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Supplemental information

Heterogeneity of human anti-viral immunity shaped by virus, tissue, age, and sex

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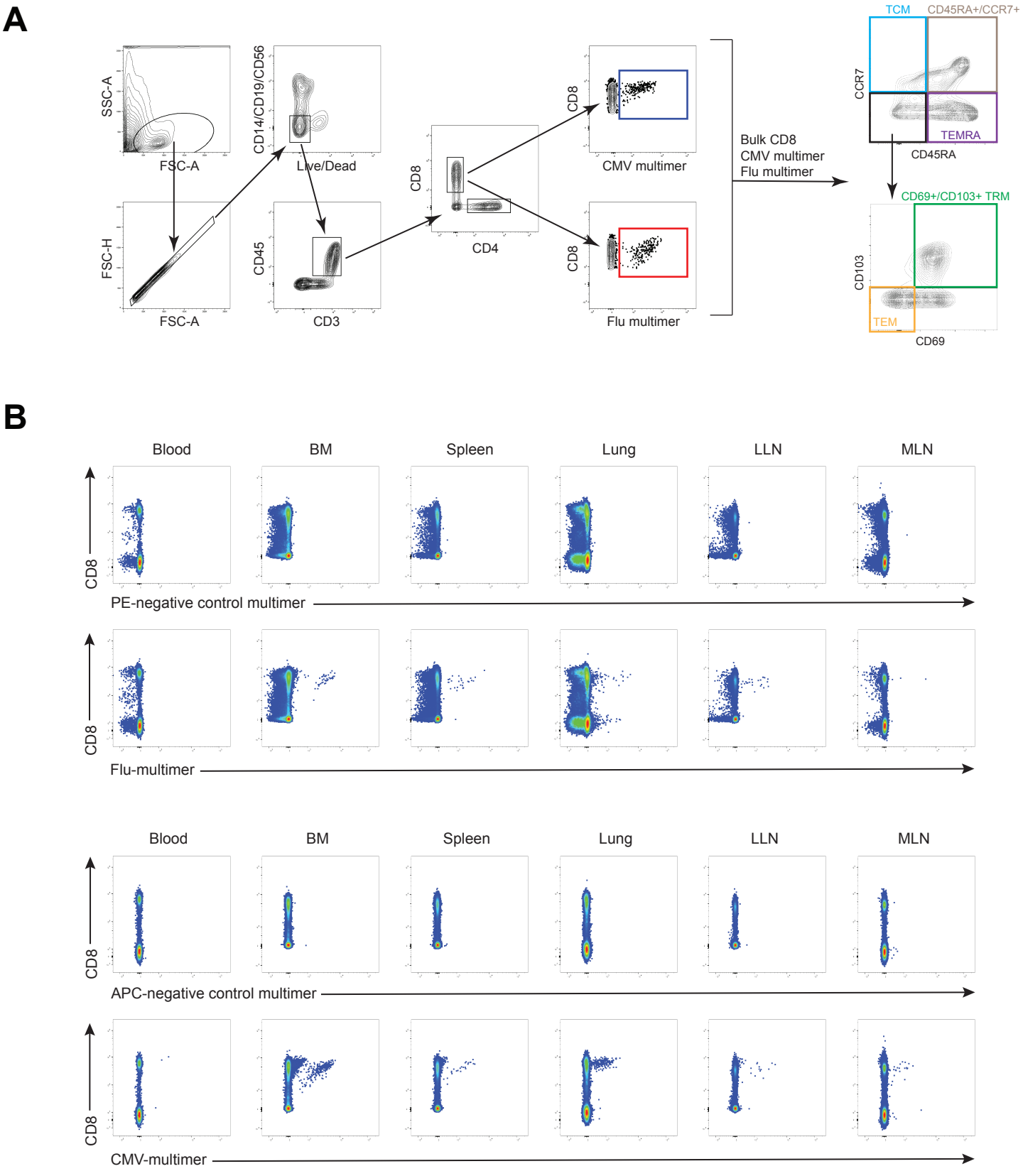

Figure S1. Gating strategy and controls for flow cytometry analysis. Related to Figures 1-6. (A) Gating strategy for multimer$^+$ virus-specific CD8$^+$ T cells gated on live CD45$^+$CD14$^-$CD19$^-$CD56$^-$CD3$^+$CD4$^-$ T cells. Flu- and CMV-specific CD8$^+$ T cells and bulk CD8$^+$ T cells were further characterized based on expression of phenotypic markers CD45RA, CCR7, CD69, and CD103 to identify T cell subsets, gated as shown. (B) Representative flow cytometry plots showing staining using negative control multimer reagents and virus-specific multimer reagents for Flu (top) and CMV (bottom). BM, bone marrow; LLN, lung-draining lymph node; MLN, mesenteric lymph node.

