

Figure S1. Gating strategy and cytokine production controls, related to Figure 1.

Representative example of data from a matched tonsil and blood sample. From singlet cells (**A**), identified with Forward Scatter-Area (FSC-A) and Forward Scatter-Height (FSC-H), lymphocytes were gated based on FSC-A and Side Scatter-Area (SSC-A). The cells were then negatively gated for dead/dying cells and dump markers (CD16, CD56, CD8, CD25, CD14), then gated on the live CD4⁺ population and CD45RA⁻. Gates to define CD4⁺ memory T cells populations were set based on the expression of PD-1, CXCR5 and CXCR3 in the naïve-enriched CD4⁺CD45RA⁺ T cell compartment (**B**).

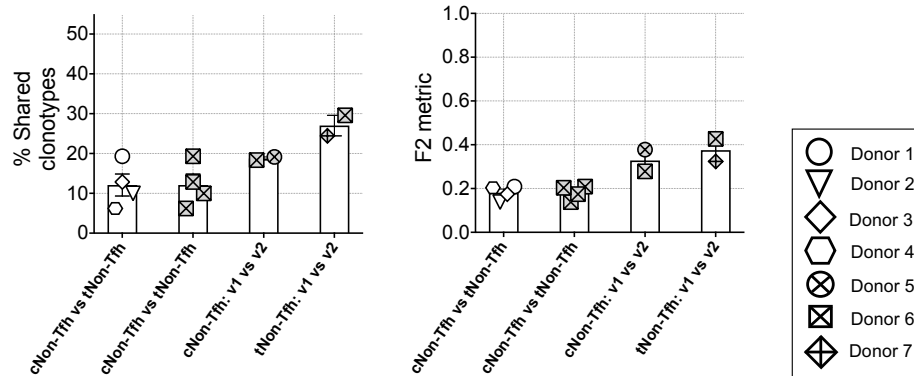
Examples of IL-21, IFN- γ (**C**) and IL-4 (**D**) production as assessed by intracellular cytokine staining after PMA/ionomycin stimulation are shown for each CD4⁺CD45RA⁻ memory T cell subset considered. The gating was set based on the IL-21, IFN- γ (**E**) and IL-4 (**F**) staining of the unstimulated control for the same subset.

A

Subset	Donor ID	N. shared clonotypes	R ²	P value
cTfh CXCR3+	1	1012	0.94	<0.0001
	2	1158	0.73	<0.0001
	3	1499	0.7	<0.0001
	4	1308	0.66	<0.0001
cTfh CXCR3-	1	896	0.84	<0.0001
	2	931	0.5	<0.0001
	3	974	0.65	<0.0001
	4	921	0.61	<0.0001
cNon-Tfh	1	995	0.99	<0.0001
	2	992	0.96	<0.0001
	3	1110	0.88	<0.0001
	4	891	0.82	<0.0001
tTfh GC	1	1365	0.97	<0.0001
	2	1452	0.98	<0.0001
	3	1199	0.98	<0.0001
	4	1412	0.94	<0.0001
tTfh CXCR3+	1	1275	0.99	<0.0001
	2	1183	0.97	<0.0001
	3	1026	0.99	<0.0001
	4	1108	0.85	2.4
tTfh CXCR3-	1	965	0.78	<0.0001
	2	964	0.77	<0.0001
	3	790	0.56	<0.0001
	4	802	0.5	<0.0001
tNon-Tfh	1	875	0.76	<0.0001
	2	788	0.73	<0.0001
	3	748	0.73	<0.0001
	4	600	0.59	<0.0001

