$^-$ CD4$^+$ T cells were cultured with autologous monocyte pulsed with SEB (1 μg/ml) or none for 6 h. Expression of CXCR5 and

intracytoplasmic CD154 was analyzed by flow cytometry. Representative data of two experiments.

(D) Expression of CD154 and cytokines. PBMCs were stimulated with Flu vaccine or inactivated influenza virus for 6 h in the presence of Brefeldin A and monensin, and the intracytoplasmic expression of CD154, IL-2, and IFN-γ in $CXCR5^+$ or $CXCR5^-$ $CD4^+$ T cells was analyzed. Expression of CXCR5 was analyzed within the $CD4^+$ T cell population expression CD154 and cytokines (bottom). Representative data of four experiments.

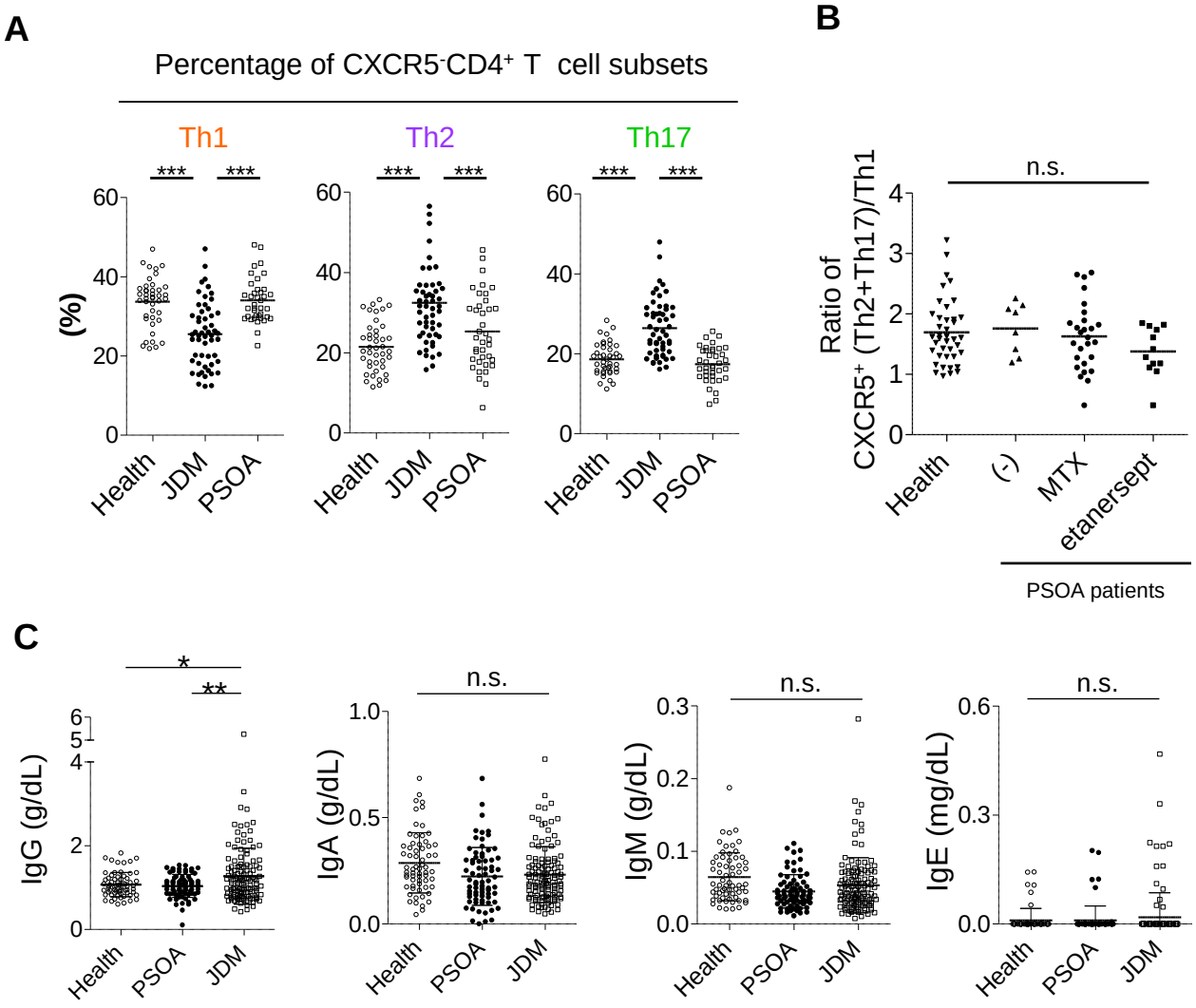
Supplemental Figure 3: Related to Figure 4