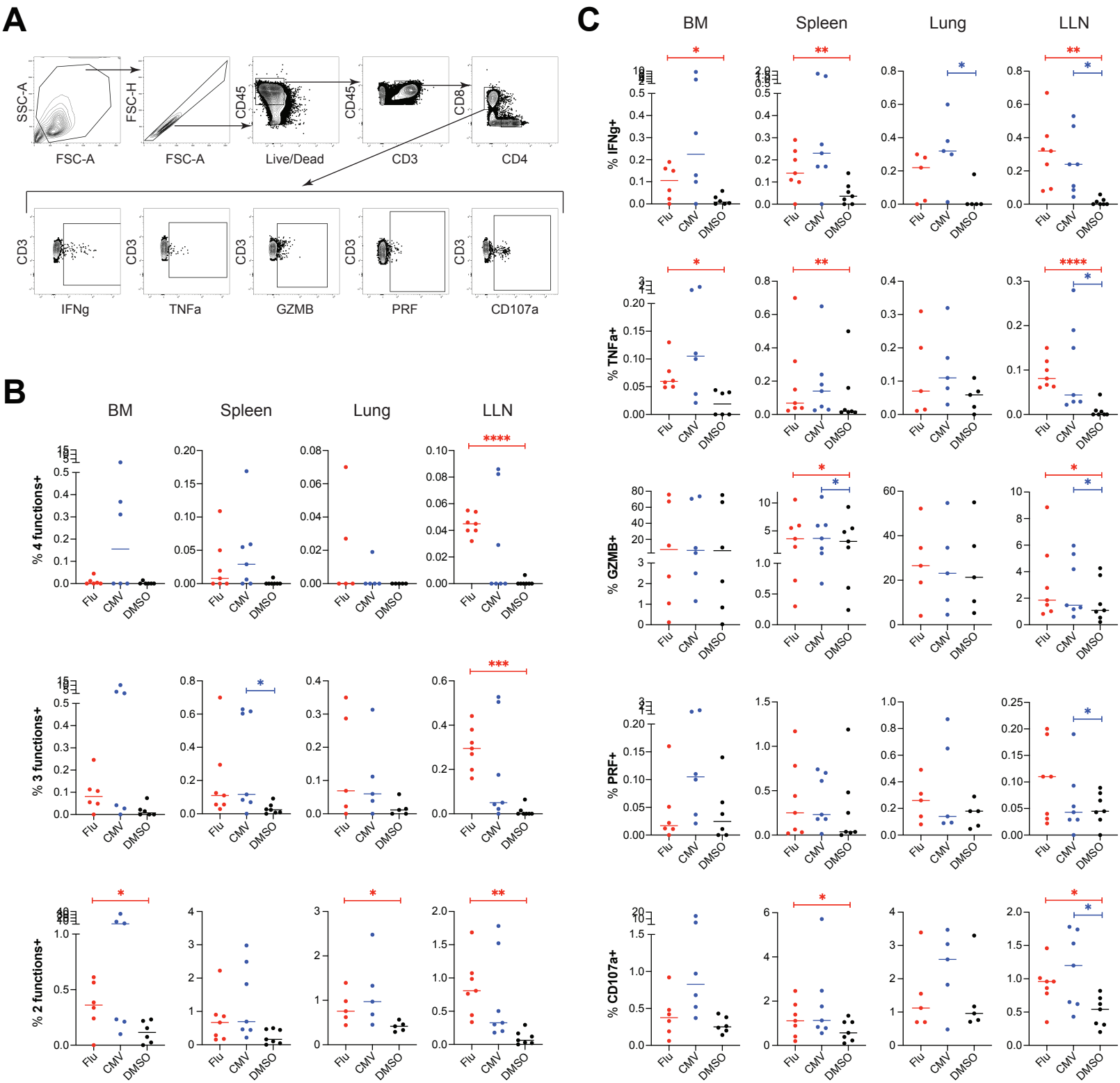

Figure S2. Virus-specific CD8+ T cell responses as measured by cytokine production following antigen stimulation in adult donors. Related to Figure 1. (A) Gating strategy for identification of antigen-responsive CD8+ T cells. CD8+ T cell response was measured via production of cytokines IFN-γ, TNF-α, GZMB, PRF and expression of functional marker CD107a. (B) Dot plots showing frequency of Flu-specific (red) and CMV-specific (blue) CD8+ T cells that exhibit either 4 functions, 3 functions, or 2 functions of CD8+ T cells effector response. (C) Dot plots showing frequency of Flu-specific (red) and CMV-specific (blue) CD8+ T cells that produce IFN-γ, TNF-α, GZMB, PRF or express CD107a. Each dot represents an individual donor. Significant significance for comparison of means was calculated by paired t test between antigen-responsive (either Flu or CMV) versus DMSO control and indicated by ****, $p \leq 0.0001$; ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$. BM, bone marrow; LLN, lung-draining lymph node.

