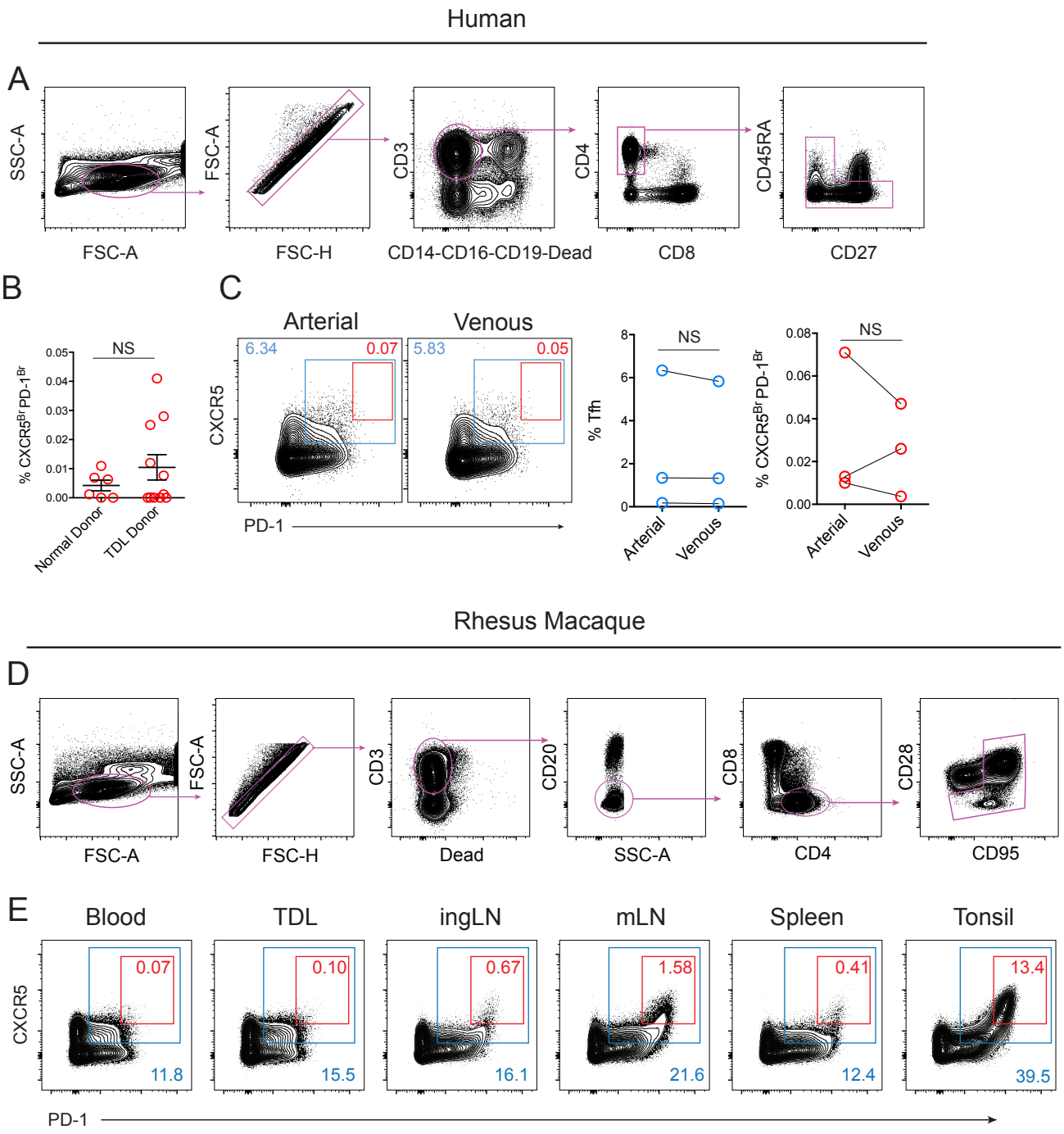
# Supplementary Materials for Vella, Buggert, *et al*.

