Supplemental Information

Supplemental Methods:

Generation of humanized mice

NOD-scid common cytokine gamma chain knockout (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) (NSG) mice were obtained from the Jackson Laboratory and housed in a specific pathogen-free microisolator environment. Discarded human fetal thymus and liver tissues (gestational age 17 to 20 weeks) were obtained from Advanced Biosciences Resource. Fetal thymus fragments were cryopreserved in 10% dimethyl sulfoxide and 90% human AB serum (Atlanta Biologicals). In Experiment 1, three NSG mice were sublethaly irradiated (100cGy) and injected i.v with 2x10^5 human fetal liver (FL)-derived CD34$^+$ cells (referred to as hematopoietic stem cells, HSCs) (Figure 1A). Autologous human fetal thymus fragments measuring about 1 mm^3 were cryopreserved, thawed and transplanted under the kidney capsule of these mice, as described [16]. In Experiment 2, six mice received i.v injection of 2x10^5 human FL-derived CD34$^+$ HSCs from another donor. Three of these mice (mice 2autoA, 2autoB and 2autoC) received autologous human fetal thymus and the other three (mice 2alloA, 2alloB and 2alloC) received an allogeneic human fetal thymus transplant (Figure 1B). In Experiment 3, two NSG mice were thymectomized, sublethaly irradiated (100cGy) and injected i.v with 2x10^5 human fetal liver (FL)-derived CD34$^+$ HSCs from a different donor than those used in Experiments 1 and 2 (Figure 1C). To ensure that the transplanted donor thymus T cells were not able to persist, we froze and thawed the thymus tissues and also physically removed residual cells by repeated pipetting up and down before transplantation. To further deplete passenger thymocytes that might migrate to the periphery and limit allogeneic HSC engraftment, an anti-human CD2 antibody was injected to the mice in 2 weekly doses (400µg/mouse, i.p) as we have described [16]. For analysis of human reconstitution, mice were bled at regular intervals for FCM analysis of human T cells, B cells and monocytes and their naïve/memory state.

FACS sorting of different subsets of grafted thymus and peripheral cells

At weeks 14, 20 and 22 after thymus transplantation, mice from Experiments 1, 2 and 3, respectively, were euthanized. Grafted thymi (for mice in all experiments) and spleen and lymph nodes (LNs) (only mice in Experiment 3) were harvested and the thymocytes and pooled spleen and LN cells were isolated by physical force (crushing the thymus tissue between two slides and crushing the spleen and LNs through a 70μm cell strainer using a syringe plunger). After counting the total number of cells, they were stained with the following antibodies for FACS sorting: anti-human CD3 (PerCP-Cy5.5), anti-human CD5 (FITC), anti-human CD4 (PE-Cy7), anti-human CD8 (APC-Cy7), anti-human CD25 (PE) and anti-human CD127 (BV421). In Experiment 2, besides staining with these antibodies, a portion of cells were stained in a separate tube with the following markers: anti-human CD3 (PerCP-Cy5.5), anti-human CD4 (PE-Cy7), anti-human CD8 (APC-Cy7), anti-human CD69 (BV650) and anti-human TCRα/β (PE). In both experiments, after gating out the dead cells and doublets, Tregs, single positive (SP) CD8 cells and Treg-depleted SP CD4 cells were sorted within a $CD3^+$ $CD5^+$ gate. Tregs were sorted as $CD8^-$ $CD25^{high}$ $CD127^-$ $CD4^+$ cells (Figure S2A). In Experiment 2, the cells in the second tube were first gated out for doublets and dead cells. Within the population of CD4 and CD8 double positive (DP) cells, $CD69^+$ $TCR\alpha/\beta^{high}$ cells were sorted as positively-selected DP cells. The remaining DP cells were sorted as non-selected DP cells (Figure S2B). Thymic SP cells in Experiment 3 were sorted with the same panel as in Experiment 2. To sort peripheral (pooled spleen and LN) CD4 and CD8 cells in Experiment 3, after gating out the dead cells and doublets, CD4 and CD8 cells were sorted within a $CD3^+$ gate. Sorting was done using a BD Influx cell sorter. The purity of sorted cells was %90-%96 for different cell subsets (Figure S2C).

Single cell TCR sequencing

Single cell TCR sequencing was performed according to the manuals provided by the 10X Genomics company (Chromium Single Cell 5' Library & Gel Bead Kit, PN-1000006). Briefly, after sorting thymic SP-CD4 cells, 17,000 cells from each thymus graft were loaded into the chip along with partitioning oil,

the Gel beads and a master mix containing RT enzyme and poly-dt RT primers. The assembled chips were placed into the Chromium Controller, where the cells are mixed with the beads, master mix reagents and oil. Gel Beads-in-emulsion (GEMs) were generated, where all generated cDNAs shared a common 10x Barcode. After cDNA amplification and TCR locus target enrichment, enriched libraries were constructed. Each sample was indexed with a unique barcode for each well of the Chromium i7 Index Plate. After quantifying the amplified DNA using a Bioanalyser, the same amount of DNA from different samples was pooled and sequenced with an Illumina NextSeq machine. The output files were converted to FASTQ files using the Cell Ranger pipeline. The Loupe V(D)J Browser was used for preliminary analysis within each sample. Further analysis to compare different samples was done in R. The vloupe files of the single cell TCR sequences are available at https://github.com/Aleksobrad/Humanized-Mouse-Data.

Computational and statistical analysis

Adaptive ImmunoSeq performs PCR amplification, read sequencing, and mapping, with bias correction and internal controls. These analyses return tabulated read counts corresponding to unique clonal CDR3 DNA sequences across all samples, and including information on the CDR3 amino acid sequence and VJ usage of these clones. From this, we normalize read counts to frequency of clonal expression for each sample on the level of distinct CDR3 nucleotide sequence, distinct CDR3 amino acid sequence, and distinct V-J pair. This repertoire characterization process is done separately for read-count tables of productive clones and nonproductive clones, which are identified as being out of frame or including a stop codon.

For each sample, then, we generate clone frequency tables at the level of non-productive nucleotide sequence, productive nucleotide sequence, amino acid sequence, and VJ usage. Template counts, clonality scores, unique clone counts and entropies for each sample were calculated. Templates are cell count estimates for each clone, derived by Adaptive ImmunoSeq in their TCR-sequencing pipeline. Each unique TCR DNA-sequence in the repertoire (unique clone) may be represented by multiple

sequenced templates, with a greater number of templates indicating a higher-frequency clone. In every sample, clonality is calculated as an inverse measure of repertoire diversity, in order to ensure that repertoires are comparable. Clonality is entropy normalized for the number of clones N, where: $\forall i\ with\ frequency\ p_i, H_{obs} = \sum p_i \log_2 p_i\ , H_{max} = \sum \frac{1}{N} \log_2 \frac{1}{N} and\ clonality = 1 - H_{obs}/H_{max}$ such that clonality of 1 indicates a single dominant clone, and clonality of 0 indicates uniform distribution of clone frequencies. Our definition of clonality is based on CDR3β sequences and not the entire TCR β chain. Entropy (H) is a measure of diversity in a system, such that high-frequency clones in the repertoire decrease entropy and entropy for a sample is maximized if all clones are present at the same frequency. It is not a normalized metric and has no upper bound. Entropy is expected to be larger for larger samples, so only samples with similar numbers of unique clones can be compared in terms of relative diversity using entropy

We compared repertoires for the same cell population across mice using shared clone fraction, a non-symmetric measure such that the shared clone fraction of repertoire p compared to repertoire q is equal to the number of clonotypes present in both repertoires divided by the total number of unique clones defined in repertoire p. We alternately compared repertoires defined by their CDR3 non-productive nucleotide sequences, CDR3 productive nucleotide sequences, and CDR3 amino acid sequences for each thymic sub-population in both experiments. We also performed systematic comparison of repertoires using the Jensen-Shannon Divergence (JSD), which accounts for clone frequencies and scales for repertoire sizes. JSD is an information theory-based measure of the divergence of TCR repertoires. This is a symmetric value defined for any two repertoires p and q as: $JSD(p,q) = H_{obs}\left(0.5 * (p+q)\right) - 0.5 * (H_{obs}(p) + H_{obs}(q))$. JSD values range between 0 and 1, where 0 indicates identical repertoires, and 1 indicates complete divergence. For both shared clone fraction and JSD, we established a statistical baseline to distinguish any observed repertoire divergences across samples from divergence due to under-sampling of rare clones. This was done by ¼ sub-sampling (with replacement) of each repertoire 100 times, and computing mean and standard deviation of divergence by JSD and clone fraction when comparing all subsamples drawn from the same sample, thus

approximating divergence due to repertoire under-sampling and capturing any potential biases towards lower divergence across thymic sub-populations due to the presence of dominant high-frequency clones. All repertoire comparisons were validated for robustness to sample size differences by sub-sampling repertoires to the same low template count (2000 templates) three times each and repeating comparisons made between whole samples across the sub-samples.

We further plot the V and J gene frequencies across samples per cell population. Mann-Whitney U-tests are performed comparing the V and J distributions of different samples, as well as the observed distributions of combined VJ frequencies to the frequencies expected by stochastic pairing of 60 possible V genes with 13 possible J genes according to the background frequency of each V and J.

To identify correlations between amino acid use at P6 or P7 and hydrophobicity, the amino acid sequence data provided by Adaptive Immunoseq were tabulated for each of the five cell populations (DPCD69^{-}, DPCD69^{+}, SP CD4, SP CD8, SP Treg) in each animal and the amino acid and the corresponding relative frequency at P6 and at P7 was recorded for each of the CDR3β lengths. These frequencies were normalized such that the sum of all the amino acids within a given cell population and given CDR3β length in each mouse is one. These frequencies were subsequently chain-length matched, and the fold-change value was obtained as the ratio of the amino acid's relative frequency in cell population 2 to its relative frequency in cell population 1. The average fold change of the amino acid was determined as the numerical average of the fold changes across the mice.