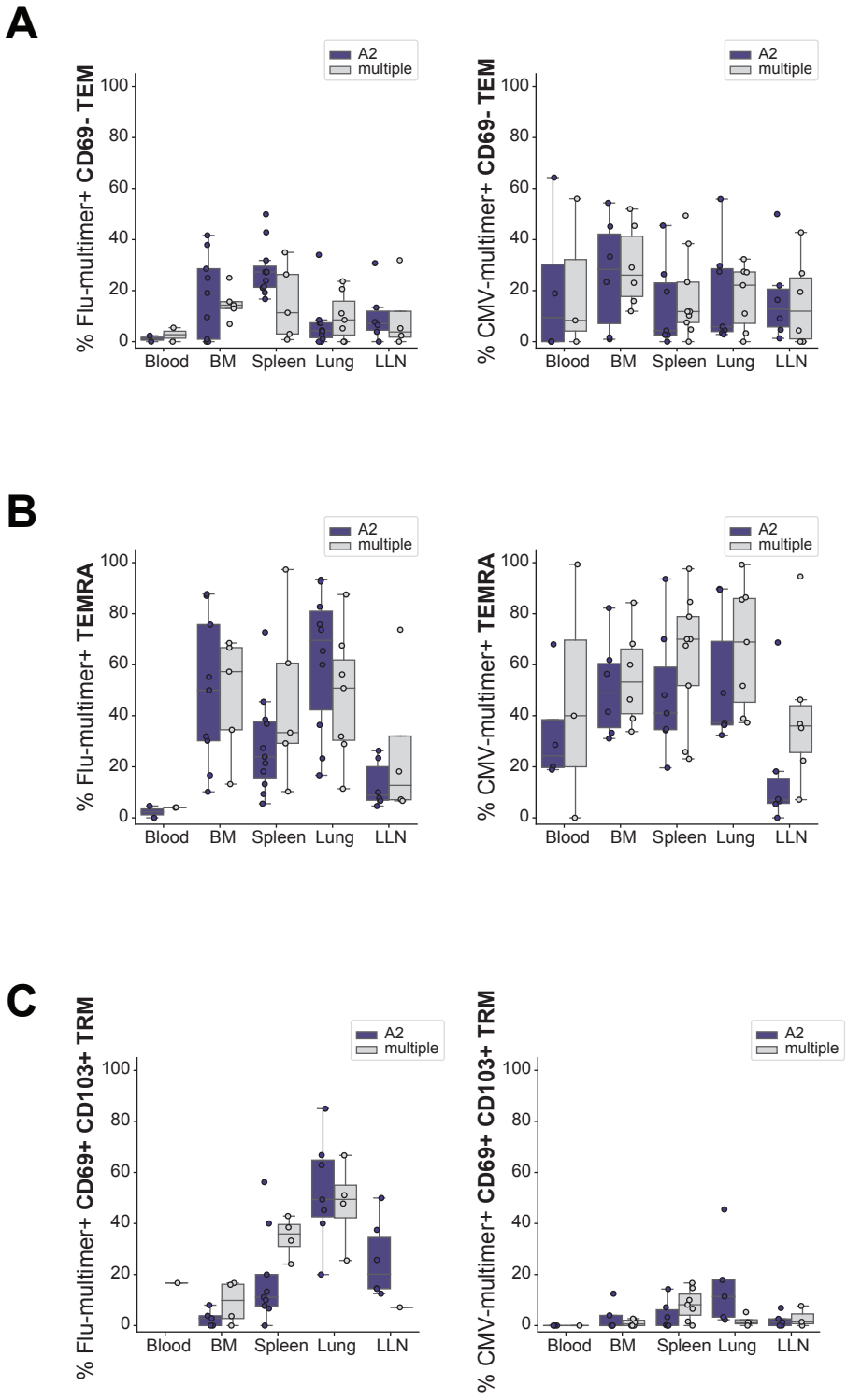

Figure S3. Subset differentiation and tissue residency of antigen-specific CD8$^+$ T cells identified using only HLA-A2 multimers versus a combination of multiple HLA-multimers. Related to Figures 1-3. (A, B) Frequencies of Flu-multimer$^+$ (left) and CMV-multimer$^+$ (right) CD8$^+$ T cells maintained as CD69$^-$ TEM (A) and TEMRA (B) obtained from donors using multimers containing HLA-A2 only (dark gray; n=2-11) compared to using a combination of multiple HLA-multimers (light gray, n=2-9). (C) Frequencies of Flu-multimer$^+$ (left) and CMV-multimer$^+$ (right) CD69$^+$ CD103$^+$ CD8$^+$ TRM obtained from donors using multimers containing HLA-A2 only (dark gray; n=3-9) compared to using a combination of multiple HLA-multimers (light gray, n=1-7). Statistical significance for comparison of means was calculated by unpaired t test. No comparisons were statistically significant. BM, bone marrow; LLN, lung-draining lymph node.