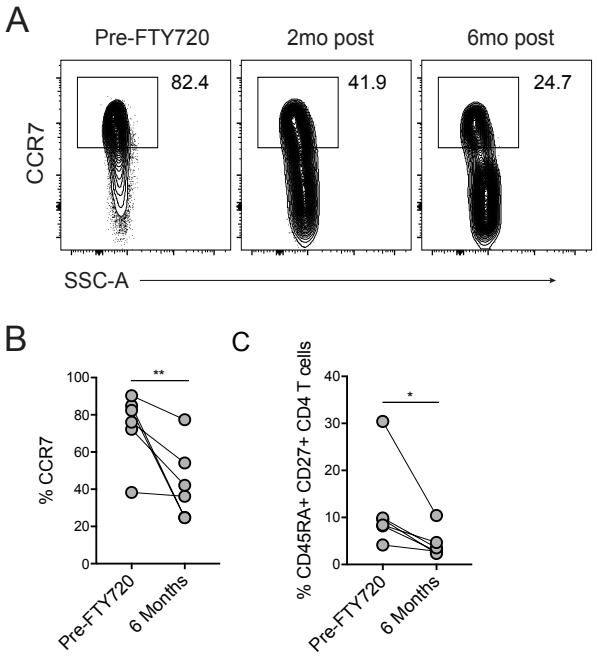
# "T Follicular Helper Cells in Human Efferent Lymph Retain Lymphoid Characteristics"