To identify motifs at the sequence level comparing sequences shared between any two mice for a given cell population and sequences unique to a single mouse, a length-matched unshared sequences dataset of the same size as the shared sequences dataset was generated for each population by randomly selecting a sequence of the same length from the unshared sequence set for each sequence in the shared sequence set. Methods from Greiff et al. [27], which successfully distinguished between public and private antibody repertoires were applied to this dataset to identify subsequence level

features which can be used to distinguish between shared and unshared sequences. This method uses normalized gapped k-mer (two subsequences of length k, separated by a gap of up to m amino acids) count as an input to a support vector machine to predict shared/unshared status. SVM analysis was run using k = 1, m = 1 and cost = 100, and 10 fold cross-validation was performed to assess performance of the classifier, using balanced accuracy (mean of sensitivity and specificity) as a performance metric. This was repeated on 10 length-matched datasets generated as described above. To analyze differential usage of amino acids at each position as defined by IMGT, Fisher's exact test was performed for all sequences in one length matched dataset of shared and unshared sequences. Frequency differences of amino acid and position combinations were analyzed and plotted for all cases where $p < 0.05$ by Fisher's exact test.

Supplementary Figures:

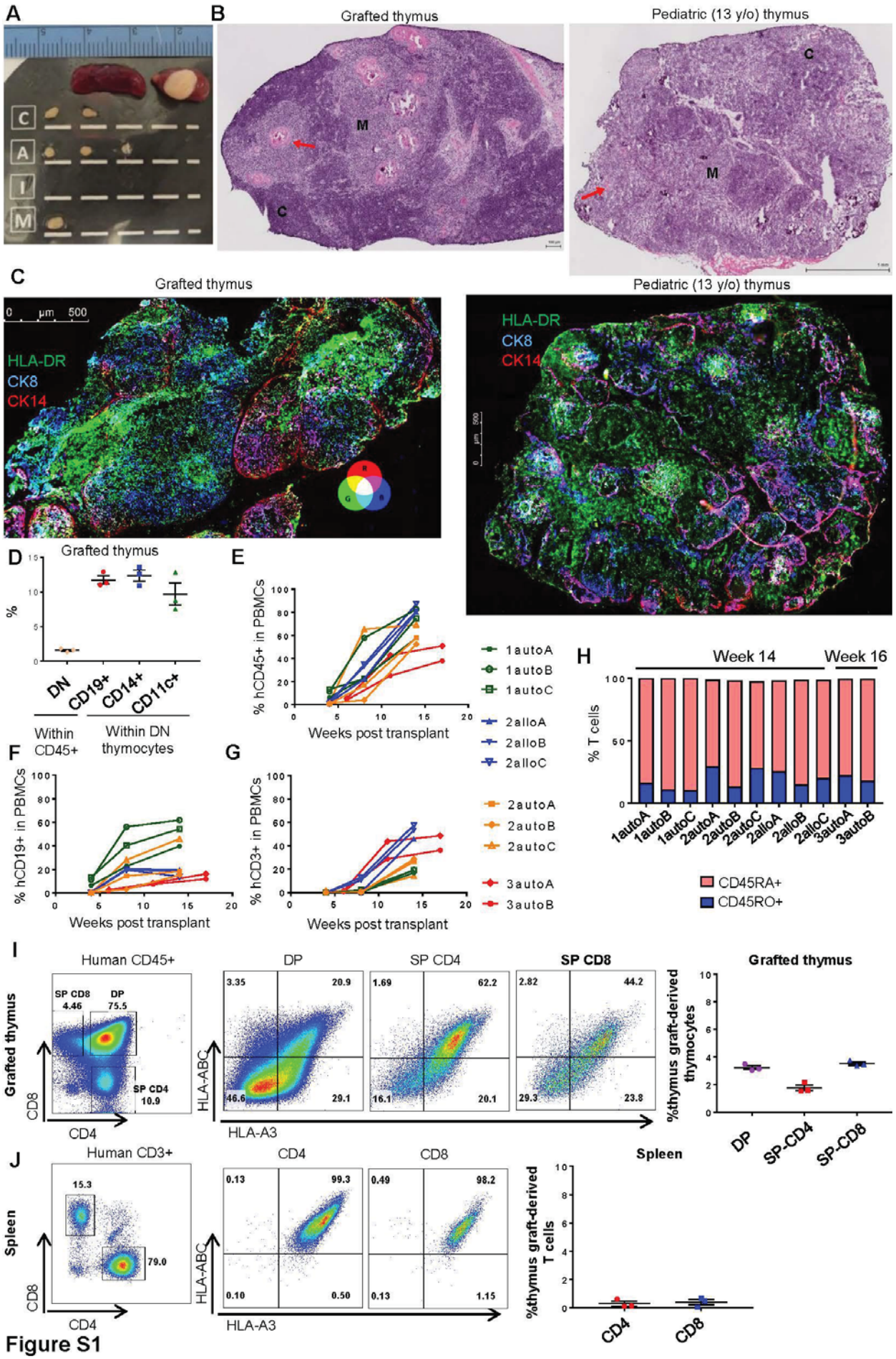

A
B
Grafted thymus
Pediatric (13 y/o) thymus
C
M
C
C
Grafted thymus
Pediatric (13 y/o) thymus
0 µm 500
HLA-DR
CK8
CK14
HLA-DR
CK8
CK14
D
Grafted thymus
%
DN
CD19+
CD14+
CD11c+
Within CD45+
Within DN thymocytes
E
% hCD45+ in PBMCs
Weeks post transplant
1autoA
1autoB
1autoC
2alloA
2alloB
2alloC
2autoA
2autoB
2autoC
3autoA
3autoB
F
% hCD19+ in PBMCs
Weeks post transplant
G
% hCD3+ in PBMCs
Weeks post transplant
H
Week 14
Week 16
% T cells
1autoA
1autoB
1autoC
2autoA
2autoB
2autoC
2alloA
2alloB
2alloC
3autoA
3autoB
CD45RA+
CD45RO+
I
Human CD45+
DP
SP CD4
SP CD8
Grafted thymus
SP CD8
4.46
DP
75.5
SP CD4
10.9
CD8
CD4
HLA-ABC
HLA-A3
3.35
20.9
46.6
29.1
1.69
62.2
16.1
20.1
2.82
44.2
29.3
23.8
Grafted thymus
%thymus graft-derived thymocytes
DP
SP-CD4
SP-CD8
J
Human CD3+
CD4
CD8
Spleen
15.3
79.0
CD8
CD4
HLA-ABC
HLA-A3
0.13
99.3
0.10
0.50
0.49
98.2
0.13
1.15
Spleen
%thymus graft-derived T cells
CD4
CD8

Figure S1

Figure S1. Grafted human thymus structure, the kinetics of development of human immune cells and the level of thymus graft-derived T cells. A shows a grafted human thymus under the kidney capsule, in addition to the spleen and LNs (cervical (C), axillary (A), Inguinal (I) and mesenteric (M)) of the same humanized mouse at the time of harvest (24 weeks post transplantation). B shows H&E staining of a grafted human thymus. Cortical (hypercellular areas (C)) and medullary (hypocellular areas (M)) areas as well as Hassall's corpuscles (shown by red arrows) are evident. C shows immunofluorescent staining of a grafted human thymus stained with antibodies to CK8, CK14 and HLA-DR. D shows the percentage of $CD4^-$ $CD8^-$ (double negative, DN) cells among human $CD45^+$ cells of each grafted human thymus and the percentage of human B cells($CD19^+$), monocytes($CD14^+$) and dendritic cells($CD11c^+$) among DN cells. E-H show the kinetics of development of human immune cells ($hCD45^+$), B cells ($CD19^+$) and T cells ($CD3^+$) in peripheral blood as well as the T cell naïve/memory phenotype. I and J show the level of thymus graft-derived T cells ($HLA\text{-}ABC^+$ $HLA\text{-}A3^-$) in the grafted thymi and spleens at 24 weeks of humanized mice generated with allogeneic fetal HSCs ($HLA\text{-}A3^+$) and thymus tissue ($HLA\text{-}A3^-$) (n=3).