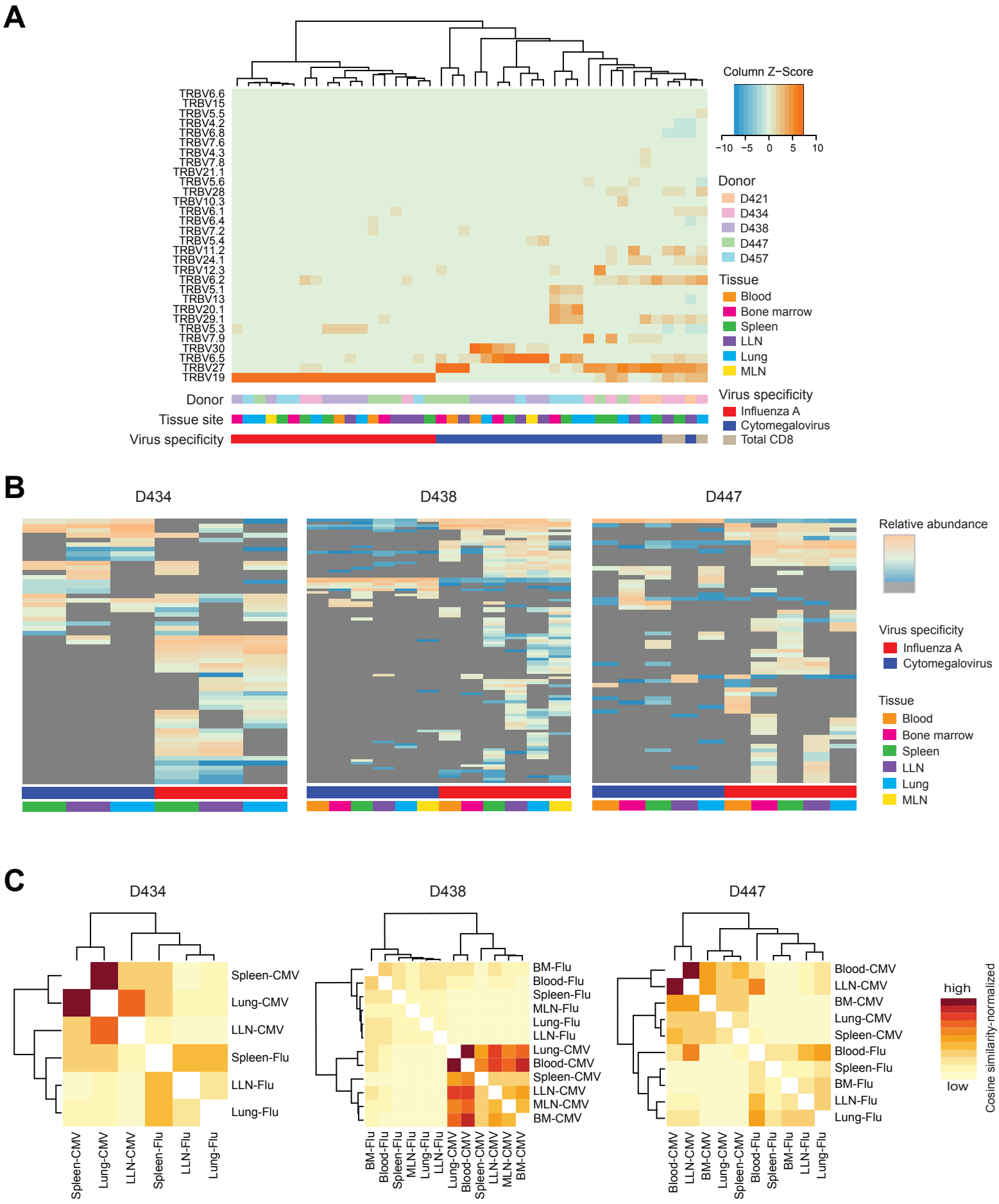
A
TRBV6.6
TRBV15
TRBV5.5
TRBV4.2
TRBV6.8
TRBV7.6
TRBV4.3
TRBV7.8
TRBV21.1
TRBV5.6
TRBV28
TRBV10.3
TRBV6.1
TRBV6.4
TRBV7.2
TRBV5.4
TRBV11.2
TRBV24.1
TRBV12.3
TRBV6.2
TRBV5.1
TRBV13
TRBV20.1
TRBV29.1
TRBV5.3
TRBV7.9
TRBV30
TRBV6.5
TRBV27
TRBV19
Donor
Tissue site
Virus specificity
Column Z−Score
−10 −5 0 5 10
Donor
D421
D434
D438
D447
D457
Tissue
Blood
Bone marrow
Spleen
LLN
Lung
MLN
Virus specificity
Influenza A
Cytomegalovirus
Total CD8
B
D434
D438
D447
Relative abundance
Virus specificity
Influenza A
Cytomegalovirus
Tissue
Blood
Bone marrow
Spleen
LLN
Lung
MLN
C
D434
Spleen-CMV
Lung-CMV
LLN-CMV
Spleen-Flu
LLN-Flu
Lung-Flu
D438
BM-Flu
Blood-Flu
Spleen-Flu
MLN-Flu
Lung-Flu
LLN-Flu
Lung-CMV
Blood-CMV
Spleen-CMV
LLN-CMV
MLN-CMV
BM-CMV
D447
Blood-CMV
LLN-CMV
BM-CMV
Lung-CMV
Spleen-CMV
Blood-Flu
Spleen-Flu
BM-Flu
LLN-Flu
Lung-Flu
high
low
Cosine similarity-normalized