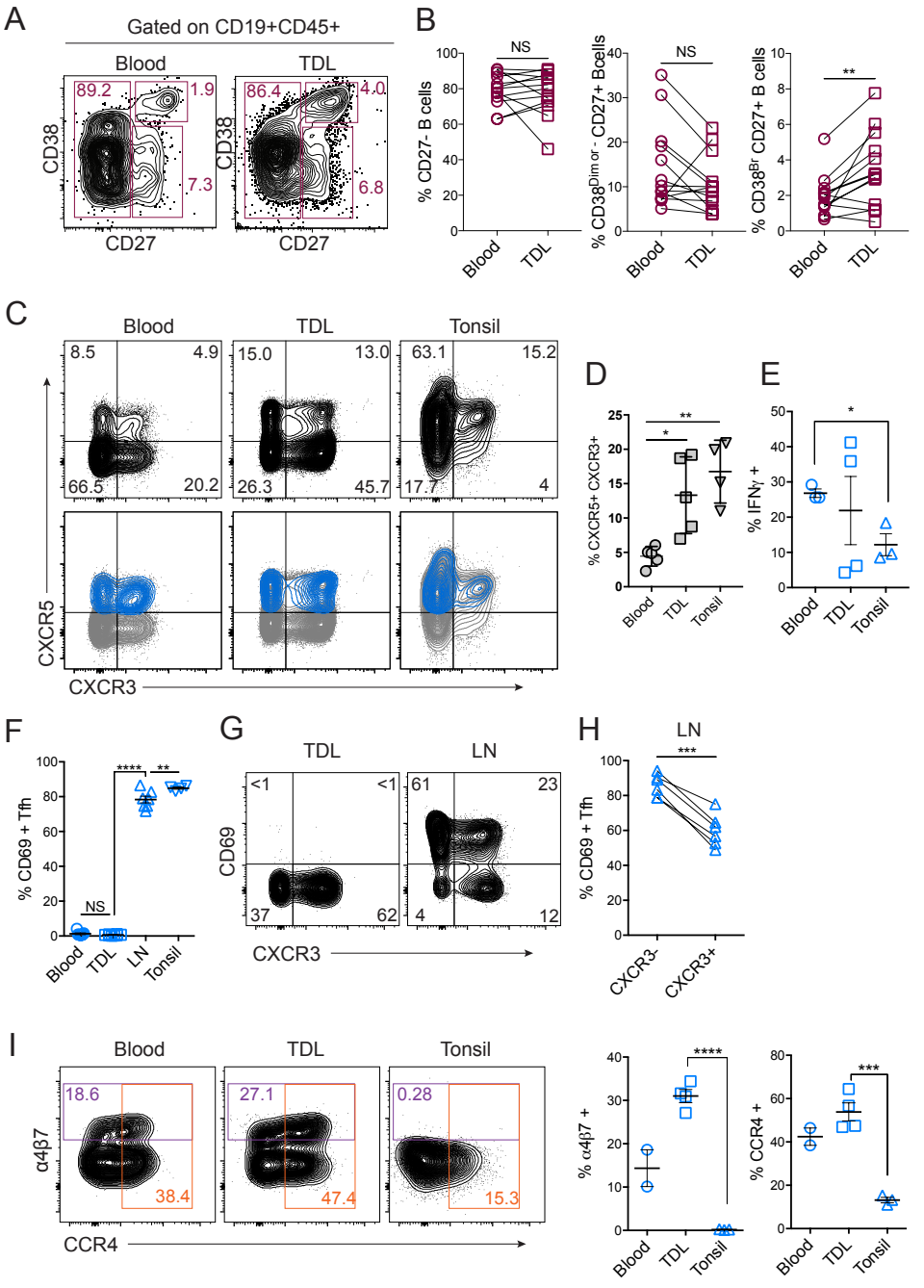

**Supplementary Figure 1**

(A) Gating strategy for human Tfh. (B) Frequency of CXCR5$^{Br}$PD-1$^{Br}$ Tfh in peripheral blood of normal donors versus donors with clinical indication for thoracic duct cannulation. (C) Frequency of all Tfh (blue boxes) and CXCR5$^{Br}$PD-1$^{Br}$ (red boxes) in paired arterial and venous blood samples (n=3). (D) Gating strategy for Rhesus Macaque (RM) Tfh. (E) Representative flow cytometric plots of RM Tfh by organ with frequencies (percent) noted on each plot (ingLN, inguinal LN; mLN, mesenteric LN). Error is reported as standard deviation. A two-tailed Student's t-test was performed in B, and a paired t-test was performed in C. Asterisks indicate statistical significance (ns P>0.05, *P < 0.05, **P<0.01).

