Evidence for a stepwise program of extrathymic T cell development within the human tonsil

Supplemental Information

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Running title: T cell development in the human tonsil

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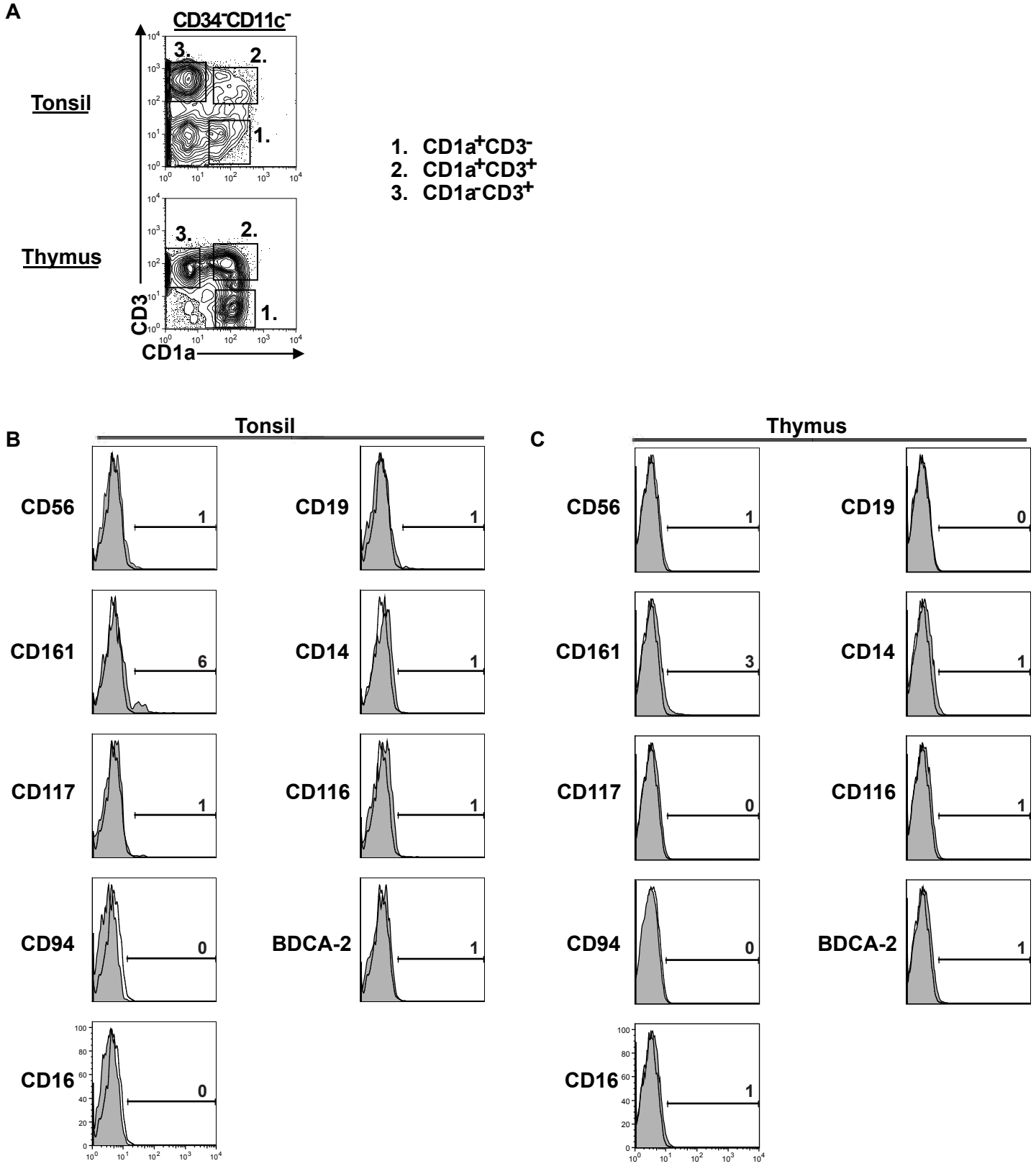

Figure S1: CD34$^-$CD11c$^-$CD1a$^+$CD3$^-$, CD34$^-$CD11c$^-$CD1a$^+$CD3$^+$, and CD34$^-$CD11c$^-$CD1a$^-$CD3$^+$ cells reside within the human tonsil and thymus. (A) Gating strategy for identifying these three subsets in the human tonsil and thymus. Tonsils were depleted of CD19$^+$ cells and were then enriched for CD1a-expressing cells (top), whereas CD1a$^+$ cells were readily apparent in CD34-depleted thymic mononuclear cells without enrichment (bottom). For the analysis shown in Figure 4, cells from each tissue were gated on CD34$^-$CD11c$^-$ events, and were then gated on the three populations shown. (B-C) Total CD34$^-$CD11c$^-$CD1a$^+$ cells from the tonsil (B) and thymus (C) were analyzed for expression of the lineage markers shown. In each histogram, the filled line represents staining with the indicated antibody, whereas the open line indicates staining with an isotype-matched control. Each histogram is a representative of 3 tonsil (B) or 2 thymic (C) donors.