B

Non-Tfh cell replicates



C

Tfh cell replicates

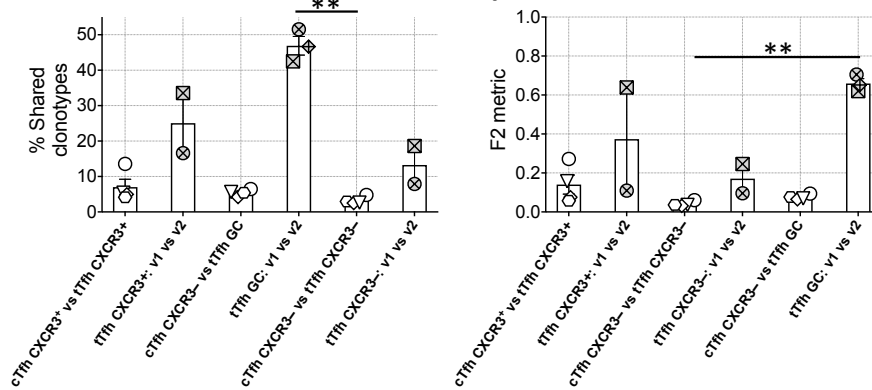
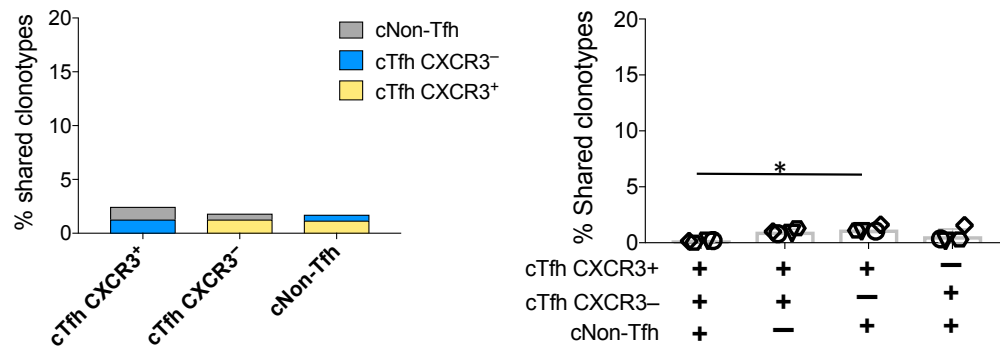


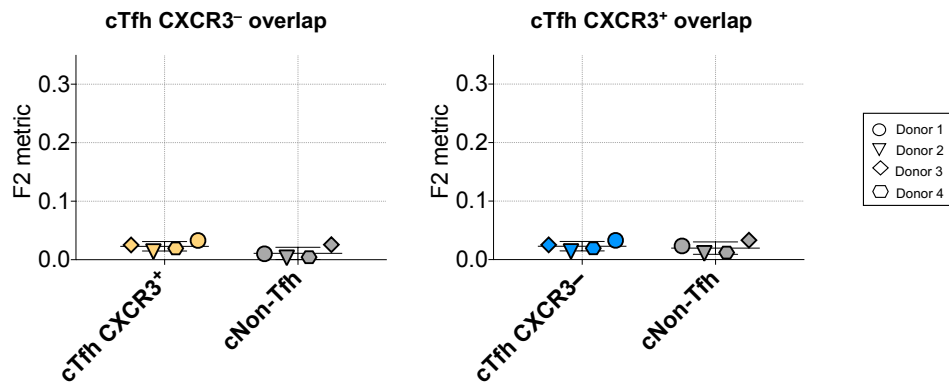
Figure S2. *In silico* resampling and experimental replicates analysis of the TCR V β CDR3 repertoire of blood and tonsil, related to Figure 4 C-D. The clonotypes of each subset were resampled using the bootstrap method. The table (A) shows for each subset the number of shared clonotypes, the square of the Pearson correlation coefficient (R^2), and the P value of the significance of the difference in the slope from zero in the 4 donors. From experimental replicates, the repertoire overlaps, percentage of shared clonotypes and normalized F2 metric (mean \pm SEM), of the top2000 clonotypes of non-Tfh cells (B) from 2 different vials of 3 additional donors (donor 5, 6 and 7) were analyzed in comparison with the observed overlap from the first 4 donors (donor 1, 2, 3 and 4). Depending on the sample availability it was possible to calculate the overlap between two vials of the same subsets (v1 vs v2) or between blood and tonsil (cNon-Tfh vs tNon-Tfh). The same repertoire overlaps and comparison were made for Tfh cells (C) for the subsets available.

A

Clonotype sharing in blood



B



C

Blood	cTfh CXCR3 ⁺ vs cTfh CXCR3 ⁻		cTfh CXCR3 ⁺ vs cNon-Tfh		cTfh CXCR3 ⁻ vs cNon-Tfh	
	Shared clonotypes	R ²	Shared clonotypes	R ²	Shared clonotypes	R ²
1	20	0.94	25	0.99	11	0.96
2	20	0.60	23	0.23	6	0.87
3	23	0.97	35	0.93	34	0.92
4	26	0.84	22	0.83	6	0.58

Figure S3. Tfh cell subsets from peripheral blood have a distinct repertoire from that of non-Tfh cells, related to Figure 5. To compare the overlap between different populations of blood cells, the percentage of total shared clonotypes in addition to the uniquely shared clonotypes was calculated for each overlap analysis in the 4 donors (mean \pm SEM) (A). The normalized F2 metric of the top2000 frequencies is also shown for cTfh CXCR3⁺ and cTfh CXCR3⁻ (B). The table shows the number of shared clonotypes and the square of the Pearson correlation coefficient (R^2) (C).