Vella et al, Supplemental Figure 2

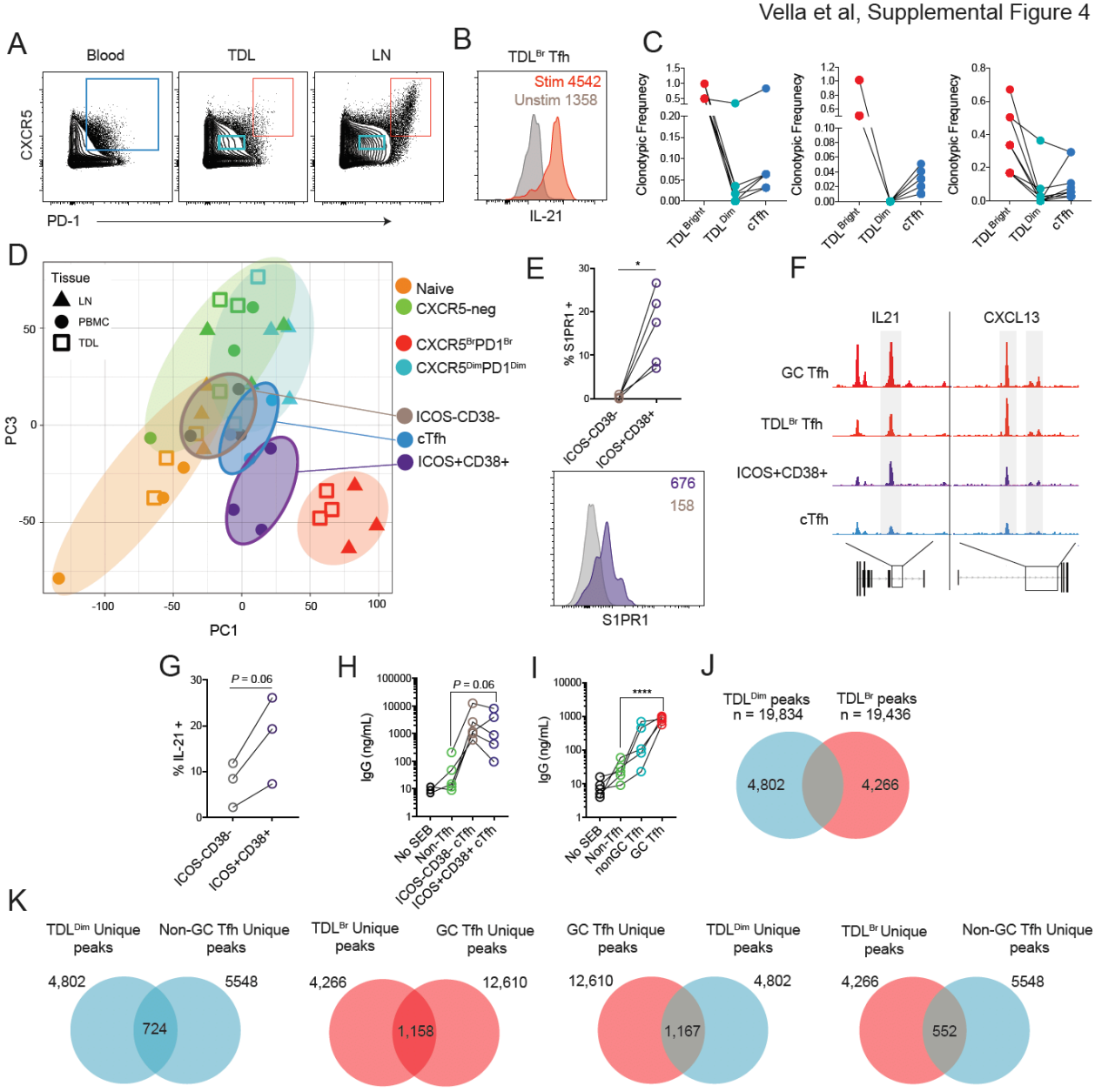



**Supplemental Figure 2**
(A) Flow cytometric analysis of CCR7 expression on non-naïve (not CD45RA+CD27+) CD4 T cells before and 2 and 6 months after FTY720 administration in patients with multiple sclerosis. Frequency (percent) noted on each plot. (B) Frequency of CCR7 and (C) CD45RA+CD27+ naïve CD4 T cells before and 6 months after FTY720 initiation. Asterisks indicate statistical significance in paired t-tests (*$P$ <0.05, **$P$ <0.01).




**Supplemental Figure 3**

(A) Representative flow cytometric plots of CD19+CD45+ B cells in TDL and blood. (B) Frequency of CD27- B cells, CD27+CD38$^{Br}$ plasmablasts, and CD27+CD38$^{Dim}$ or - memory B cells in paired TDL and blood (n=13). (C) Representative flow cytometric plots of CXCR5 versus CXCR3 in all non-naïve CD4 T cells, with CXCR5+PD-1+ Tfh overlaid in blue, bottom row. (D) Frequency of CXCR5+CXCR3+ non-naïve CD4 T cells in blood (n=5), TDL (n=5), and tonsil (n=4). (E) Percentage of Tfh producing IFNγ after PMA/ionomycin stimulation in blood (n=3), TDL (n=4), and tonsil (n=3). (F) Frequency of CD69+ Tfh in blood (n=7), TDL (n=8), LN (n=7), and tonsil (n=4). (G) Representative flow cytometric plots of CD69 versus CXCR3 in TDL and LN, with (H) individual frequencies of CXCR3+ versus CXCR3- CD69+ Tfh in LN (n= 6). (I) Representative flow cytometric plots and frequencies of α4β7 and CCR4 in blood (n=2), TDL (n=4), and tonsil (n=3). Error is reported as standard deviation. Multiple comparisons in D and E were performed using ANOVA with Šídák's post-test. Comparisons in E and I were performed with a two-tailed Student's t-test. Asterisks indicate statistical significance (NS $P$ >0.05, *$P$ <0.05, ***$P$ <0.001, ****$P$ <0.0001).