Figure S4. *TRBV* gene usage, clonal overlap, and cosine similarity of virus-specific CD8$^+$ T cell clones across multiple tissue sites. Related to Figure 6. (A) *TRBV* gene usage of CD8$^+$ T cell clones from individual samples with donor, tissue, and virus specificity indicated by color bars beneath each heatmap. Color intensity of each cell is based on column z-score. Each unique clone is counted once per donor. (B) Heatmaps showing the relative abundance of shared clones among Flu-specific and CMV-specific CD8$^+$ T cells in indicated tissues obtained from donor D434 (left), D438 (middle), and D447 (right). Color intensity of each cell is based on the relative abundance of a particular T cell clone within a tissue site. (C) Heatmaps showing cosine similarity between pairwise cell populations of Flu-specific and CMV-specific CD8$^+$ T cell clones in indicated tissues of donors D434 (left), D438 (middle), and D447 (right). Color intensity of each cell is based on the normalization of (min-max scaled) values of samples for a given donor. BM, bone marrow; LLN, lung-draining lymph node; MLN, mesenteric lymph node.

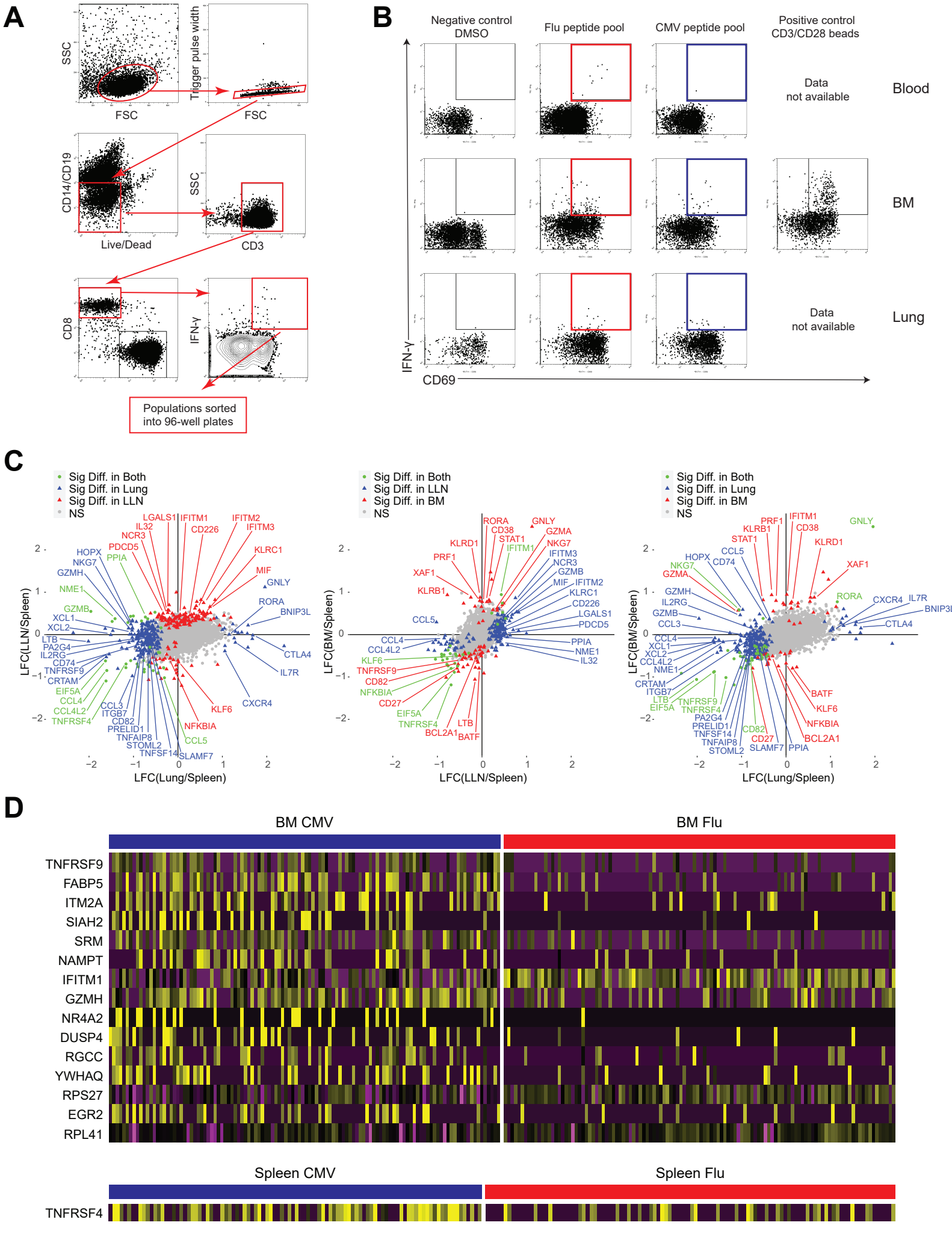
A
SSC
FSC
Trigger pulse width
FSC
CD14/CD19
Live/Dead
SSC
CD3
CD8
IFN-γ
Populations sorted into 96-well plates
B
Negative control DMSO
Flu peptide pool
CMV peptide pool
Positive control CD3/CD28 beads
Data not available
Blood
BM
Data not available
Lung
IFN-γ
CD69
C
Sig Diff. in Both
Sig Diff. in Lung
Sig Diff. in LLN
NS
LFC(LLN/Spleen)
LFC(Lung/Spleen)
Sig Diff. in Both
Sig Diff. in LLN
Sig Diff. in BM
NS
LFC(BM/Spleen)
LFC(LLN/Spleen)
Sig Diff. in Both
Sig Diff. in Lung
Sig Diff. in BM
NS
LFC(BM/Spleen)
LFC(Lung/Spleen)
D
BM CMV
BM Flu
TNFRSF9
FABP5
ITM2A
SIAH2
SRM
NAMPT
IFITM1
GZMH
NR4A2
DUSP4
RGCC
YWHAQ
RPS27
EGR2
RPL41
Spleen CMV
Spleen Flu
TNFRSF4

Figure S5. PLATE-seq analysis of Flu- and CMV-specific CD8$^+$ T cell function following stimulation with viral peptide pools. Related to Figure 7. (A) Strategy for sorting viral antigen-responsive T cells in different sites (live CD14$^-$CD19$^-$CD3$^+$CD4$^-$CD8$^+$CD69$^+$IFN-γ^+ cells) isolated after 24-hour *in vitro* stimulation with Flu- or CMV-specific peptide pools (see methods). (B) Antigen-responding CD69$^+$ IFN-γ^+ CD8$^+$ T cells in the blood, BM, and lung of donor D481 following stimulation by DMSO negative control, Flu antigen-specific peptide pool, CMV antigen-specific peptide pool, and anti-CD3/CD28 bead positive control, shown in representative flow cytometry plots. Colored gates (red: Flu; blue: CMV) indicate populations that were subsequently sorted for sequencing. (C) Biplots showing logFC of LLN/Spleen versus logFC of Lung/Spleen (left), logFC of BM/Spleen versus logFC of LLN/Spleen (middle), and logFC of BM/Spleen versus logFC of Lung/Spleen (right) (see methods). Significantly differentially expressed genes (padj < 0.1) are colored based on tissue site as indicated. See Table S6 for information about differentially expressed genes highlighted in biplots. (D) Heatmaps showing all differentially expressed genes (padj < 0.1) between CMV- and Flu-specific CD69$^+$ IFN-γ^+ CD8$^+$ T cells in BM (top) and spleen (bottom). See Table S7. BM, bone marrow; LLN, lung-draining lymph node.