(A) CXCR5^{+} Th1 (CXCR3^{+}CCR6^{-}) cells are unable to help memory B cells to produce Igs. Th subsets within blood CXCR5^{+} Th cells were co-cultured with SEB-pulsed memory B cells (IgD^{-}CD27^{+}CD19^{+} B cells), and the produced Igs were measured at day 12. n=3-4, Mean ± s.d. Representative data of three independent experiments.

(B) Production of IgG subclasses by naïve B cells co-cultured with CXCR5^{+} Th2 and CXCR5^{+} Th17 cells. Blood CXCR5^{+} Th2 or Th17 cells were co-cultured with SEB-pulsed naive B cells, and the produced IgG subclasses were measured at day 12. n=3, Mean ± s.d. Representative data of two experiments.

Supplemental Figure 4: Related to Figure 5



(A) CXCR5$^-$ Th subsets are skewed towards Th2 and Th17 in JDM. Percentage of the within CXCR5$^-$ CD4$^+$ T cells in JDM patients, age-matched healthy controls, and PSOA patients. One way ANOVA test. *** $p<0.001$

(B) The composition of blood CXCR5$^+$ Th subsets was not altered by treatments in PSOA. Ratio of Th2+Th17/Th1 in CXCR5$^+$ CD4$^+$ T cells in healthy controls and PSOA patients receiving different therapies is shown. One way ANOVA test.

(C) JDM patients display higher levels of serum IgG. Serum Ig levels were analyzed by ELISA. One-way ANOVA. * $p<0.05$, ** $p<0.01$.

Supplemental Experimental Procedures

Blood samples

PBMCs were purified by Ficoll gradient centrifugation from apheresis blood samples obtained from adult healthy volunteers and kept frozen in 10% DMSO at -80°C. Fresh blood samples were collected also from JDM patients (10.8 ± 4.2 years old, Mean ± s.d. n=52) who fulfilled criteria of Bohan and Peter (Bohan and Peter, 1975), PSOA patients (7.7 ± 4.7 years old, n=31) and age-matched pediatric controls (n=43). Detailed clinical characteristics, clinical lab data, and treatment at the time of analysis are shown in **Table S1**. Patients were classified as active if they had systemic symptoms (skin rash and/or muscular weakness [measured by the Childhood Myositis Assessment Scale (CMAS), where the degree of increased muscular weakness is shown by lower scores with a cut-off value 48 of the full score 52]). Patients and pediatric controls were recruited at Texas Scottish Rite Hospital for Children in Dallas. The study was approved by the Institutional Review Boards (IRBs) of UT Southwestern Medical Center, Texas Scottish Rite Hospital, and Baylor Health Care System. Informed consent was obtained from parents or legal guardians.

Antigen Specificity

CFSE-labeled $CXCR5^-$ and $CXCR5^+$ memory $CD4^+$ T cells (1 x 10^5 cells/well) were cultured with autologous monocytes (1 x 10^5 cells/well) pre-incubated for 15 h with heat-inactivated influenza virus (PR8) or CMV lysate (Advanced Biotechnologies). At day 5, cells were stained with anti-CD4-PE and anti-CD14-APC, and proliferation of $CD4^+$ T cells were assessed by flow cytometry. The intracytoplasmic CD154 assay was performed according to the previous reports

(Chattopadhyay et al., 2005; Frentsch et al., 2005). Briefly, PBMCs (1 x 10^6 cells/200 μl/well) were incubated with Flu-vaccine (Fluzone) or heat-inactivated flu virus for 6 h in the presence of brefeldin A/monensin. The cells were stained with anti-CXCR5-Alexa488 (RF8B2), CD8-PE (RPA-T8), CD3-PerCP Cy5.5 (UCHT1), CD4-PE-Cy7 (RPA-T4) for cell surface molecules, and with anti-CD154-APC eF780 (24-31), IL-2-APC (5344.111), and IFN-γ-PB (4S.B3) for intracytoplasmic molecules.