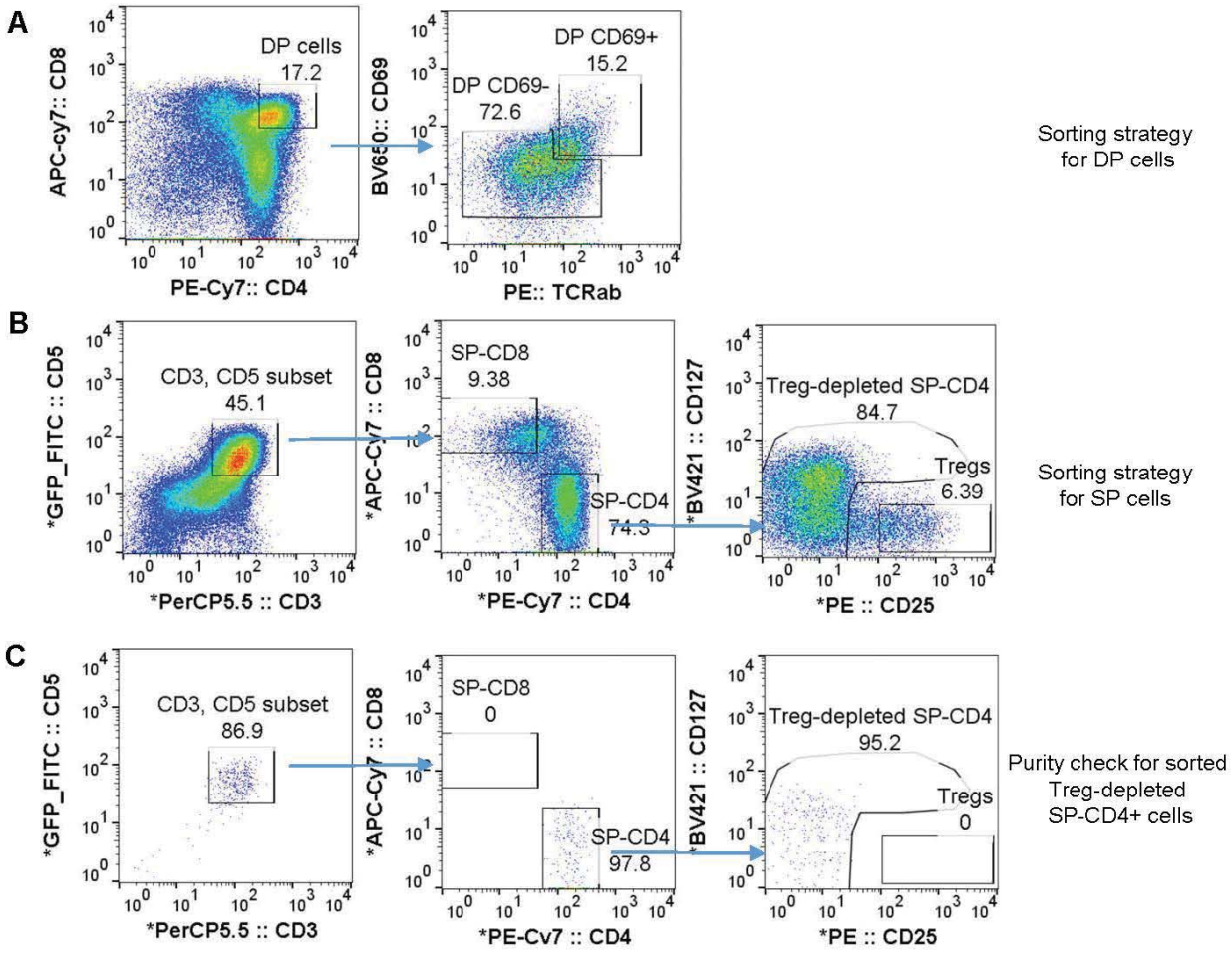


Figure S2. Sorting strategy and purity check. A shows the sorting strategy for DP cells. After gating out the dead cells and doublets, within the population of double positive (DP) CD8 and CD4 cells, $CD69^{+}$ $TCR\alpha/\beta^{high}$ cells were sorted as positively selected DP cells. The remaining DP cells were sorted as non-selected-DP cells as shown. B shows the sorting strategy for SP cells. First, dead cells and doublets were gated out. Tregs, SP CD8 cells and Treg-depleted SP CD4 cells were sorted within a $CD3^{high}$ $CD5^{high}$ gate. Tregs were sorted as $CD25^{high}$ $CD127^{-}$ $CD4^{+}$ cells. C shows the purity of sorted Treg-depleted SP-CD4 cells.

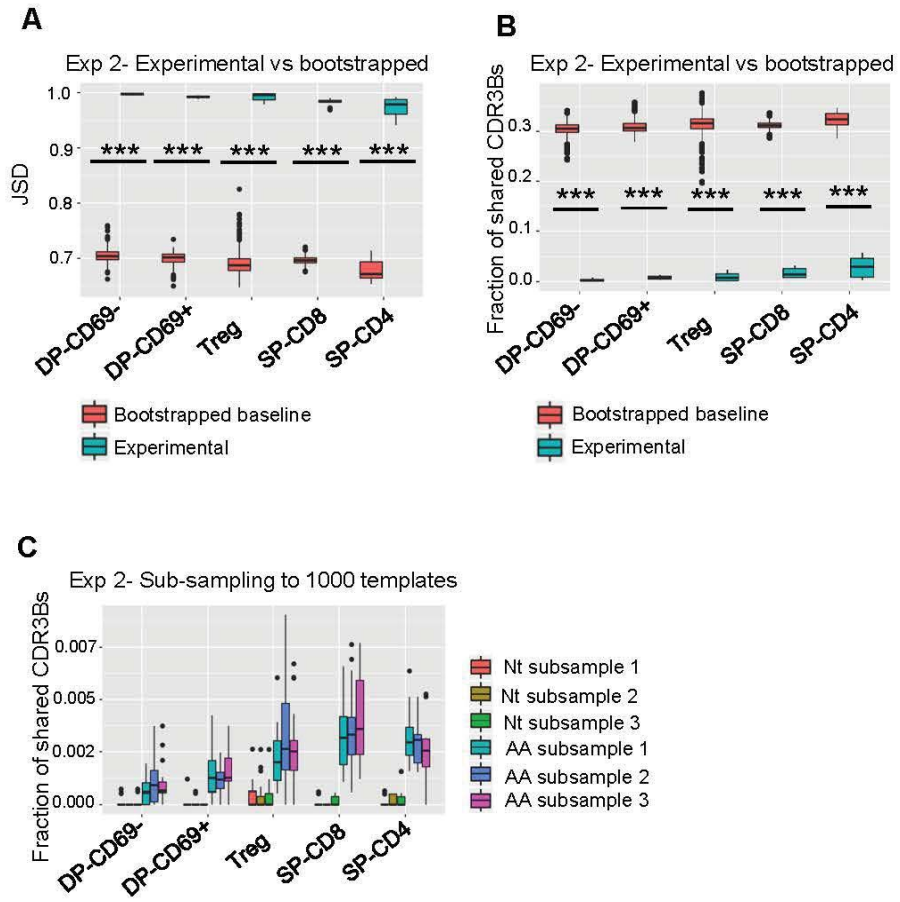


Figure S3. Bootstrapped baseline and effect of sample size normalization on fraction of shared CDR3βs. A and B show pairwise JSD and fraction of shared CDR3βs between experimental mouse samples of Experiment 2 (n=6) compared to a boot-strapped statistical baseline derived by ¼ sub-sampling (with replacement) of identical repertoires 100 times each and calculating JSD and fraction of shared CDR3βs of pairs derived from identical repertoires. C shows pairwise shared CDR3β fraction between mice in Experiment 2 after randomly subsampling each sample (with replacement) to 1000 templates three times and computing shared CDR3β fraction at nucleotide and amino acid level to ensure results observed in Figure 3 are robust to under-sampling and initial differences in sample size. Statistically significant differences are shown with * (* 0.01<p-value<0.05, **0.001<p-value<0.01, ***p-value<0.001).

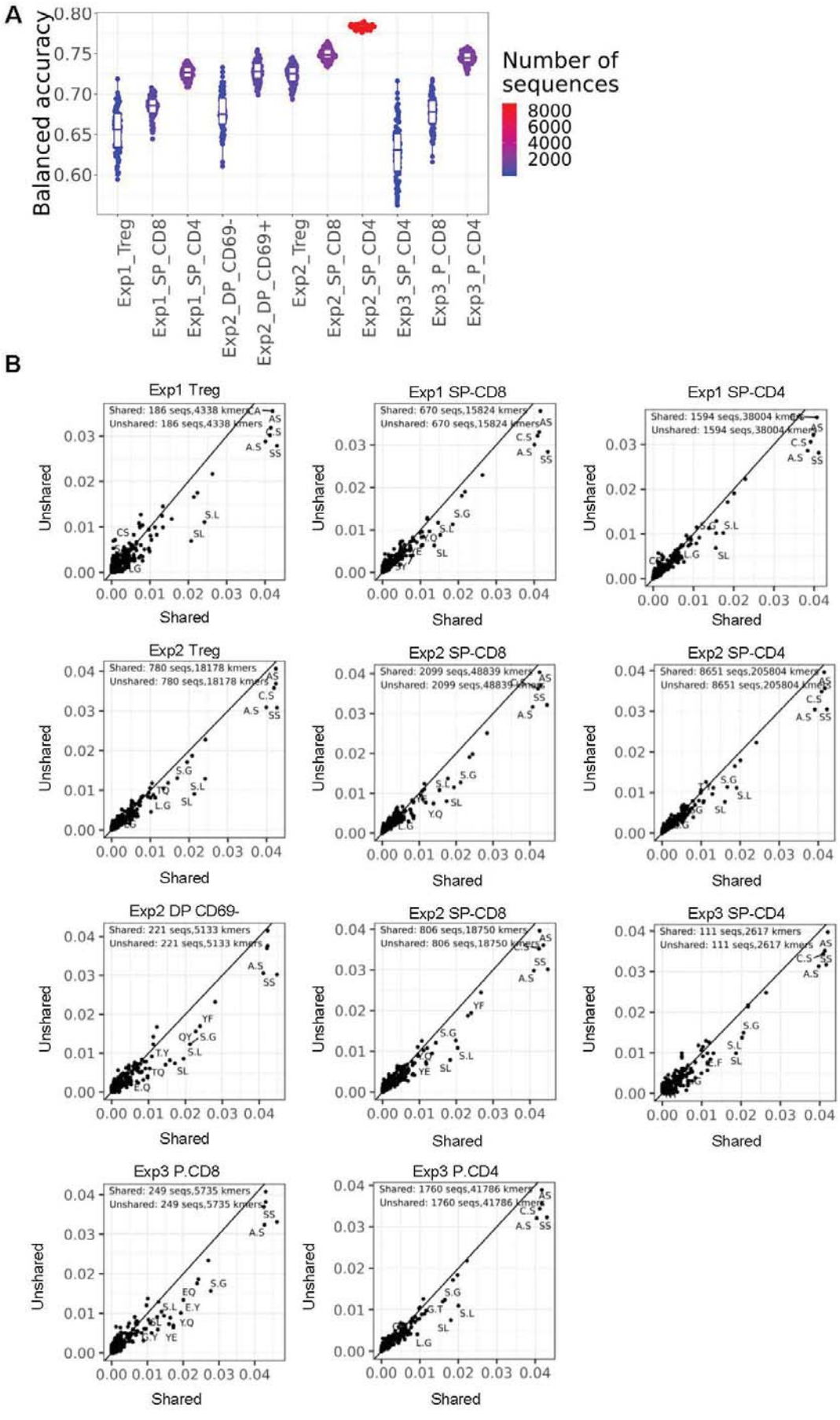

A
Balanced accuracy
0.80
0.75
0.70
0.65
0.60
Number of sequences
8000
6000
4000
2000
Exp1_Treg
Exp1_SP_CD8
Exp1_SP_CD4
Exp2_DP_CD69-
Exp2_DP_CD69+
Exp2_Treg
Exp2_SP_CD8
Exp2_SP_CD4
Exp3_SP_CD4
Exp3_P_CD8
Exp3_P_CD4
B
Exp1 Treg
Shared: 186 seqs,4338 kmers
Unshared: 186 seqs,4338 kmers
Exp1 SP-CD8
Shared: 670 seqs,15824 kmers
Unshared: 670 seqs,15824 kmers
Exp1 SP-CD4
Shared: 1594 seqs,38004 kmers
Unshared: 1594 seqs,38004 kmers
Exp2 Treg
Shared: 780 seqs,18178 kmers
Unshared: 780 seqs,18178 kmers
Exp2 SP-CD8
Shared: 2099 seqs,48839 kmers
Unshared: 2099 seqs,48839 kmers
Exp2 SP-CD4
Shared: 8651 seqs,205804 kmers
Unshared: 8651 seqs,205804 kmers
Exp2 DP CD69-
Shared: 221 seqs,5133 kmers
Unshared: 221 seqs,5133 kmers
Exp2 SP-CD8
Shared: 806 seqs,18750 kmers
Unshared: 806 seqs,18750 kmers
Exp3 SP-CD4
Shared: 111 seqs,2617 kmers
Unshared: 111 seqs,2617 kmers
Exp3 P.CD8
Shared: 249 seqs,5735 kmers
Unshared: 249 seqs,5735 kmers
Exp3 P.CD4
Shared: 1760 seqs,41786 kmers
Unshared: 1760 seqs,41786 kmers
Unshared
Shared
0.00 0.01 0.02 0.03 0.04

Figure S4. Subsequence features in shared vs unshared CDR3βs. A, Support vector machine (SVM) analysis using normalized count of gapped k-mers showing that these features can be used to predict shared or unshared status of sequences with a median balanced accuracy of ~62-78% for all cell subsets and developmental stages. B, Frequency of gapped k-mers in shared sequences plotted against the frequency in unshared sequences.