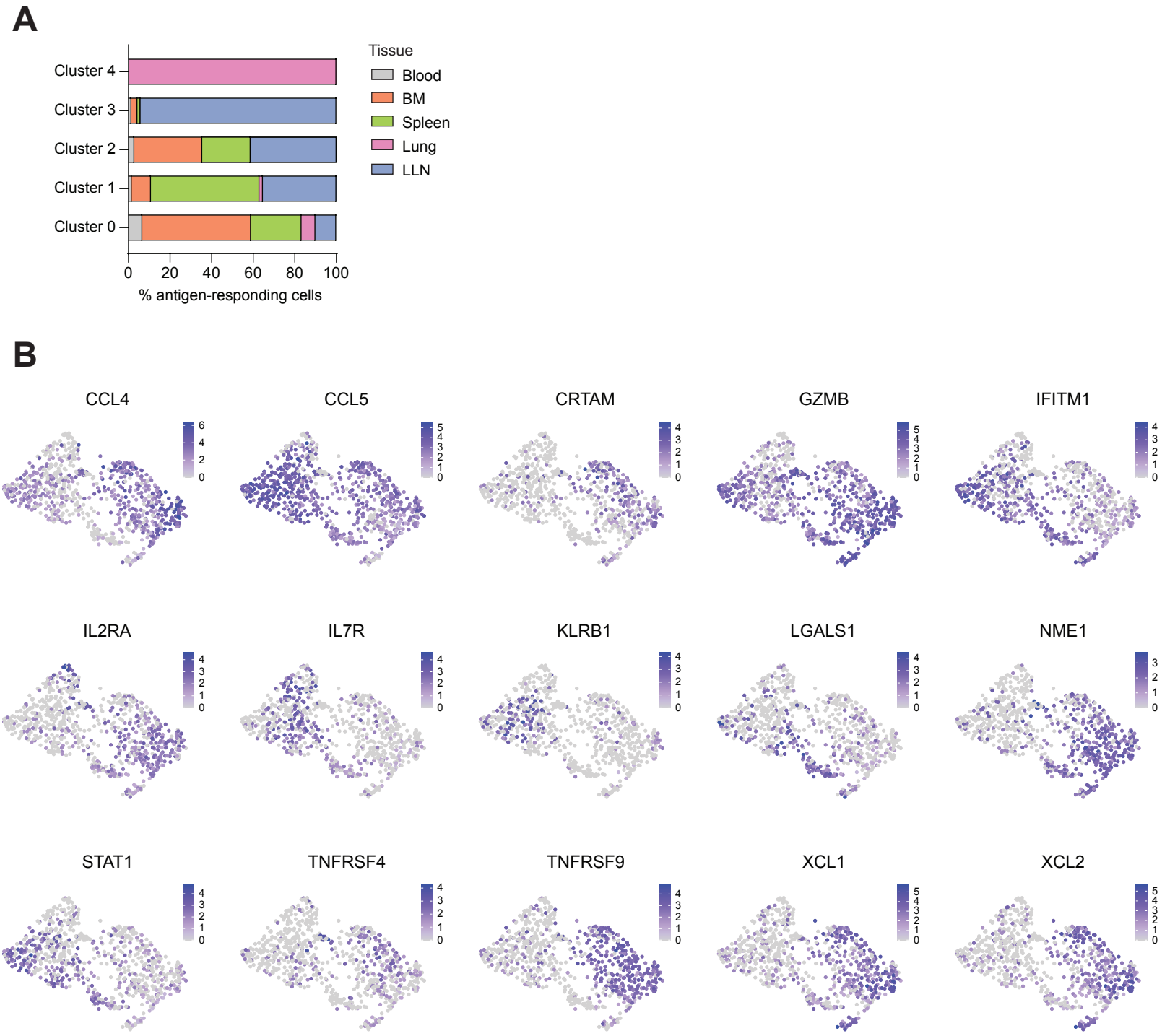

Figure S6. Tissue distribution and gene expression across clusters identified in PLATE-seq analysis of antigen-responding CD8$^+$ T cells. Related to Figure 7. (A) Tissue origins of antigen-responding CD8$^+$ T cells within clusters identified through differential expression analysis and unsupervised clustering. Related to Figure 7C. (B) Separate UMAP embeddings showing indicated gene expression (based on logFC) from antigen-responding CD8$^+$ T cells.