**Supplementary Figure 4**

(A) Gating strategy for sort purification for blood (n=3), TDL (n=3), and LN (n=3). (B) Representative histogram and MFI of IL-21 production in CXCR5$^{Br}$PD-1$^{Br}$ TDL Tfh (TDL$^{Br}$) when unstimulated (gray) and after PMA/ionomycin stimulation (red). (C) Frequency of CXCR5$^{Br}$PD-1$^{Br}$ CDR3 sequences of overlapping clones between compartments (TDL and blood) and gates (Bright and Dim) in 3 additional paired samples included in Figure 6F. (D) Principal component analysis of RNA-seq data from Figure 5B, with the addition of ICOS+CD38+ and ICOS-CD38- cTfh populations. (E) Frequency of S1PR1+ cells in ICOS-CD38- versus ICOS+CD38+ cTfh, with representative histogram and MFI comparison (n=5). (F) ATAC-seq tracks of loci in Figure 6D, with ICOS+CD38+ cTfh added in purple (n=1). (G) Percentage of ICOS-CD38- versus ICOS+CD38+ cTfh producing IL-21 after PMA/Ionomycin stimulation in blood (n=3). (H) Allogeneic B cell help assay for IgG production after co-culture with the indicated populations from blood (n=5) and (I) LN (n=5). (J) Unique peaks for CXCR5$^{Br}$PD-1$^{Br}$ TDL Tfh versus CXCR5$^{Dim}$PD-1$^{Dim}$ TDL Tfh. (K) Determination of overlapping peaks between TDL and LN subsets. Comparisons in H and I differed in normality of distribution; a Wilcoxon matched pairs signed rank test was performed in H and a paired t-test was performed in I. Asterisks indicate statistical significance (*$P$ < 0.05, ***$P$ <0.001, ****$P$ <0.0001).