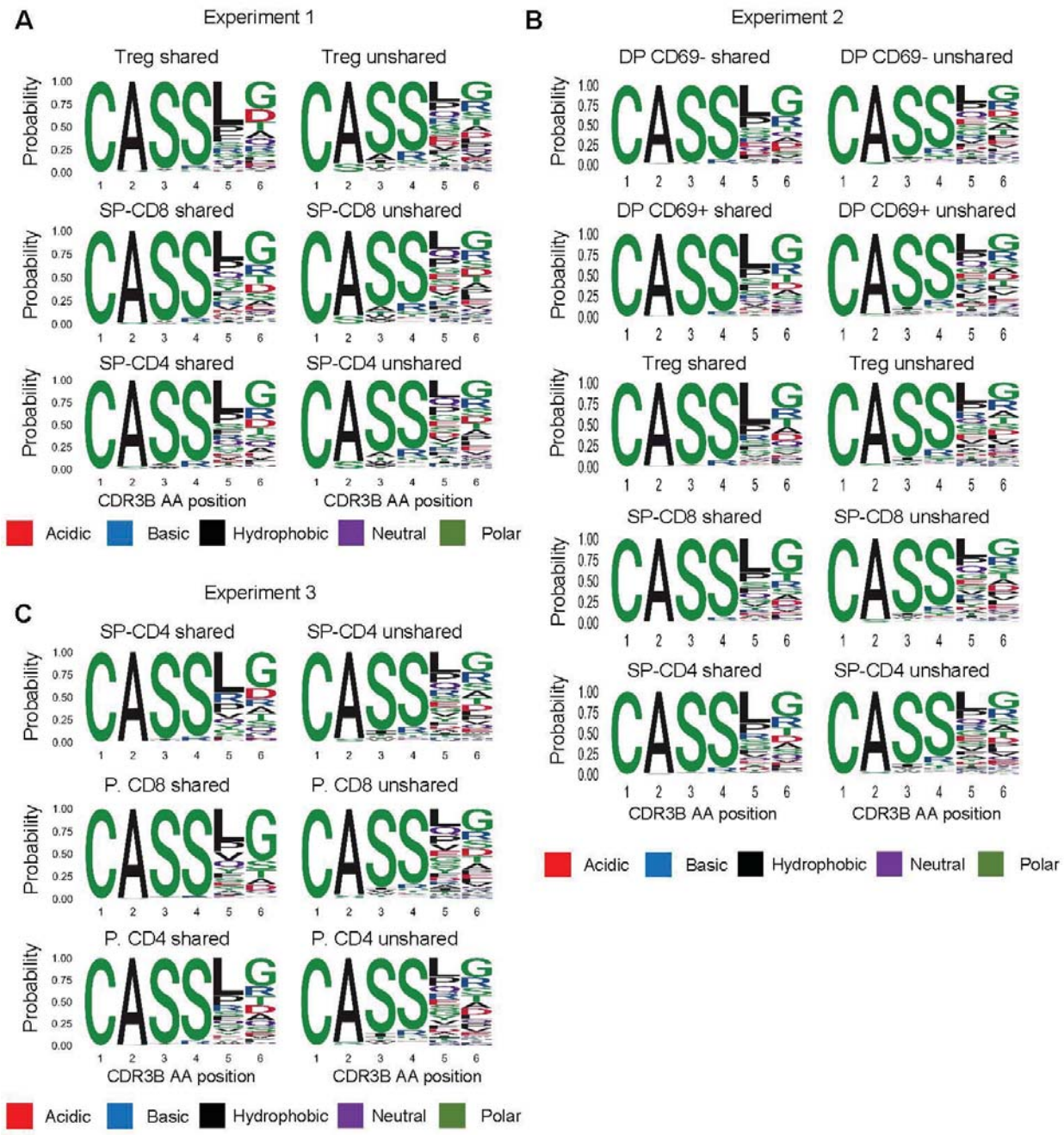


Figure S5. Amino acid usage in shared and unshared CDR3βs. Stacked amino acid usage plots showing frequency of each amino acid in the first six positions of CDR3β, for the set of all shared and all unshared sequences within each cell population in Experiments 1, 2 and 3. Amino acids are stacked such that the highest-usage amino acid is on top, the height of each amino acid represents its frequency, and the total frequencies of all amino acids at a given position sum to 1.

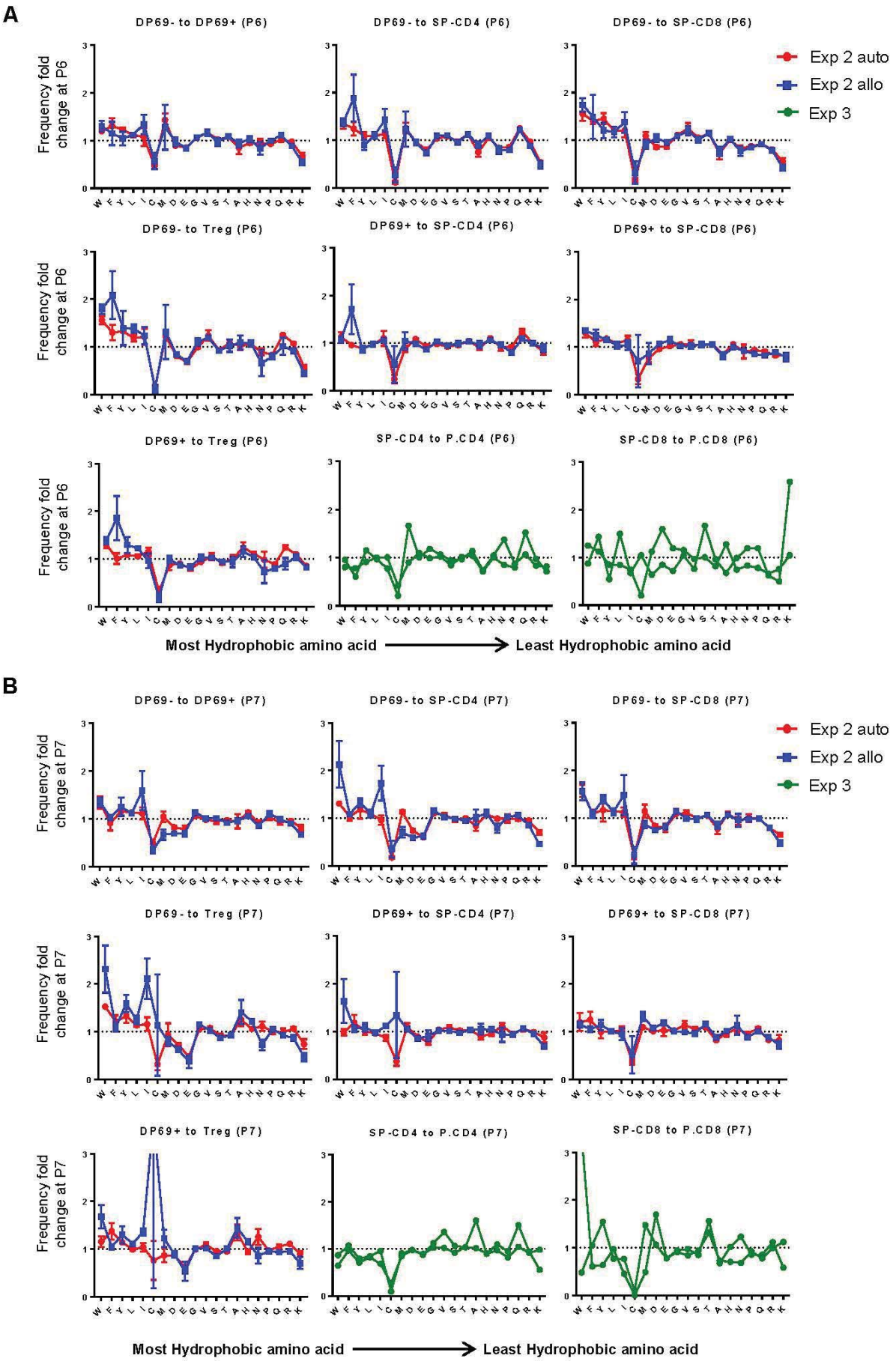
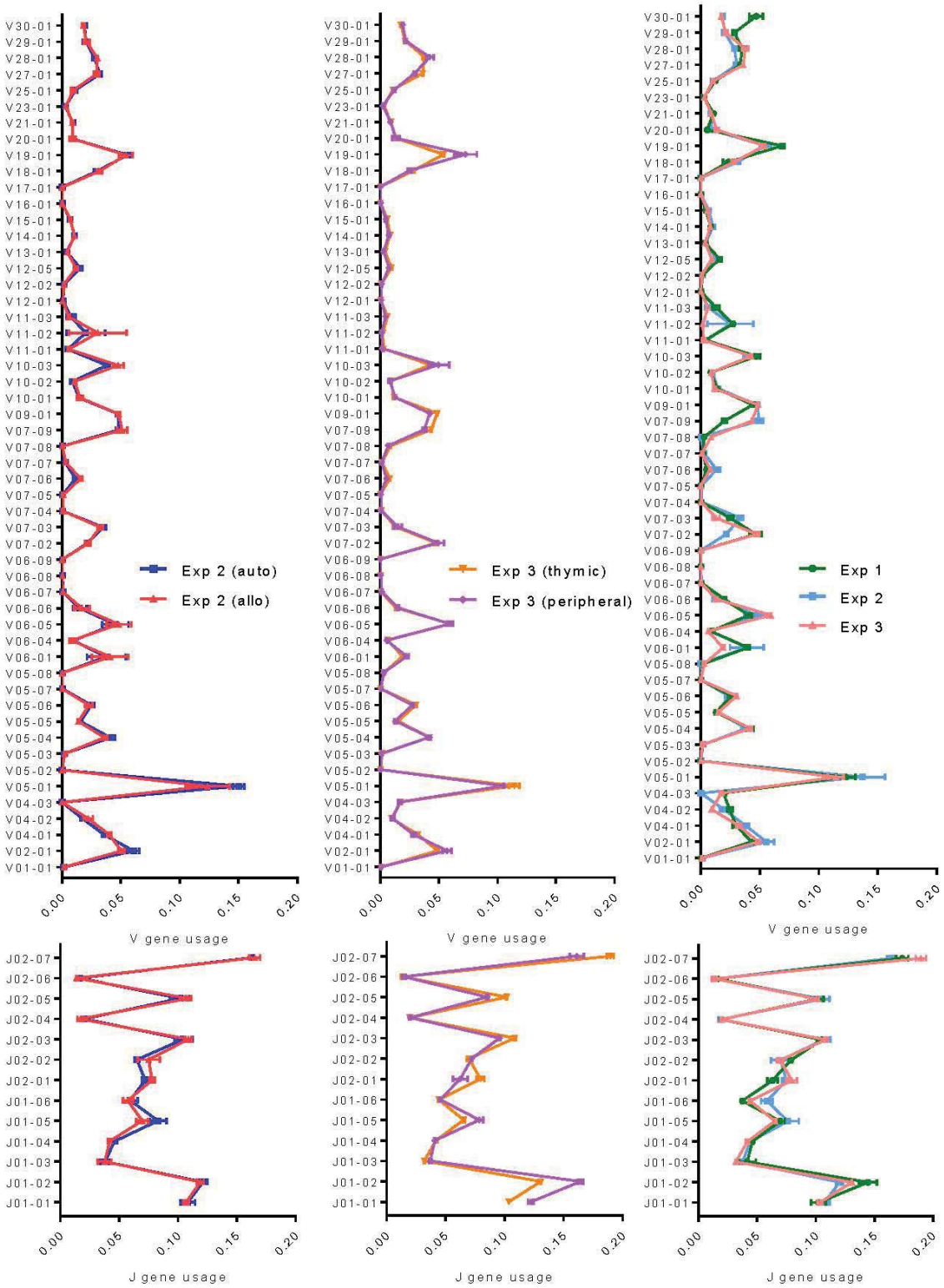