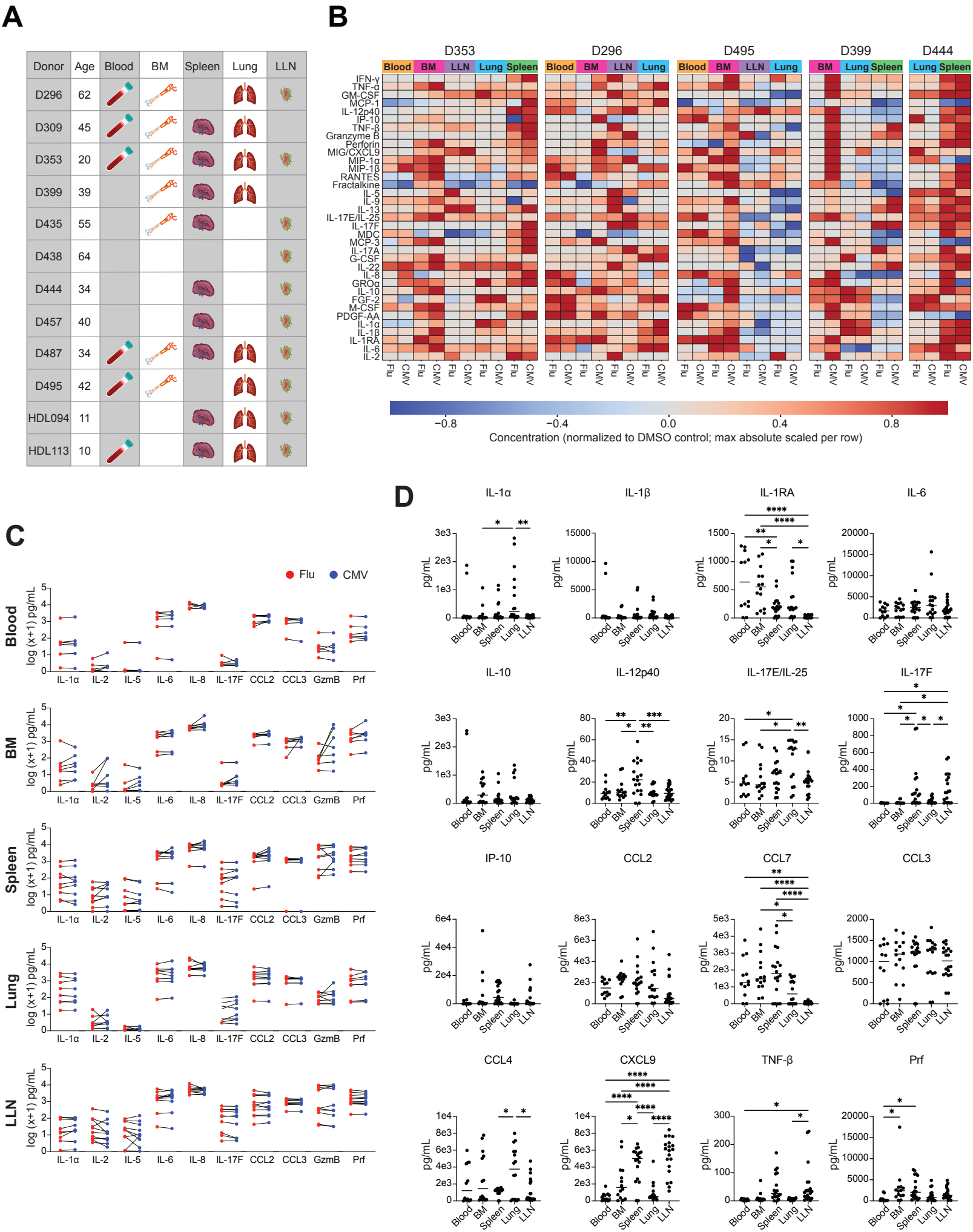
A
Donor
Age
Blood
BM
Spleen
Lung
LLN
D296 62
D309 45
D353 20
D399 39
D435 55
D438 64
D444 34
D457 40
D487 34
D495 42
HDL094 11
HDL113 10
B
D353
D296
D495
D399
D444
Blood
BM
LLN
Lung
Spleen
IFN-γ
TNF-α
GM-CSF
MCP-1
IL-12p40
IP-10
TNF-β
Granzyme B
Perforin
MIG/CXCL9
MIP-1α
MIP-1β
RANTES
Fractalkine
IL-5
IL-9
IL-13
IL-17E/IL-25
IL-17F
MDC
MCP-3
IL-17A
G-CSF
IL-22
IL-8
GROα
IL-10
FGF-2
M-CSF
PDGF-AA
IL-1α
IL-1β
IL-1RA
IL-6
IL-2
Flu
CMV
−0.8
−0.4
0.0
0.4
0.8
Concentration (normalized to DMSO control; max absolute scaled per row)
C
Flu
CMV
Blood
BM
Spleen
Lung
LLN
log (x+1) pg/mL
IL-1α
IL-2
IL-5
IL-6
IL-8
IL-17F
CCL2
CCL3
GzmB
Prf
D
IL-1α
IL-1β
IL-1RA
IL-6
IL-10
IL-12p40
IL-17E/IL-25
IL-17F
IP-10
CCL2
CCL7
CCL3
CCL4
CXCL9
TNF-β
Prf
pg/mL
Blood
BM
Spleen
Lung
LLN

Figure S7. Cytokine production by antigen-responding T cells following stimulation with Flu and CMV CD8 peptide pools. Related to Figure 7. (A) Diagram outlining supernatant samples collected following *in vitro* stimulation with Flu or CMV CD8 peptide pools of single-cell suspensions from the blood, BM, spleen, lung, and LLN of 12 individual donors. (B) Heatmaps showing cytokine production profiles across multiple tissue sites within individual donors for five representative donors. Color intensity of each cell represents cytokine concentration normalized to DMSO negative control and max absolute scaled per row within each donor, with values ranging from -1 to 1 across all analytes. (C) Pairwise comparisons of log(x+1) normalized cytokine levels in supernatants from following *in vitro* stimulation of single-cell suspensions from blood (n=6), BM (n=7), spleen (n=9), lung (n=8), and LLN (n=10) with Flu (red) or CMV (blue) peptide pools. No comparison shown was statistically significant. (D) Cytokine levels in blood, BM, spleen, lung, LLN supernatant samples. Statistical significance was calculated using one-way ANOVA followed by Tukey's multiple comparisons test indicated by ****, $p \leq 0.0001$; ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p \leq 0.05$. BM, bone marrow; LLN, lung-draining lymph node.