Cytokine Quantification

The concentrations of IL-4, IL-5, IL-13, IL-17A, IL-21 (Schmitt et al., 2009), and IFN-γ were determined by a bead-based multiplex cytokine assay (Luminex™). Mouse monoclonal antibodies specific for IL-5, IL-13, IL-17A, and IL-21 were generated in the Institute, and conjugated to microbeads. Antibody pairs for cytokine measurement were validated for the measurement by Luminex. The concentrations of IL-22 were determined with ELISA (R&D Systems).

Reverse Transcription-PCR

Total RNA was extracted from sorted $CD4^+$ T cells with an RNeasy Mini Kit (Qiagen) according to the manufacture's instruction. Single-strand cDNA was synthesized with Superscript reverse transcriptase III and oligo(dT) primers (Invitrogen). PCR was performed in 35 cycles for transcription factors and in 25 cycles for β-actin with the following primer pairs. T-bet; forward 5'-CACTACAGGATGTTTGTGGACGTG-3' and reverse 5'-CCCCTTGTTGTTTGTGAGCTTTAG-3'; GATA-3; forward 5'-TGT CTGCAGCCAGGAGAGC-3' and reverse 5'-ATGCATCAAACAACTGTGGCCA-3'; RORγt;

forward 5'-TCTGGAGCTGGCCTTTCATCATCA-3' and reverse 5'-TCTGCTCACTTCCAAAGAGCTGGT-3'; β-actin; forward 5'-TCCTGTGGCATCCACGAAACT-3' and reverse 5'-GAAGCATTTGCGGTGGACGAT-3'. PCR products were separated through 1% agarose gel, stained with ethidium bromide and visualized with an UV transilluminator.

Real time RT-PCR

Total RNA was extracted from blood Th subpopulations or cultured B cells using RNeasy mini kit (QIAGEN), and reverse-transcribed into cDNA in a 96-well plate using the High Capacity cDNA Archive kit (Applied Biosystems). The primer pairs (Integrated DNA Technology) used in this study was designed using the Roche Primer Design Program. Primer sequences were as follows: *Bcl-6* **(**Accession number: NM_001706.2) forward primer: 5' ttccgctacaagggcaac-3', reverse primer: 5'- tgcaacgatagggtttctca-3'; *Prdm1* (Accession number: NM_001198.2) forward primer: 5' gtggtgggttaatcggtttg -3', reverse primer: 5'-gaagctcccctctggaataga-3'; and *AICDA* (Accession number: NM_020661.2) forward primer: 5' gactttggttatcttcgcaataaga-3', reverse primer: 5'- aggtcccagtccgagatgta-3'.

Real-time PCR was set up with Roche Probes Master reagents and Universal Probe Library hydrolysis probes. PCR reaction was performed on the LightCycler 480 (Roche Applied Science) followed these conditions: step 1 (denaturation) at 95°C for 5 min, step 2 (amplification) at 60°C for 30 min, step 3 (cooling) at 40°C for 30 seconds. The expression level of each gene was normalized to the levels of housekeeping gene *ACTB*.

In the measurement of *Bcl6*, *Prdm1*, and *AICDA* mRNA expression in naïve B cells cultured with blood $CD4^+$ T cell subsets, expression levels of each mRNA were normalized to

those of *ACTB* mRNA in naïve B cells before culture and B cells purified after 7 d co-culture with $CXCR5^-$ or $CXCR5^+$ $CD4^+$ T cell subsets. Then, the expression levels of each molecule were normalized to those in naïve B cells before culture.