A
DP69- to DP69+ (P6)
DP69- to SP-CD4 (P6)
DP69- to SP-CD8 (P6)
DP69- to Treg (P6)
DP69+ to SP-CD4 (P6)
DP69+ to SP-CD8 (P6)
DP69+ to Treg (P6)
SP-CD4 to P.CD4 (P6)
SP-CD8 to P.CD8 (P6)
Frequency fold change at P6
Exp 2 auto
Exp 2 allo
Exp 3
Most Hydrophobic amino acid
Least Hydrophobic amino acid
B
DP69- to DP69+ (P7)
DP69- to SP-CD4 (P7)
DP69- to SP-CD8 (P7)
DP69- to Treg (P7)
DP69+ to SP-CD4 (P7)
DP69+ to SP-CD8 (P7)
DP69+ to Treg (P7)
SP-CD4 to P.CD4 (P7)
SP-CD8 to P.CD8 (P7)
Frequency fold change at P7
Exp 2 auto
Exp 2 allo
Exp 3
Most Hydrophobic amino acid
Least Hydrophobic amino acid

Figure S6. Fold changes in amino acid frequency at Position 7 of the CDR3βs. Fold changes in relative amino acid frequencies at Positions 6 (A) and 7 (B) of CDR3βs are shown in transition from DP $CD69^-$ to DP $CD69^+$ cells and from there to SP cell subsets for mice with allogeneic vs autologous thymus in Experiment 2 (shown as mean±SEM). Also the fold changes in transition from SP-CD4 to peripheral CD4 cells and from SP-CD8 to peripheral CD8 cells is shown for mice in Experiment 3. Amino acids are listed in decreasing order of hydrophobicity from left to right.



	Exp 2 auto vs allo	p-value
J genes	n.s.	n.s.
V genes	n.s.	n.s.

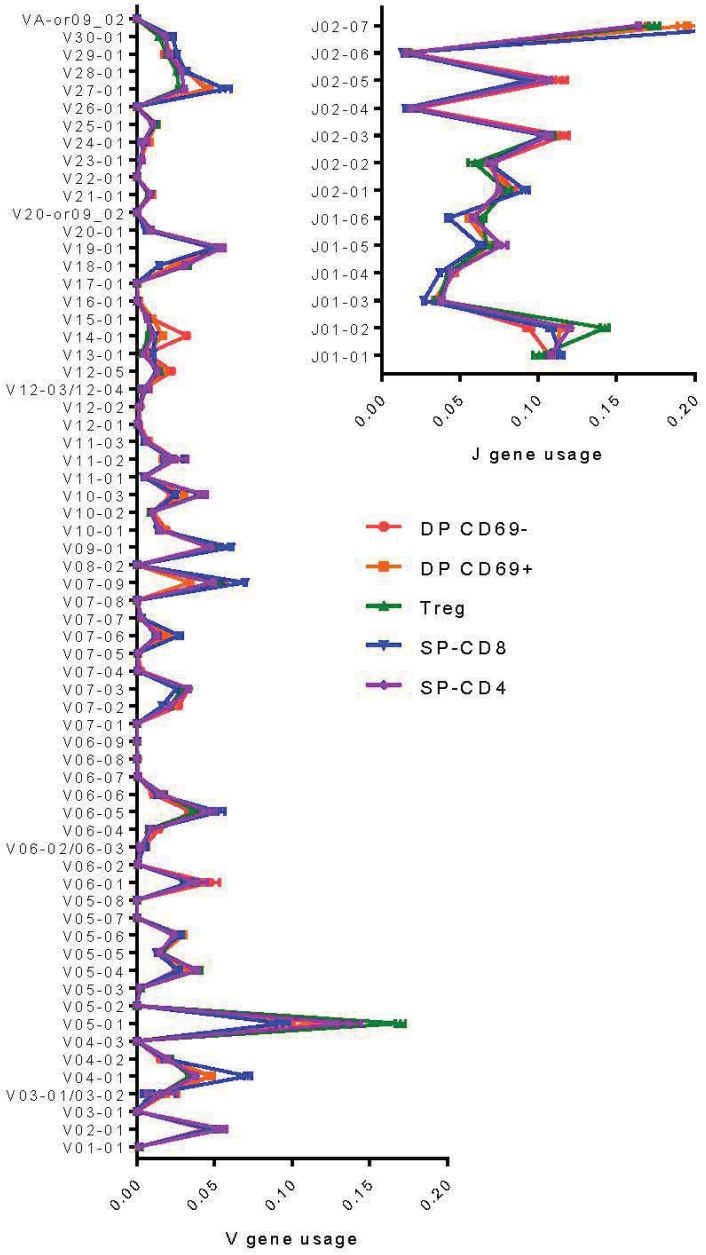
	Exp 3 thymic vs peripheral	p-value
J genes	n.s.	n.s.
V genes	n.s.	n.s.

	Exp 1 vs Exp 2	p-value
J genes	J01-06	0.01068645
V genes	V04-03	0.0254639
	V07-09	0.000869265
	V17-01	0.009298685

	Exp 1 vs Exp 3	p-value
J genes	n.s.	n.s.
V genes	V06-01	0.02936416
	V07-09	0.04591294
	V11-02	0.01275489

	Exp 2 vs Exp 3	p-value
J genes	n.s.	n.s.
V genes	V07-03	0.0173452
	V07-08	0.01892448

Figure S7. Vβ and Jβ gene usage of thymic and peripheral CD4 cells. Vβ and Jβ gene usage distributions of SP-CD4 thymocytes of mice with allogeneic (n=3) vs autologous (n=3) thymus in Experiment 2 are shown in the left graphs. The Vβ and Jβ gene usage distributions of thymic (n=2) vs peripheral (n=2) CD4 T cells in Experiment 3 are shown in the middle graphs. The Vβ and Jβ gene usage distributions of thymic SP-CD4 T cells comparing mice in Experiments 1 (n=3), 2 (n=6) and 3 (n=2) are shown in the right graphs. Results are shown as mean±SEM. Unpaired t-test with Bonferroni correction for multiple testing was performed to compare the Vβ and Jβ gene usages for each gene. Genes with statistically significant differences in usage are shown in the tables below. p-value<0.05 was considered significant.