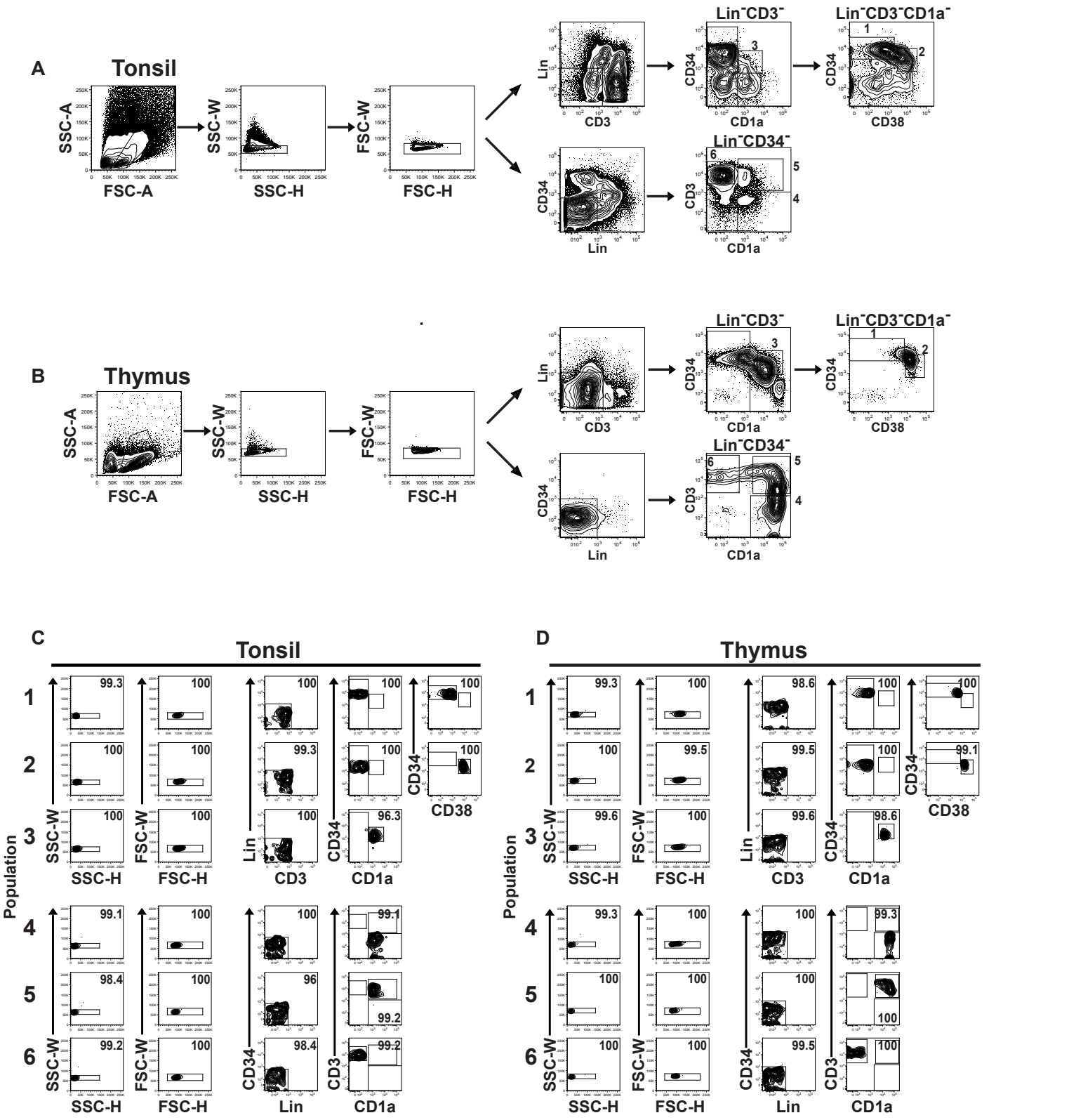

Figure S2: Sorting strategy for isolating 6 populations from tonsillar and thymic cells. (A) Tonsillar mononuclear cells were magnetically depleted of $CD19^+$ cells, and were simultaneously enriched for CD34 and CD1a-expressir cells. Cells were gated and sorted into the six populations as shown. First, we gated on lymphocytes and excluded doublets by gating on SSC-W and FSC-W low events. Next, all populations were gated on CD11c, BDCA-2, CD56 CD161, CD117, CD19 (Lin^-) negative events, and populations 1 and 2 were additionally gated on CD3 and CD1a negative events. For sorting populations 4, 5, and 6, events were gated on Lin^-CD34^- events. (B) Thymic mononuclear cells were magnetically enriched for CD34-expressing cells. Populations 1-3 were sorted from the CD34 enriched fraction, and populations 4-6 were sorted from CD34 depleted fraction. (C-D) Representative sort purities from the tonsil (C) and thymus (D). Sort purity for a representative donor is shown for each gate, and overall purity was routinely greater than 95%, and often in excess of 98%

Table S1: Frequency of putative extrathymic T cell precursors in the human tonsil

	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop 6
Mean Frequency (%)[A]	0.016	0.016	0.004	0.083	2.42	94.38
Range (%)	0.006-0.032	0.005-0.031	0.0003-0.015	0.006-0.242	0.083-11.24	80.60-98.30
Mean Number per Tonsil[B]	21,052	19,323	3,915	81,954	$2.1x10^6$	$1.1x10^8$
Range	3,575-57,750	6,340-50,989	216-15,338	3,331-245,909	77,571-$1.15x10^6$	$4.6x10^7$-$2.16x10^8$

[A] Mean frequency is expressed as a percentage of Lin^- (CD19, CD117, CD56, CD161, CD11c, BDCA-2) cells as determined by flow cytometry.

[B] The absolute number of precursors in each population was calculated by multiplying the number of CD19-depleted mononuclear cells in each tonsil (determined by trypan blue exclusion) by the proportion of cells making up each subset (determined by flow cytometry).