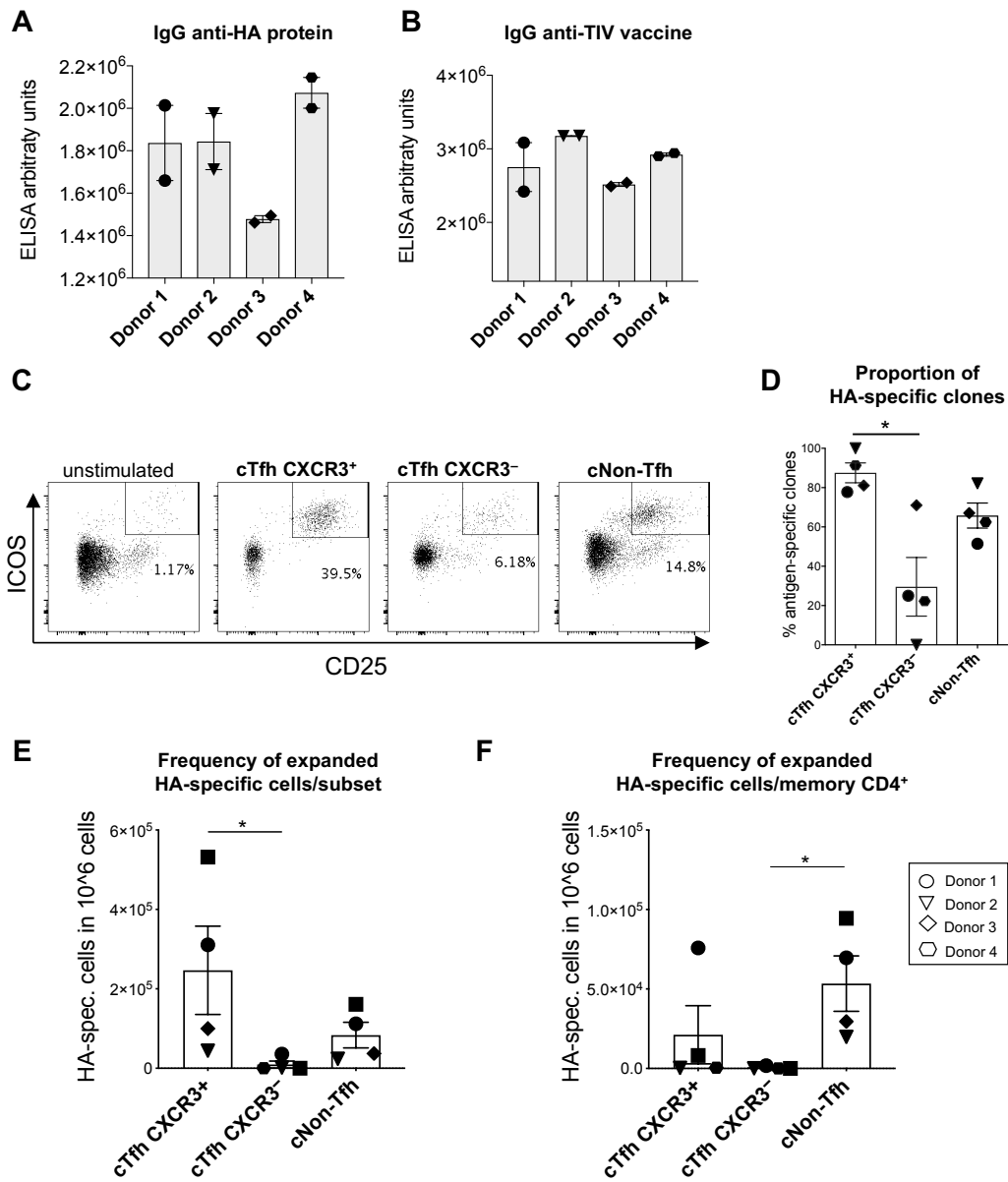


Figure S4. Influenza antibody analysis and generation of HA-specific CD4⁺ T cell clones, related to Figure 6. IgG reactive with HA/California (A) and TIV (Trivalent Influenza Vaccine, from 2017) (B) in plasma samples from the 4 donors was analyzed by ELISA and is expressed in ELISA arbitrary units. Data presented are results from duplicate wells, and have been background subtracted (mean \pm SEM). To generate HA-specific T cell clones from these 4 donors, memory CD4⁺ T cell subsets sorted from peripheral blood were co-cultured with autologous monocytes (CD14⁺) and overlapping peptides from HA/California, and activated memory CD4⁺ T cells identified on the basis of co-expression of ICOS and CD25 were isolated by cell sorting after 7 days, as shown in the examples (from donor 3) in (C). A negative control (unstimulated cells from the cTfh CXCR3⁺ subset) is also shown. The bar graph (D) shows the percentage of clones generated that were confirmed to be HA-specific on re-screening after expansion. Estimation of the number of HA-specific cells per 10⁶ total cells within each subset (E) or within memory CD4⁺ (F) after 7 days expansion is represented from 4 donors (mean \pm SEM).