	DP69- vs DP69+	P-value
J genes	n.s.	
V genes	TCRBV07-09	0.006875296
	TCRBV12-05	0.020999554
	TCRBV14-01	0.029324548
	DP69+ vs Treg	**P-value**
J genes	TCRBJ02-07	0.03670862
V genes	TCRBV14-01	0.016555176
	TCRBV27-01	0.001426217
	DP69+ vs SP-CD8	**P-value**
J genes	TCRBJ02-07	7.81E-03
V genes	TCRBV04-01	0.002171162
	TCRBV07-09	0.000318317
	DP69+ vs SP-CD4	**P-value**
J genes	TCRBJ02-07	1.00E-02
V genes	TCRBV07-09	0.01939807
	TCRBV27-01	0.02853274
	Treg vs SP-CD8	**P-value**
J genes	TCRBJ01-02	1.15E-02
	TCRBJ01-06	9.56E-03
	TCRBJ02-07	1.84E-03
V genes	TCRBV04-01	2.88E-03
	TCRBV05-01	5.49E-05
	Treg vs SP-CD4	**P-value**
J genes	n.s.	
V genes	n.s.	
	SP-CD8 vs SP-CD4	**P-value**
J genes	TCRBJ01-03	1.08E-02
	TCRBJ01-06	2.26E-02
	TCRBJ02-01	2.61E-03
	TCRBJ02-07	0.001111738
V genes	TCRBV04-01	0.02541822
	TCRBV07-06	0.03352391
	TCRBV07-09	0.01988107

Figure S8. Vβ and Jβ gene usage distributions during thymic selection. The left and right plots show the Vβ and Jβ gene usage distributions of different thymic cell populations (DP CD69$^-$, DP CD69$^+$, SP CD4, SP CD8 and Treg cell populations) of the 6 mice in Experiment 2. Paired t-test with Bonferroni correction for multiple testing was performed to compare the V and J gene usage between DP CD69$^-$ and DP CD69$^+$ cells and also between DP CD69$^+$ cells and SP cell populations for each gene. Genes with statistically significant differences in usage are shown in the table to the right. p-value<0.05 was considered significant. All results are shown as mean±SEM.

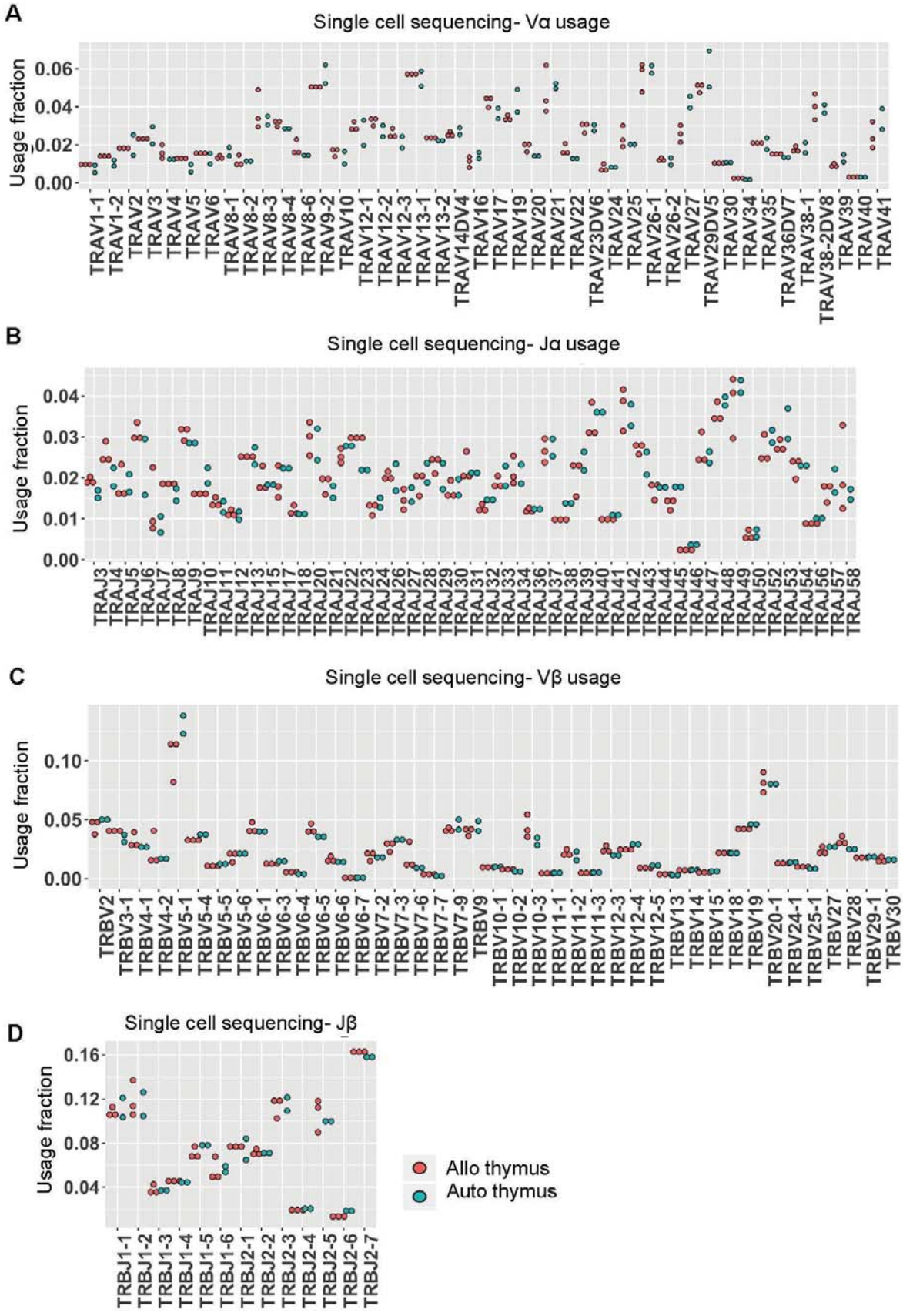
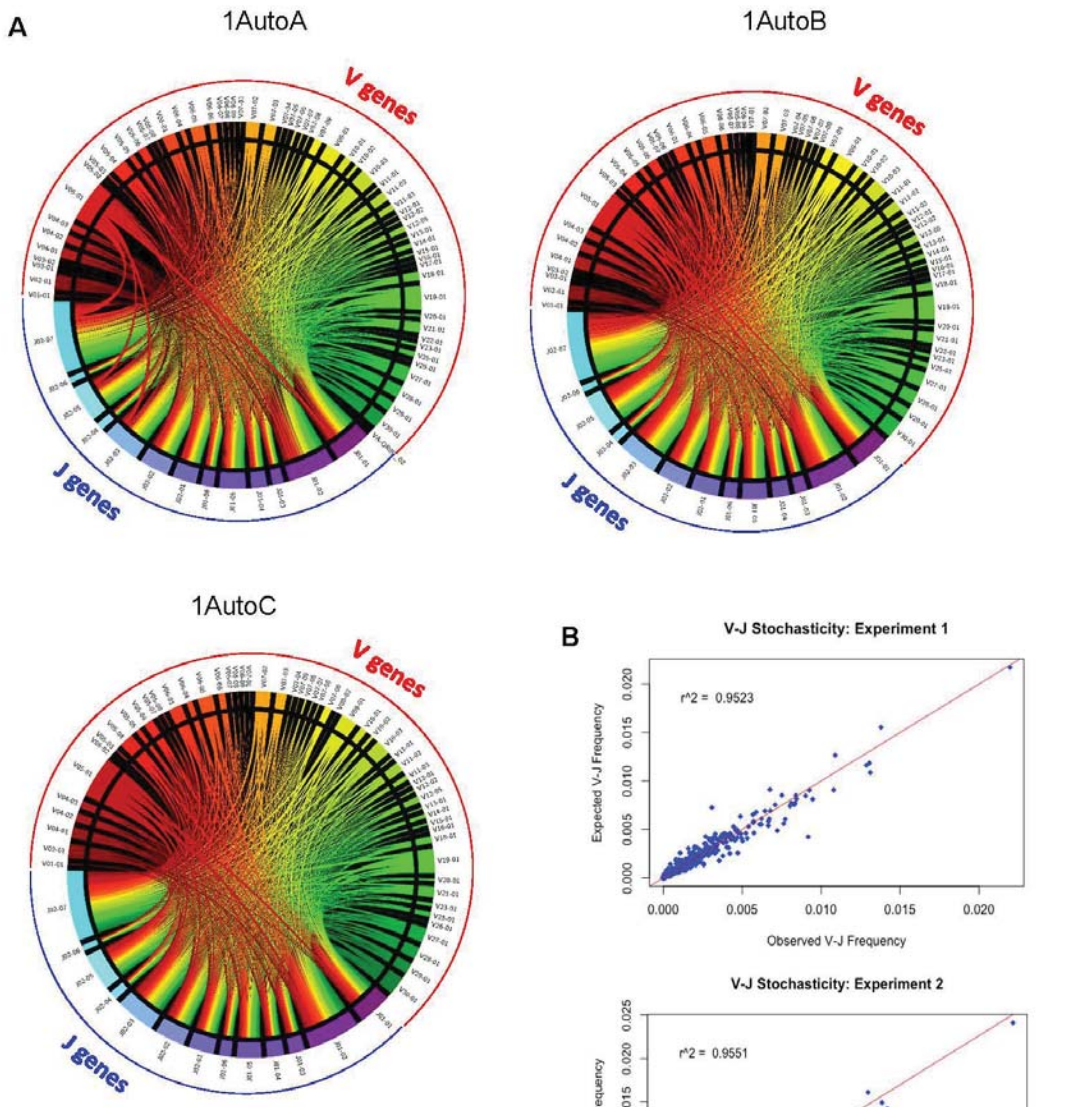