| Sample Type | Age (yrs) | Sex | Indication for Procedure and/or Clinical Diagnosis | Flow Cytometry | ATAC -seq | RNA-seq | Paired blood available for flow cytometry | B cell co-culture | B cell stains only |
|---|---|---|---|---|---|---|---|---|---|
| Mesenteric Lymph Node | 76 | Male | Normal stricture | x | x | | | | |
| Mesenteric Lymph Node | 28 | Female | Normal | x | x | | | | |
| Mesenteric Lymph Node | 3 | Female | Colitis | x | x | | | | |
| Mesenteric Lymph Node | 49 | Female | Diverticulitis | x | | | | | |
| Mesenteric Lymph Node | 66 | Female | Normal | x | | | | | |
| Mesenteric Lymph Node | 68 | Female | Normal | x | | | | | |
| Mesenteric Lymph Node | 69 | Male | Diverticulitis | x | | x | | | |
| Mesenteric Lymph Node | 72 | Female | Diverticulitis | x | | x | | | |
| Mesenteric Lymph Node | 76 | Female | Colovaginal Fistula | x | | x | | | |
| Mesenteric Lymph Node | 22 | Female | Crohn's disease | x | | | | | |
| Mesenteric Lymph Node | 22 | Male | Ulcerative Colitis | | | | | x | |
| Mesenteric Lymph Node | 27 | Female | Ulcerative Colitis | | | | | x | |
| Mesenteric Lymph Node | 52 | Female | Ulcerative Colitis | | | | | x | |
| Mesenteric Lymph Node | 25 | Female | Ulcerative Colitis | | | | | x | |
| Mesenteric Lymph Node | 34 | Female | Fibrous serosal adhesions | | | | | x | |
| | | | | | | | | | |
| Thoracic Duct Lymph | 57 | Female | Chylous ascites | x | x | | x | | |
| Thoracic Duct Lymph | 74 | Male | Right chylothorax after esophagectomy | x | | | x | | |
| Thoracic Duct Lymph | 11 | Male | Chylous pericardium | x | x | | x | | |
| Thoracic Duct Lymph | 52 | Female | Plastic bronchitis | x | x | | x | | |
| Thoracic Duct Lymph | 11 | Female | Plastic bronchitis, history of Fontan procedure | x | | | x | | |
| Thoracic Duct Lymph | 11 | Female | Chylothorax (Gorham's disease) | x | | | x | | |
| Thoracic Duct Lymph | 12 | Male | Plastic bronchitis | x | | x | | | |
| Thoracic Duct Lymph | 67 | Male | Left pleural effusion after left lower lobectomy | x | | | x | | |
| Thoracic Duct Lymph | 4 | Male | Plastic bronchitis, history of Fontan procedure | x | | | x | | |
| Thoracic Duct Lymph | 61 | Male | Lymphedema after pacemaker placement | x | | x | x | | |
| Thoracic Duct Lymph | 46 | Male | Right chylothorax after esophagectomy | x | | | x | | |
| Thoracic Duct Lymph | 61 | Male | Plastic bronchitis | x | | | | | |
| Thoracic Duct Lymph | 66 | Male | Right chylothorax after esophagectomy | x | | | | | |
| Thoracic Duct Lymph | 11 | Male | Plastic bronchitis, history of Fontan procedure | x | | x | | | |
| Thoracic Duct Lymph | 5 | Male | Plastic bronchitis, history of Fontan procedure | x | | | | | |
| Thoracic Duct Lymph | 49 | Male | Right chylous effusion after lymphangioma resection (Noonan's syndrome) | x | | | | | |
| Thoracic Duct Lymph | 0.4 | Female | Dilated TD, elevated R-sided atrial pressure | x | | | | | |
| Thoracic Duct Lymph | 60 | Female | Plastic bronchitis | x | | | | | |
| Thoracic Duct Lymph | 50 | Male | Right chylous effusion after thoracic surgery | x | | | | | |
| Thoracic Duct Lymph | unknown | unknown | unknown | x | | | | | |
| Thoracic Duct Lymph | 15 | Female | Plastic bronchitis | | | | | | x |
| Thoracic Duct Lymph | 61 | Male | Plastic bronchitis | | | | | | x |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Thoracic Duct Lymph | 49 | Male | Chylothorax | | | | | | x |
| Thoracic Duct Lymph | 7 | Female | Plastic bronchitis | | | | | | x |
| Thoracic Duct Lymph | 12 | Male | Plastic bronchitis s/p Fontan | | | | | | x |
| Thoracic Duct Lymph | 10 | Female | Chylopericardium | | | | | | x |
| Thoracic Duct Lymph | 6 | Female | Plastic bronchitis | | | | | | x |
| Thoracic Duct Lymph | 6 | Male | Plastic bronchitis | | | | | | x |
| Thoracic Duct Lymph | 4 | Male | Plastic bronchitis, pleural effusions | | | | | | x |
| Thoracic Duct Lymph | 47 | Male | Chylothorax | | | | | | x |
| Thoracic Duct Lymph | 8 | Female | Pleural effusions, lymphangiomatosis | | | | | | x |
| Thoracic Duct Lymph | 54 | Male | Plastic bronchitis | | | | | | x |
| Thoracic Duct Lymph | 31 | Male | Chylothorax | | | | | | x |
| | | | | | | | | | |
| Tonsil | adult | unknown | unknown | x | | | | | |
| Tonsil | adult | unknown | unknown | x | | | | | |
| Tonsil | adult | unknown | unknown | x | | | | | |
| Tonsil | adult | unknown | unknown | x | | | | | |
| Tonsil | adult | unknown | unknown | x | | | | | |
| Tonsil | adult | unknown | unknown | x | | | | | |
| Tonsil | adult | unknown | unknown | x | | | | | |