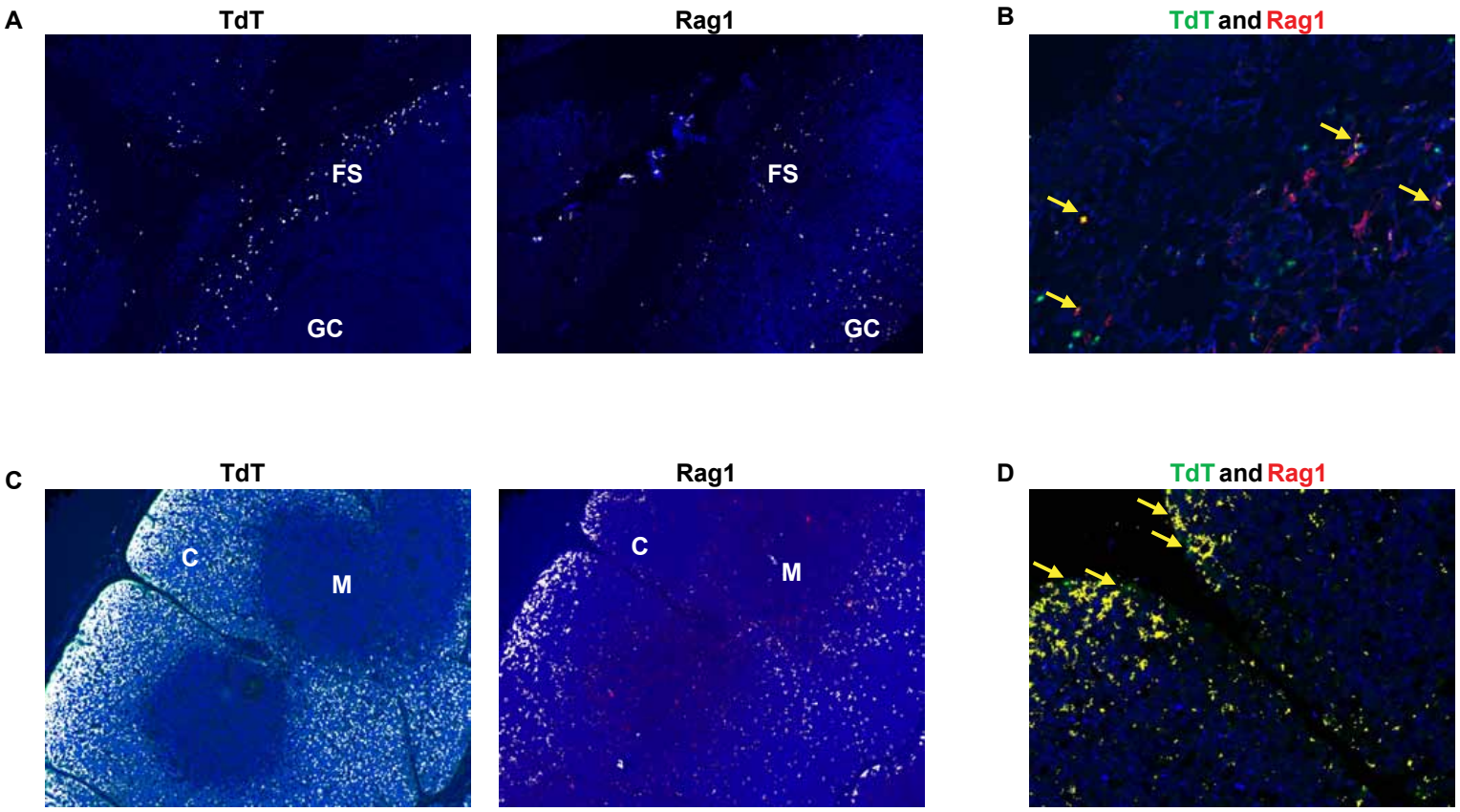

Figure S3: TdT$^+$Rag1$^+$ cells reside near the fibrous scaffold of the human tonsil. (A) Immunohistochemical staining of paraffin-embedded tonsillar sections localizes TdT$^+$ cells to the fibrous scaffold (FS) region (left image). White signal indicates co-localization of TdT protein with the nuclear stain hematoxylin. A serial section of the same tonsillar region localizes Rag1$^+$ cells to the fibrous scaffold and germinal center (GC) regions (right image). Again, co-localization of nuclear Rag1 and hematoxylin is indicated by a white signal. (B) A serial section of the same tonsillar region at a magnification of 400X that has been co-stained with antibodies for both TdT (green) and Rag1 (red) demonstrates that within the FS region of the tonsil, there are cells that express only Rag1 or TdT, and cells that co-express both proteins (yellow signal and arrows). (C) Immunohistochemical staining of paraffin-embedded human thymic sections localizes TdT$^+$ and Rag1$^+$ cells to the cortex (c). In both images, white signal indicates co-localization of the protein of interest (TdT or Rag1) with nuclear hematoxylin. The thymic medulla (M) is indicated for reference. (D) A serial section of the same thymic region at a magnification of 400X that has been co-stained for TdT (green) and Rag1 (red) proteins demonstrates the presence of Rag1$^+$TdT$^+$ (yellow) cells within the thymic cortex. Images in A and C were taken at a Magnification of 200X.

Table S2: Mean fold change[A] of total cells following 26 day OP9-DL1 co-cultures

	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop 6
Tonsil	93.6±39.7	107.2±32.8	96.8±69.4	2.8±1.7	1.8±0.66	2.8±0.66
Thymus	270.5±150.3	83.0±20.7	61.0±30.2	0.75±0.25	3.0±2.7	5.8±2.4

[A] The number of human cells obtained from each culture was determined by counting total harvested cells (using trypan blue exclusion), and multiplying this number by the fraction of live cells defined as GFP(-)CD45(+). Data is represented as mean fold increase ± SEM.