A

Clones specific for peptide 313-330: HPITIGKCPKYVKSTKLK

DONOR ID	CLONE TYPE	CLONE ID	TRAV	AA α CDR3	TRAJ	TRBV	AA β CDR3	TRBJ
Donor 1	cTfh CXCR3+	21	26-1 29/DV5	CIVRVGREQGGKLI CAAPEGTYKYI	23 40	2	CAKQGTGYNEQF	2-1
Donor 1	cTfh CXCR3-	1	13-1	CAARTGAQKLV	54	11-2	CASTRTSGGANTGELF	2-2
Donor 1	cTfh CXCR3-	2	25	CAGRGPNSNSGYALN	41	18	CASSQGYEQY	2-7

B

CLONE TYPE	CLONE ID	AA α CDR3	TRAV	AA β CDR3	TRBV	V α frequency	V β frequency
cTfh CXCR3 ⁺	21	CAVRVGTGRRALT	21	CASSAGQATTGEQY	5-1	7.42E-05	1.17E-04
cTfh CXCR3 ⁺	44	CALKTGANNLF	24	CSAKAPGATQY	20-1	2.47E-05	3.91E-05
cTfh CXCR3 ⁺	48	CAVLLFMDSNYQLI	22	CAISEGGGSYGRKNIQY	10-3	2.47E-05	2.61E-05

C

CLONE TYPE	CLONE ID	AA α CDR3	TRAV	AA β CDR3	TRBV	V α frequency	V β frequency
cTfh CXCR3 ⁺	21	CAVRVGTGRRALT	21	CASSAGQATTGEQY	5-1	1.35E-04	5.35E-04
cTfh CXCR3 ⁺	57	CAVLISSGSARQLT	22	CASSSHPTGTYGRNTEAF	12-3	6.75E-04	7.49E-04

Figure S5. TCR CDR3 sequences of additional HA-specific cTfh clones from donor 1 and CDR3 sequences of cTfh CXCR3⁺ clones detected in the tTfh CXCR3⁺ subset from tonsil and blood in donor 2, related to Figure 7.

The table lists additional clones from donor 1, specific for HA H1/California peptide 313-330 generated from cTfh CXCR3⁺ and cTfh CXCR3⁻ (**A**). For each clone, the CDR3 region of the α and β chains of the TCR and the family classification of the J region (TRAJ and TRBJ) and the V region (TRAV and TRBV) are shown.

The tables in **B** and **C** list the clones from donor 2 for which matching sequences were found in tonsil tTfh CXCR3⁺ (**B**) and blood cTfh CXCR3⁺ repertoires (**C**). The amino acid (AA) sequences of the CDR3 regions of the TCR α and β chains, family classification of the V regions (TRAV and TRBV) and the clonotype frequency within the relevant population are shown.

Donor ID	Age	Gender	Indication for surgery	Regular medication
1	42	M	Sleep apnea	none
2	22	F	Chronic recurrent tonsillar infection	none
3	19	M	Chronic recurrent tonsillar infection	none
4	21	M	Chronic recurrent tonsillar infection	none
5	41	M	Chronic recurrent tonsillar infection	Ventolin inhaler
6	42	M	Quinsy	none
7	23	F	Chronic recurrent tonsillar infection	none
8	26	M	Chronic recurrent tonsillar infection	none
9	25	F	Chronic recurrent tonsillar infection	none
10	35	F	Quincy and chronic recurrent tonsillar infection	none
11	23	F	Chronic recurrent tonsillar infection	Ventolin, rigevidon
12	23	F	Quincy and chronic recurrent tonsillar infection	sertraline
13	24	F	Chronic recurrent tonsillar infection	none
14	36	F	Chronic recurrent tonsillar infection	none
15	23	M	Chronic recurrent tonsillar infection	none
16	21	F	Chronic recurrent tonsillar infection	none

Table S6. List of donors. The table lists the donor ID, age, gender, indication for surgery and any regular medication taken at the time of the surgery for the donors from which tonsil and blood samples were collected.