**Table S1: Clinical Characteristics of Thoracic Duct Lymph and Tissue Donors**

| Antibody Target | Clone(s) | Supplier(s) |
|---|---|---|
| | | |
| **HUMAN** | | |
| CD3 | UCHT1, OKT3 | BioLegend, BD Biosciences |
| CD4 | RPA-T4, S3.5, SK3 | BioLegend, Invitrogen, BD Biosciences |
| CD8 | RPA-T8 | BioLegend |
| CD45RA | HI100 | BD Biosciences |
| CD14 | M5E2 | BioLegend |
| CD16 | 3G8 | BioLegend |
| CD19 | HIB19 | BioLegend |
| CD27 | O323 | BioLegend |
| CXCR5 | RF8B2 | BD Biosciences, Fisher |
| PD-1 | EH12.1, EH12.2H7, EH12.2 | BD Biosciences, BioLegend |
| ICOS | C398.4a, DX29, ISA-3 | BD Biosciences, BioLegend, eBioscience |
| CD38 | HIT2 | BioLegend, BD Biosciences |
| CXCR3 | GO25H7 | BioLegend |
| CCR7 | GO43H7 | BioLegend |
| CD200 | MRC OX-104 | BD Biosciences, BioLegend |
| CD57 | HNK-1 | BioLegend |
| Bcl6 | K112-91 | BD Biosciences |
| CCR6 | GO34E3 | BioLegend |
| CD69 | FN50, TP1.55.3 | BioLegend, Beckman Coulter |
| CCR4 | 1G1 | BD Biosciences |
| CCR6 | G034E3 | Biolegend |
| IFNg | B27 | BD Biosciences |
| IL-21 | 3A3-N2.1 | BD Biosciences |
| TNF-α | MAb11 | Biolegend |
| Dead | LIVE/DEAD | Invitrogen |
| S1PR1 (EDG-1) | 218713 | R&D Biosystems |
| CD45 | HI30 | BD Biosciences |
| α4β7 | Act-1 | This reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-Human α4-β7 integrin Monoclonal (Act-1) (cat#11718) from Dr. A. A. Ansari. |
| | | |
| **NON-HUMAN PRIMATE** | | |
| CD3 | SP34-2 | BD Biosciences |
| CD20 | 2H7 | BioLegend |
| CD8 | RPA-T8 | BioLegend |
| CD4 | S3.5 | Invitrogen |
| CD28 | CD28.2 | Beckman Coulter |
| CD95 | DX2 | BD Biosciences |
| CXCR5 | MU5UBEE | eBiosciences |
| PD-1 | EH12.2H7 | BioLegend |
| Dead | LIVE/DEAD | Invitrogen |

**Table S2: Antibody clones**

**Supplemental Materials and Methods:**

**Mathematical modeling of lymphocyte residency in the blood**.
Given a continuous input of lymphocytes from the lymph to the blood it is possible to estimate the average residency time of lymphocytes in the blood before they enter lymph nodes (LNs), Peyer's patches (PPs), or nonlymphoid peripheral tissues (e.g., lung, liver, skin) that contain lymphatics. Because cells migrating to the spleen exit the spleen into circulation, these calculations provide an estimate of the average residency time of lymphocytes in the blood excluding migration to the spleen. Change in the total number of lymphocytes of a specific type (e.g., Tfh cells) in the blood $x$ can be described by the following differential equation:

$$\frac{dx}{dt} = \lambda - dx, \quad (1)$$

where $\lambda$ is the number of lymphocytes entering the blood from the lymph per unit of time and $d$ is the rate at which lymphocytes exit the blood for LNs, PPs, nonlymphoid tissues, or die. Residency time of lymphocytes in the blood can then be defined as $T = 1/d$. At the steady state $x = x^*$, and the residency time can be calculated as

$$T = \frac{x^*}{\lambda}. \quad (2)$$

Cells exiting lymph nodes enter the blood via right and left (thoracic) lymphatic ducts with the thoracic duct draining approximately ¾ of the total body lymph(1). Therefore, if $\lambda_T$ cells enter the blood via the thoracic duct per unit of time, then the total number of lymphocytes entering the blood is approximately $\lambda = 4/3 \times \lambda_T$. We applied eqn. (2) to calculate the average residency time of resting lymphocytes in blood of rats. Given that for rats $x^* = 10^8$ (2) and $\lambda_T = 30 \times 10^6$ cell/h (or $7.2 \times 10^8$ cell/day, (3)) the average residency time of lymphocytes in the blood in rats is $T = 2.5\ h$. This is in line with what is known about time that lymphocytes spend in the blood (about 15-25 min) and in the spleen (about 2-3 h, (1)). Residency times provided by eqn. (2) are likely to be an upper bound estimate given that it ignores the process of lymphocyte division in the blood or spleen.