Donor	Age (yrs)	Sex	Ethnicity/race	HLA-A	HLA-B	CMV serostatus	Assays performed*
D25	50	F	White	2, 11	7, 65	+	Flu/CMV multimer
D97	40	M	Black/African American	2, 30	42, 58	+	Flu/CMV multimer
D117	32	M	Hispanic/Latino	1, 2	35, 51	+	Flu/CMV multimer
D178	51	M	Black/African American	2, 30	52, 63	-	Flu multimer
D194	53	M	Asian	24, 29	7, 52	+	Flu/CMV multimer
D210	37	M	Hispanic/Latino	2, 24	35, 51	+	Flu/CMV multimer
D217	49	M	Black/African American	68, 68	7, 42	+	Flu/CMV multimer
D226	66	F	White	1, 3	13, 35	+	Flu/CMV multimer
D238	21	M	White	1, 2	7, 8	-	Flu multimer
D245	50	F	Black/African American	1, 74	7, 8	+	Flu/CMV multimer
D282	32	F	Hispanic/Latino	24, 32	35, 49	+	CMV multimer
D289	58	M	White	2, 24	44, 49	+	Flu/CMV multimer
D296	62	F	Black/African American	2, 3	7, 13	+	Flu/CMV multimer, cytokine profile
D309	45	F	Black/African American	Not given	Not given	+	Cytokine profile
D324	56	M	White	2, 32	13, 35	+	Flu/CMV multimer
D331	32	M	Black/African American	2, 24	51, 60	-	Flu multimer
D353	20	M	Hispanic/Latino	24, 24	35, 37	+	Cytokine profile
D362	37	F	White	2, 2	7, 62	+	Flu/CMV multimer
D374	62	F	White	2, 2	8, 78	+	Flu/CMV multimer
D376	28	F	Black/African American	1, 6601	44, 44	-	Flu multimer
D382	62	F	Asian	2, 11	46, 56	+	Flu/CMV multimer
D393	55	F	Hispanic/Latino	2, 2	18, 44	+	Flu/CMV multimer
D399	39	F	White	2, 66	7, 58	+	Flu/CMV multimer, cytokine profile
D400	18	M	White	2, -	44, 57	+	Flu/CMV multimer
D416	37	F	White	24, 26	7, 38	+	Flu/CMV multimer
D417	78	M	Black/African American	2, 24	41, 57	+	Flu/CMV multimer
D418	52	M	Black/African American	2, 68	35, 50	-	Flu multimer
D420	34	M	White	2, 30	13, 27	+	Flu/CMV multimer
D421	18	F	Hispanic/Latino	3, 29	7, 44	+	Flu/CMV multimer
D426	43	F	Hispanic/Latino	3, 68	7, 27	+	Flu/CMV multimer
D431	64	F	Black/African American	2, 33	53, 65	+	Flu/CMV multimer
D434	46	F	White	2, 24	7, 44	+	Flu/CMV multimer
D435	55	F	White	1, 3	7, 44	+	Flu/CMV multimer, cytokine profile
D438	64	M	Black/African American	2, 23	35, 44	+	Flu/CMV multimer, cytokine profile, TCRseq
D444	34	F	Black/African American	2, 34	64, 35	+	Flu/CMV multimer, cytokine profile, TCRseq
D445	47	M	Black/African American	2, 33	63, 53	+	Flu/CMV multimer
D447	40	F	Hispanic/Latino	2, 68	39, 44	+	Flu/CMV multimer, TCRseq
D457	40	M	Asian	2, 24	62, 77	+	Flu/CMV multimer, cytokine profile, TCRseq
D459	52	F	Black/African American	1, 3	53, 53	+	Flu/CMV multimer
D481	29	M	Hispanic/Latino	3, 29	35, 44	+	PLATE-seq
D487	34	M	Black/African American	2, 30	45, 65	+	Flu/CMV multimer, cytokine profile
D495	42	M	Black/African American	23, 30	72. 45	+	Cytokine profile
Pediatric Donors							
D143	6	M	White	2, 3	7, 57	-	Flu multimer
D258	1	M	White	1, 31	8, 60	-	Flu multimer
P003	9	F	White	1, 24	7, 8	+	Flu multimer
HDL052	9	M	White	2, 24	7, 64	-	Flu multimer
HDL053	2	M	White	2, 11	35, 60	+	Flu multimer
HDL060	3	M	White	2, 24	18, 44	-	Flu multimer
HDL062	4	F	White	2, 3	62, 47	-	Flu multimer
HDL069	6	M	Hispanic/Latino	1, 2	8, 53	-	Flu multimer
HDL072	5	M	White	1, 24	7, 50	-	Flu multimer
HDL081	8	M	White	2, 3	7, 62	+	Flu multimer
HDL094	11	M	White	1, 2	27, 37	+	Flu multimer, cytokine profile
HDL098	10	M	Hispanic/Latino	2, 2	35, 52	+	Flu multimer
HDL099	4	M	White	1, 2	13, 62	+	Flu multimer
HDL101	9	M	Unknown	2, 3	7, 45	-	Flu multimer
HDL102	2	F	Hispanic/Latino	2, 24	44, 61	+	Flu multimer
HDL113	10	M	Hispanic/Latino	2, -	51, 52	+	Flu multimer, cytokine profile
*Multimer assays include phenotyping for T cell subset differentiation and tissue residency							

Table S1. Related to Figures 1- 7. Information about donors in this study.

Sample name	# of cells sorted	# of clones	DNA (ng)
D421_Spleen_CMV	4000	324	5.93
D421_LLN_CMV	3000	1407	14.43
D421_Lung_CMV	54000	1017	89.70
D434_Spleen_CMV	361	47	1.03
D434_Spleen_Flu	444	61	1.47
D434_Spleen_BulkCD8	10000	1672	19.37
D434_LLN_CMV	343	90	<1
D434_LLN_Flu	838	108	1.69
D434_LLN_BulkCD8	10000	2484	18.07
D434_Lung_CMV	296	30	<1
D434_Lung_Flu	1233	102	3.04
D434_Lung_BulkCD8	10000	1144	18.20
D438_Blood_CMV	572	31	<1
D438_Blood_Flu	3769	86	2.78
D438_BM_CMV	646	51	<1
D438_BM_Flu	1366	56	1.68
D438_Spleen_CMV	1575	78	2.13
D438_Spleen_Flu	6096	155	7.80
D438_LLN_CMV	159	39	<1
D438_LLN_Flu	2906	107	1.94
D438_Lung_CMV	494	59	<1
D438_Lung_Flu	17071	184	17.16
D438_MLN_CMV	1842	68	1.74
D438_MLN_Flu	8049	199	8.45
D447_Blood_CMV	330	28	<1
D447_Blood_Flu	108	33	<1
D447_BM_CMV	2300	58	2.98
D447_BM_Flu	1185	134	<1
D447_LLN_CMV	180	38	<1
D447_LLN_Flu	240	56	<1
D447_Lung_CMV	366	66	<1
D447_Lung_Flu	2448	63	2.33
D447_Spleen_CMV	199	70	<1
D447_Spleen_Flu	633	140	<1
D457_BM_CMV	38683	350	12.98
D457_BM_Flu	3249	285	6.83
D457_LLN_CMV	15175	429	27.00
D457_LLN_Flu	9251	1000	4.80
D457_Lung_CMV	31935	975	9.30
D457_Lung_Flu	47451	691	8.93
D457_Spleen_CMV	17818	565	31.73
D457_Spleen_Flu	3022	286	39.75

Table S2. Related to Figure 6. TCR sequencing sample names, cells sorted, number of clones, and DNA.