N° cells sequenced and % CD4 ⁺ CD45RA ⁻	Peripheral blood			Tonsil			
DONOR ID	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh	Tfh GC	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh
1	23,000	50,000	50,000	100,000	100,000	100,000	100,000
	(2.7%)	(5.0%)	(57.3%)	(14.0%)	(4.6%)	(29.1%)	(15.8%)
2	13,000	20,000	50,000	100,000	100,000	100,000	100,000
	(1.3%)	(2.0%)	(62.8%)	(4.4%)	(5.0%)	(21.3%)	(27.7%)
3	8,000	17,000	80,000	123,000	119,000	120,000	120,000
	(0.7%)	(3.6%)	(84.0%)	(17.6%)	(7.5%)	(21.2%)	(21.4%)
4	12,000	30,000	50,000	100,000	100,000	100,000	100,000
	(0.6%)	(1.6%)	(78.1%)	(5.8%)	(2.7%)	(22.2%)	(38.3%)

N° cells sequenced and % CD4 ⁺ CD45RA ⁻	Peripheral blood			Tonsil			
DONOR ID	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh	Tfh GC	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh
5 (vial 1)	4,000	n.d.	100,000	100,000	100,000	100,000	n.d.
	(0.5%)	n.d.	(79.5%)	(4.28%)	(3.7%)	(16.2%)	n.d.
5 (vial 2)	3,000	n.d.	100,000	100,000	100,000	100,000	100,000
	(0.5%)	n.d.	(79.5%)	(4.28%)	(3.7%)	(16.2%)	(26.7%)
6 (vial 1)	n.d.	n.d.	50,000	50,000	80,000	100,000	100,000
	n.d.	n.d.	(55.6%)	(5.5%)	(4.6%)	(18.5%)	(15.8%)
6 (vial 2)	n.d.	n.d.	50,000	50,000	100,000	100,000	100,000
	n.d.	n.d.	(55.6%)	(5.5%)	(4.6%)	(18.5%)	(15.8%)
7 (vial 1)	n.d.	n.d.	n.d.	100,000	n.d.	n.d.	100,000
	n.d.	n.d.	n.d.	(9.15%)	n.d.	n.d.	(23.8%)
7 (vial 2)	n.d.	n.d.	n.d.	100,000	n.d.	70,000	100,000
	n.d.	n.d.	n.d.	(9.15%)	n.d.	(12.4%)	(23.8%)

Table S7. T cells sorted for TCR deep-sequencing. Number of cells sorted per subset and percentage of CD4⁺CD45RA⁻ (n.d.= not determined).

Total number of TRAV reads	Peripheral blood			Tonsil			
DONOR ID	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh	Tfh GC	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh
1	10,716	25,113	17,549	33,395	44,337	27,188	31,720
2	13,296	7,400	23,114	46,986	40,421	29,304	28,475
3	3,097	7,505	27,692	23,257	27,665	22,810	23,779
4	3,204	11,483	14,411	33,157	35,303	25,067	21,326
5 (vial 1)	18,740	n.d.	18,755	49,386	9,664	14,038	n.d.
5 (vial 2)	7,618	n.d.	22,249	49,642	10,862	14,792	23,590
6 (vial 1)	n.d.	n.d.	11,319	70,121	53,037	41,493	40,617
6 (vial 2)	n.d.	n.d.	11,559	75,371	31,591	55,729	37,073
7 (vial 1)	n.d.	n.d.	n.d.	46,869	n.d.	n.d.	28,912
7 (vial 2)	n.d.	n.d.	n.d.	51,496	n.d.	7,121	27,729

Total number of TRBV reads	Peripheral blood			Tonsil			
DONOR ID	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh	Tfh GC	Tfh CXCR3 ⁺	Tfh CXCR3 ⁻	Non-Tfh
1	16,782	36,333	31,742	94,745	97,883	65,386	60,354
2	9,345	17,143	27,817	125,249	76,651	63,444	44,380
3	5,354	13,179	38,453	94,155	78,065	60,526	48,934
4	6,466	19,239	27,921	114,448	95,522	51,691	39,164
5 (vial 1)	38,483	n.d.	31,894	154,330	23,369	38,466	n.d.
5 (vial 2)	23,591	n.d.	40,708	156,435	32,702	39,315	45,630
6 (vial 1)	n.d.	n.d.	18,799	187,665	137,741	108,946	84,030
6 (vial 2)	n.d.	n.d.	20,037	195,133	81,657	148,885	83,690
7 (vial 1)	n.d.	n.d.	n.d.	151,640	n.d.	n.d.	55,830
7 (vial 2)	n.d.	n.d.	n.d.	155,682	n.d.	21,668	57,690

Table S8. Number of TRAV and TRBV reads per subset. (n.d.=not determined).