In our case it was not possible to estimate i) the total number of lymphocytes in the blood because the total weight of the patients was not recorded at the time of blood and lymph sampling, limiting estimates of total blood volume and ii) the rate at which lymphocytes enter the blood via the thoracic duct because the overall lymph flow rate was not recorded. Thus, we could only provide approximate estimates of the residency times using previously published average values of the blood volume and lymph flow rates in humans. Yet, even if these parameters are unknown it is possible to provide relative residency time of two different subsets of lymphocytes, e.g., total CD4 T cells and GC Tfh cells. The total number of lymphocytes in the blood is $x^* = f_B V_B$ where $f_B$ is the concentration of lymphocytes in the blood (in cells/ml) and $V_B$is blood volume (in ml). The total number of lymphocytes entering blood via thoracic duct is $\lambda_T = v f_L$ where $v$ is the lymph flow rate (in ml/day) and $f_L$ is the concentration of lymphocytes in the lymph (in cells/ml). Then the ratio of residency times of CD4 T cells ($T_{CD4}$) to that of Tfh cells ($T_{T\text{fh}}$) is

$$\frac{T_{CD4}}{T_{T\text{fh}}} = \frac{f_B^{CD4}}{f_L^{CD4}} \times \frac{f_L^{T\text{fh}}}{f_B^{T\text{fh}}} \quad (3)$$

which is independent of $V_B$ and $v$ but only depends on cell frequencies in the blood and lymph. However, eqn. (3) may not hold if there is proliferation of lymphocytes in the blood or spleen.

Calculations were performed using the typical minimum and maximum mononuclear cells/mL of each fluid, estimated minimum and maximum lymph and blood volumes, and the mean frequencies of each T cell subset, as listed below.

| Thoracic Duct Lymph | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Mononuclear cells per mL TDL** | **mL TDL into blood per day** | **Proportion Lymphocytes** | **proportion T cells** | **proportion CD4+** | **proportion Non-naive** | **proportion Tfh** | **proportion $CXCR5^{BR}$ $PD\text{-}1^{BR}$** |
| **min** | 1,000,000 | 3,000 | 1 (typically >0.95) | 0.7 | 0.7 | 0.8 | 0.08 | 0.003 |
| **max** | 2,000,000 | 9,000 | 1 (typically >0.95) | 0.7 | 0.7 | 0.8 | 0.08 | 0.003 |
| Blood | | | | | | | | |
| | **Mononuclear cells per mL blood** | **Total volume of blood (mL)** | **Proportion Lymphocytes** | **proportion T cells** | **proportion CD4+** | **proportion Non-naive** | **proportion Tfh** | **proportion $CXCR5^{BR}$ $PD\text{-}1^{BR}$** |
| **min** | 1,000,000 | 4,500 | 0.8 | 0.7 | 0.7 | 0.9 | 0.04 | 0.001 |
| **max** | 2,000,000 | 5,500 | 0.8 | 0.7 | 0.7 | 0.9 | 0.04 | 0.001 |

## Supplementary References


1. Ganusov VV, Tomura M. Experimental and mathematical approaches to quantify recirculation kinetics of lymphocytes. *bioRxiv* 2018;:1–21.

2. Trepel F. Number and distribution of lymphocytes in man. A critical analysis. *Klin Wochenschr* 1974;52(11):511–515.

3. Gowans JL. The effect of the continuous re-infusion of lymph and lymphocytes on the output of lymphocytes from the thoracic duct of unanaesthetized rats. *Br J Exp Pathol* 1957;38(1):67–78.