A
Single cell sequencing- Vα usage
Usage fraction
0.06
0.04
0.02
0.00
TRAV1-1
TRAV1-2
TRAV2
TRAV3
TRAV4
TRAV5
TRAV6
TRAV8-1
TRAV8-2
TRAV8-3
TRAV8-4
TRAV8-6
TRAV9-2
TRAV10
TRAV12-1
TRAV12-2
TRAV12-3
TRAV13-1
TRAV13-2
TRAV14DV4
TRAV16
TRAV17
TRAV19
TRAV20
TRAV21
TRAV22
TRAV23DV6
TRAV24
TRAV25
TRAV26-1
TRAV26-2
TRAV27
TRAV29DV5
TRAV30
TRAV34
TRAV35
TRAV36DV7
TRAV38-1
TRAV38-2DV8
TRAV39
TRAV40
TRAV41
B
Single cell sequencing- Jα usage
Usage fraction
0.04
0.03
0.02
0.01
0.00
TRAJ3
TRAJ4
TRAJ5
TRAJ6
TRAJ7
TRAJ8
TRAJ9
TRAJ10
TRAJ11
TRAJ12
TRAJ13
TRAJ15
TRAJ17
TRAJ18
TRAJ20
TRAJ21
TRAJ22
TRAJ23
TRAJ24
TRAJ26
TRAJ27
TRAJ28
TRAJ29
TRAJ30
TRAJ31
TRAJ32
TRAJ33
TRAJ34
TRAJ36
TRAJ37
TRAJ38
TRAJ39
TRAJ40
TRAJ41
TRAJ42
TRAJ43
TRAJ44
TRAJ45
TRAJ46
TRAJ47
TRAJ48
TRAJ49
TRAJ50
TRAJ52
TRAJ53
TRAJ54
TRAJ56
TRAJ57
TRAJ58
C
Single cell sequencing- Vβ usage
Usage fraction
0.10
0.05
0.00
TRBV2
TRBV3-1
TRBV4-1
TRBV4-2
TRBV5-1
TRBV5-4
TRBV5-5
TRBV5-6
TRBV6-1
TRBV6-3
TRBV6-4
TRBV6-5
TRBV6-6
TRBV6-7
TRBV7-2
TRBV7-3
TRBV7-6
TRBV7-7
TRBV7-9
TRBV9
TRBV10-1
TRBV10-2
TRBV10-3
TRBV11-1
TRBV11-2
TRBV11-3
TRBV12-3
TRBV12-4
TRBV12-5
TRBV13
TRBV14
TRBV15
TRBV18
TRBV19
TRBV20-1
TRBV24-1
TRBV25-1
TRBV27
TRBV28
TRBV29-1
TRBV30
D
Single cell sequencing- Jβ
Usage fraction
0.16
0.12
0.08
0.04
TRBJ1-1
TRBJ1-2
TRBJ1-3
TRBJ1-4
TRBJ1-5
TRBJ1-6
TRBJ2-1
TRBJ2-2
TRBJ2-3
TRBJ2-4
TRBJ2-5
TRBJ2-6
TRBJ2-7
Allo thymus
Auto thymus

Figure S9. V and J gene usage for both α and β chains obtained in single cell TCR sequencing. Usage of Vα, Jα, Vβ and Jβ genes for SP-CD4 cells of the mice in Experiment 2 with autologous (n=2) vs allogeneic (n=3) thymus obtained in single cell TCR sequencing are plotted in panels A-D.



JSD top 100	1AutoA CD4	1AutoB CD4	1AutoC CD4
1AutoA CD4	0	0.006438535	0.010341141
1AutoB CD4	0.006438535	0	0.009205221
1AutoC CD4	0.010341141	0.009205221	0

JSD top 100	1AutoA CD8	1AutoB CD8	1AutoC CD8
1AutoA CD8	0	0.008773661	0.005618316
1AutoB CD8	0.008773661	0	0.010230808
1AutoC CD8	0.005618316	0.010230808	0

JSD top 100	1AutoA Treg	1AutoB Treg	1AutoC Treg
1AutoA Treg	0	0.014437412	0.021581936
1AutoB Treg	0.014437412	0	0.026194587
1AutoC Treg	0.021581936	0.026194587	0

	Mann-Whitney U-Test of observed vs expected
Experiment 1	p-value=0.3992
Experiment 2	p-value=0.8055426

Figure S10. Pairing of V and J genes and comparison of observed vs expected VJ frequency distributions. A, Circos plots of VJ usage for SP-CD4 repertoires of the 3 mice in Experiment 1 are

shown. V genes are shown at the top and J genes are shown at the bottom. Width of a line connecting the top and bottom represents the relative frequency of a given VJ pair, and the widths of bars on the circumference represent the relative frequencies of each V and J. The color of each connecting line corresponds to a particular V gene, such that range of colors along the bottom of the plot shows diversity of Vs connecting to Js. The tables show JSD values comparing the V-J distribution between each pair of mice in Experiment 1 for different cell populations. B, Linear regressions of observed vs. expected frequency of each V-J pair are shown for the cumulative total of all samples in Experiments 1 and 2. Expected frequency is calculated as the product of V and J frequency. The correlation between the observed frequencies and the expected frequencies were plotted. Mann-Whitney U-Tests were performed with the null hypothesis that the VJ combination is stochastic.

Supplementary Tables:

Table S1. Grafted thymus cell counts, FACS-sorted cell counts, template and unique cell counts and clonality scores for each mouse in Experiments 1, 2 and 3 and the donor thymus for Experiment 2.

Mouse/Tissue	Thymus cell count	Cell population	No. of FACS-sorted cells	nucleotide nonproductive			nucleotide productive			amino acid	
				Template count	Clonality	Unique clone count	Template count	Clonality	Unique clone count	Clonality	Unique clone count
1AutoA	33x10e6	Treg	87,355	4,209	0.01	3,416	17,085	0.01	13,019	0.013	12,707
		SP CD4	583,226	19,578	0.0099	15,375	74,649	0.014	53,952	0.023	49,836
		SP CD8	491,310	13,921	0.0074	11,907	49,776	0.0085	41,270	0.012	38,209
1AutoB	58x10e6	Treg	53,789	2,136	0.0066	1,875	7,722	0.006	6,531	0.0077	6,382
		SP CD4	427,728	13,671	0.0048	12,288	49,949	0.0058	43,227	0.0096	41,134
		SP CD8	314,351	4,535	0.0022	4,352	16,098	0.0044	15,195	0.0049	14,352
1AutoC	25x10e6	Treg	34,048	1,801	0.0068	1,575	6,431	0.0076	5,456	0.0097	5,340
		SP CD4	201,086	6,046	0.0076	5,298	21,577	0.011	17,937	0.016	17,121
		SP CD8	228,890	8,090	0.0065	7,070	30,152	0.0074	25,695	0.012	24,624
2AutoA	150x10e6	DP_CD69-	792,889	6,534	0.0018	6,266	18,536	0.0021	17,576	0.0031	17,080
		DP_CD69+	204,108	5,520	0.0012	5,382	17,783	0.0011	17,286	0.0025	16,652
		Treg	222,288	7,547	0.0022	7,228	25,318	0.0031	24,001	0.005	22,904
		SP_CD4	820,703	29,597	0.0035	27,539	104,688	0.0041	95,847	0.0066	89,788
		SP_CD8	337,912	8,237	0.0019	7,879	29,185	0.0021	27,693	0.0034	26,853
2AutoB	400x10e6	DP_CD69-	433,613	4,465	0.0015	4,329	11,661	0.0013	11,295	0.0022	11,046
		DP_CD69+	111,822	6,518	0.0011	6,355	18,638	0.0011	18,127	0.0025	17,473
		Treg	121,528	4,344	0.0055	3,865	14,422	0.0066	12,457	0.0071	12,290
		SP_CD4	821,448	10,798	0.0015	10,462	33,300	0.0015	32,034	0.0036	30,382
		SP_CD8	238,420	10,195	0.0017	9,797	33,243	0.0018	31,748	0.0033	30,661
2AutoC	100x10e6	DP_CD69-	200,000	1,691	0.0025	1,606	4,915	0.0024	4,644	0.0028	3,913
		DP_CD69+	300,000	2,052	0.0029	1,928	6,460	0.0026	6,079	0.0028	5,194
		Treg	70,000	656	0.0032	618	2,038	0.0035	1,915	0.0037	1,647
		SP_CD4	300,000	3,397	0.0022	3,236	11,193	0.0026	10,531	0.0032	9,012
		SP_CD8	248,000	2,202	0.0029	2,070	7,471	0.0029	6,932	0.0035	6,127
2AlloA	220x10e6	DP_CD69-	489,849	3,764	0.0019	3,616	9,754	0.0016	9,409	0.0025	9,183
		DP_CD69+	103,440	4,812	0.0014	4,666	15,408	0.0013	14,909	0.0021	14,587
		Treg	195,514	6,611	0.003	6,336	19,930	0.0049	18,792	0.0067	17,987
		SP_CD4	779,000	22,054	0.002	21,011	69,169	0.011	64,132	0.014	60,352
		SP_CD8	302,336	7,012	0.0014	6,803	21,439	0.0023	20,551	0.0045	19,545
2AlloB	150x10e6	DP_CD69-	400,317	628	0.00094	618	1,597	0.00099	1,566	0.0019	1,540
		DP_CD69+	101,851	2,913	0.0044	2,629	9,496	0.0046	8,357	0.0053	8,214
		Treg	178,315	2,300	0.002	2,212	7,178	0.0042	6,812	0.0051	6,661
		SP_CD4	800,000	30,088	0.0034	27,698	97,477	0.0061	87,590	0.0085	82,340
		SP_CD8	330,315	2,385	0.0013	2,318	7,358	0.0013	7,161	0.0029	6,892
2AlloC	168x10e6	DP_CD69-	200,000	1,047	0.0033	982	2,957	0.0026	2,796	0.0028	2,358
		DP_CD69+	300,000	1,940	0.0026	1,834	5,972	0.0026	5,619	0.0031	4,766
		Treg	42,000	150	0.005	141	397	0.0017	386	0.0017	332
		SP_CD4	300,000	1,744	0.0044	1,619	5,596	0.0056	5,110	0.0065	4,347
		SP_CD8	156,000	2,399	0.0034	2,268	7,495	0.0039	7,004	0.0045	6,147
Fetal Thymus	15x10e6	Treg	23,904	278	0.0071	248	725	0.0069	633	0.0089	609
		SP_CD4	414,877	18,082	0.0052	16,030	44,541	0.017	33,829	0.049	23,388
		SP_CD8	111,449	8,357	0.0067	6,945	21,015	0.013	15,901	0.04	11,816
3AutoA	107x10e6	SP_CD4	748,000	6,269	0.0023	5,930	23,178	0.0027	21,558	0.0036	18,056
		SP_CD8	245,000	1,066	0.0029	1,010	4,176	0.0028	3,942	0.0031	3,436
		P. CD4	800,000	11,396	0.05	8,082	48,326	0.067	31,767	0.07	26,553
		P. CD8	800,000	7,423	0.097	5,052	30,741	0.13	18,866	0.14	16,420
3AutoB	175x10e6	SP_CD4	437,000	2,124	0.0021	2,033	7,752	0.0029	7,326	0.0036	6,133
		SP_CD8	144,000	765	0.0037	714	2,477	0.0032	2,307	0.0039	1,954
		P. CD4	800,000	33,554	0.06	22,077	124,806	0.06	80,609	0.06	65,157

		P. CD8	600,000	4,417	0.14	2,587	15,888	0.14	9,011	0.15	7,823

Table S2. HLA typing of fetal tissues used to generate humanized mice in all three experiments.