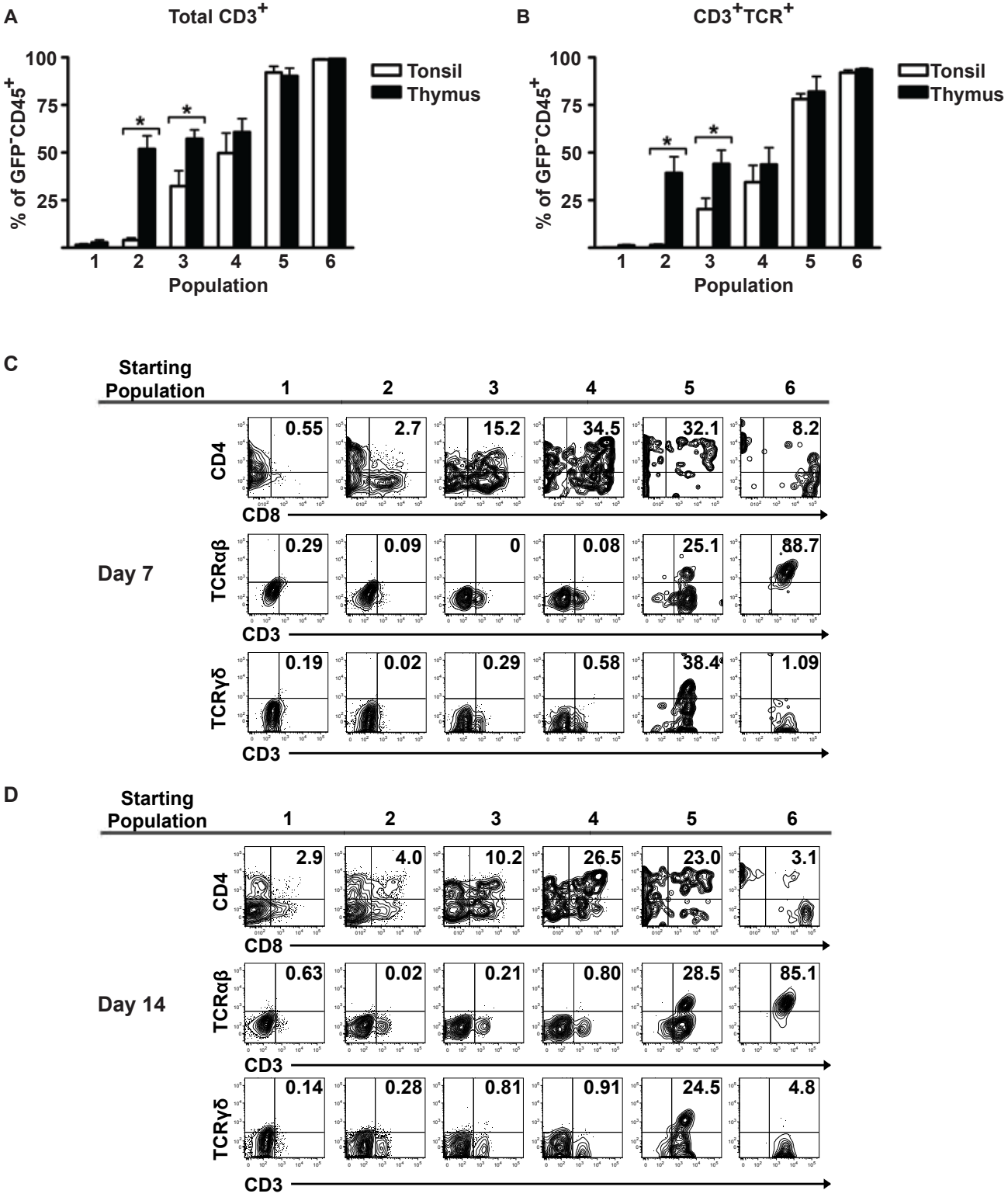

Figure S4: Kinetics of T cell development from putative tonsillar T cell precursors: Tonsillar and thymic populations 1-6 were sorted as shown in Figure 5A and S2A-D, and were then cultured on the OP9-DL1 cell line with the cytokines FL and IL-7. (A) Comparison of the mean percentage of GFP^-CD45^+ cells that stained positive for CD3 after 26 days of culture. (B) Comparison of the mean percentage of GFP^-CD45^+ cells that stained positive for both CD3 and a TCR (either TCRγδ or TCRαβ). For (A-B) progeny of each population from the human tonsil (white bars) was compared to the same population sorted from the human thymus (black bars). Data represents mean percentages of GFP^-CD45^+ cells ± SEM; n=7 for the tonsil and n=4 for the thymus. (C) Phenotype of cells harvested after 7 days from tonsillar populations 1-6. (D) Phenotype of cells harvested after 14 days from tonsillar populations 1-6. Dot plots in (C-D) are from a representative donor; n=3 tonsils. The number in the upper right hand corner of each plot represents the mean percentage of events staining double positive for the antigens shown.

Table S3: Single cell analysis of T and NK cell potential in populations 1-4 of the human tonsil[A]

Average	% Wells Analyzed	% CD5+ CD3- CD56- Pre-T cells	% CD3+ T cells	% CD56+CD3- NK cells
Population 1	24.58	90.48	0.00	81.25
Population 2	22.50	60.51	13.92	62.50
Population 3	67.50	97.53	50.55	27.38
Population 4	15.83	100.00	57.78	11.11

[A]All numbers represent the mean percentage from two independent experiments.