Typing for some MHCs are not done (shown as ND).

HLA	Experiment 1 thymus and HSCs	Experiment 2 HSCs and auto thymus	Experiment 2 allo thymus	Experiment 3 thymus and HSCs
A1	**02**	**02:01:01**	**31:01:02**	**68:01:01:01**
A2	**24**	**23:01:01**	**24:02:01**	**34:02:01**
B1	**ND**	**49:01**	**35:12:02**	**49:01**
B2	**ND**	**35:08**	**39:06:02**	**81:01**
C1	**ND**	**07:01:01**	**04:01:01**	**07:01:01**
C2	**ND**	**04:01:01**	**07:02:01**	**08:04:01**
DRB1-1	**4:01**	**11:04:01**	**08:02:01**	**08:02:01**
DRB1-2	**4:02**	**04:05:01**	**14:06:01**	**14:06:01**
DQB1-24	**3:02**	**03:01:01**	**04:02:01**	**03:01:01:01**
DQB1-24	**ND**	**03:02:01**	**03:01:01:01**	**06:09:01**
DQA1	**3:01**	**ND**	**04:01:01**	**04:01:01**
DQA2	**ND**	**ND**	**05:01:01:01**	**01:02:01:01**

Table S3. Jenson-Shannon Divergence (JSD) scores comparing each pair of mice in Experiment 2 for different cell subsets (amino acid level).

JSD	Experiment 2: amino acid level					
DP69-	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	0	0.99431849	0.99554971	0.99515414	0.99841448	0.99731111
2autoB	0.99431849	0	0.99830573	0.99534656	0.99876902	0.99712877
2autoC	0.99554971	0.99830573	0	0.99692997	0.99919302	1
2alloA	0.99515414	0.99534656	0.99692997	0	0.99930653	0.99734988
2alloB	0.99841448	0.99876902	0.99919302	0.99930653	0	0.99890367
2alloC	0.99731111	0.99712877	1	0.99734988	0.99890367	0
DP69+	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	0	0.98814838	0.99251902	0.98885698	0.99198406	0.99382505
2autoB	0.98814838	0	0.99179387	0.98878889	0.99057187	0.99351998
2autoC	0.99251902	0.99179387	0	0.9945415	0.99315126	0.99330556
2alloA	0.98885698	0.98878889	0.9945415	0	0.99239498	0.99356011
2alloB	0.99198406	0.99057187	0.99315126	0.99239498	0	0.99536621
2alloC	0.99382505	0.99351998	0.99330556	0.99356011	0.99536621	0
Treg	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	0	0.98747171	0.99497389	0.97855264	0.98574743	0.9986055
2autoB	0.98747171	0	0.99559425	0.98666388	0.9930674	0.99762974
2autoC	0.99497389	0.99559425	0	0.99612371	0.99612923	0.99888098
2alloA	0.97855264	0.98666388	0.99612371	0	0.98481821	0.99807573
2alloB	0.98574743	0.9930674	0.99612923	0.98481821	0	0.9990455
2alloC	0.9986055	0.99762974	0.99888098	0.99807573	0.9990455	0
SP-CD8	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	0	0.96974267	0.98418995	0.97236814	0.98337473	0.98485022
2autoB	0.96974267	0	0.98420004	0.96871132	0.9833744	0.98465465
2autoC	0.98418995	0.98420004	0	0.98618989	0.99020655	0.99030095
2alloA	0.97236814	0.96871132	0.98618989	0	0.9822219	0.98472348
2alloB	0.98337473	0.9833744	0.99020655	0.9822219	0	0.99007559
2alloC	0.98485022	0.98465465	0.99030095	0.98472348	0.99007559	0
SP-CD4	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	0	0.96057632	0.97953517	0.95198027	0.94699361	0.98754906
2autoB	0.96057632	0	0.98143646	0.96208864	0.96339328	0.98903941
2autoC	0.97953517	0.98143646	0	0.97866014	0.97861591	0.99206847
2alloA	0.95198027	0.96208864	0.97866014	0	0.94102448	0.98845932
2alloB	0.94699361	0.96339328	0.97861591	0.94102448	0	0.98718573
2alloC	0.98754906	0.98903941	0.99206847	0.98845932	0.98718573	0

Table S4. Fraction of shared CDR3βs comparing each pair of mice in Experiment 2 for different cell subsets (amino acid level).

Shared CDR3B Fraction	Experiment 2: amino acid level					
DP69-	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	1	0.00431003	0.00241069	0.00343341	0.00043831	0.00124187
2autoB	0.00671752	1	0.00113856	0.00375726	0.00045543	0.00136628
2autoC	0.00843343	0.00255558	1	0.00383338	0.00051112	0
2alloA	0.00645427	0.00453172	0.00205987	1	0.00027465	0.00151057
2alloB	0.00494234	0.00329489	0.00164745	0.00164745	1	0.00164745
2alloC	0.0072095	0.00508906	0	0.00466497	0.00084818	1
DP69+	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	1	0.01134866	0.00461268	0.00966467	0.00541807	0.00366086
2autoB	0.01091396	1	0.00471765	0.00936488	0.00647796	0.00366146
2autoC	0.01212938	0.0128995	1	0.00827878	0.00750866	0.00616095
2alloA	0.01126857	0.01135394	0.00367082	1	0.00571965	0.00401229
2alloB	0.01100045	0.01367623	0.00579753	0.00995986	1	0.00371637
2alloC	0.01049098	0.01091062	0.00671423	0.00986152	0.00524549	1
Treg	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	1	0.00883925	0.00169375	0.01503202	0.00529297	0.00037051
2autoB	0.01668832	1	0.00179874	0.01438993	0.00479664	0.00059958
2autoC	0.01942927	0.01092896	1	0.01275046	0.00667881	0.00060716
2alloA	0.0194774	0.00987587	0.00144023	1	0.00596667	0.00054866
2alloB	0.01842639	0.00884467	0.0020269	0.01603096	1	0.00036853
2alloC	0.02108434	0.01807229	0.00301205	0.02409639	0.0060241	1
CD8	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	1	0.02638279	0.00763828	0.018964	0.00759438	0.00741879
2autoB	0.02315635	1	0.00755182	0.02053633	0.00716653	0.00732064
2autoC	0.02839889	0.03198955	1	0.02072793	0.00897666	0.00930308
2alloA	0.02617547	0.0322952	0.0076951	1	0.00872516	0.0084222
2alloB	0.03007127	0.03233096	0.00956023	0.02503042	1	0.0100817
2alloC	0.02749309	0.03090939	0.00927282	0.02261266	0.0094355	1
CD4	2autoA	2autoB	2autoC	2alloA	2alloB	2alloC
2autoA	1	0.01967026	0.00701926	0.0316547	0.0401159	0.0033736
2autoB	0.05815637	1	0.01033623	0.04464286	0.05003218	0.00494691
2autoC	0.05725699	0.02851753	1	0.04727031	0.05614736	0.00554816
2alloA	0.04780099	0.0228015	0.00875085	1	0.0457468	0.00382079
2alloB	0.0444354	0.01874454	0.00762439	0.03355634	1	0.00361631
2alloC	0.05705084	0.02829538	0.01150219	0.04278813	0.05521049	1

Table S5. Mean NT/AA ratio for shared vs unshared CDR3βs.

mean NT/AA	shared	unshared
DP69-	2.171946	1.024848
DP69+	2.380893	1.028829
Treg	2.421795	1.038576
CD8	2.71512	1.03049
CD4	2.954687	1.053213

Table S6. Comparison of NT/AA ratio of shared CDR3βs between different cell subsets.

NT/AA ratio (shared sequences)	p-value
DP69- vs DP69+	0.0002077
DP69- vs CD4	< 2.2e-16
DP69- vs CD8	< 2.2e-16
DP69- vs Treg	1.06E-05
DP69+ vs CD4	< 2.2e-16
DP69+ vs CD8	3.73E-13
DP69+ vs Treg	0.3618

Table S7. Number of shared and unshared CDR3βs at the Nt/non-productive, Nt/productive and amino acid levels for different cell subsets of the mice in Experiment 1.

Experiment 1	Treg			SP-CD8			SP-CD4		
CDR3B Unique Sequences	Nt Non-productive	Nt productive	Amino acid	Nt Non-productive	Nt productive	Amino acid	Nt Non-productive	Nt productive	Amino acid
1autoA	3,415	13,007	12,544	11,906	41,238	37,674	15,372	53,583	48,543
1autoB	1,874	6,520	6,254	4,352	15,168	13,997	12,285	43,140	39,877
1autoC	1,575	5,451	5,249	7,069	25,276	24,157	5,296	17,895	16,408
1autoA and 1autoB	0	9	95	0	18	203	2	70	881
1autoA and 1autoC	0	3	58	0	14	315	1	25	337
1autoB and 1autoC	0	2	23	0	9	135	1	13	301

1autoA and 1autoB and 1autoC	0	0	10	0	0	17	0	4	75

Table S8. Number of shared and unshared CDR3βs at amino acid level for different cell subsets of the mice in Experiment 2.

CDR3B unique sequences (amino acid level)	DP CD69-	DP CD69+	SP CD8	SP CD4	Treg
2autoA	17,080	16,652	26,853	89,788	22,904
2autoB	11,046	17,473	30,661	30,382	12,290
2autoC	3,913	5,194	6,127	9,012	1,647
2alloA	9,183	14,587	19,545	60,352	17,987
2alloB	1,540	8,214	6,892	82,340	6,661
2alloC	2,358	4,766	6,147	4,347	332
shared between all three auto	3	9	51	128	2
shared between all three allo	1	6	23	80	1
shared between all six	0	1	5	8	0

Table S9. Number of unshared CDR3βs and also CDR3βs that are shared between all mice in each experiment at the nucleotide and amino acid levels for each cell subset.

# unique CDR3B sequences		Nucleotide		Amino acid	
experiment	**cell subset**	**non-shared**	**shared**	**non-shared**	**shared**
Exp1	**SP CD4**	114,888	112	104,828	1,594
	SP CD8	82,078	41	75,828	670
	Treg	24,978	14	24,047	186
Exp2	**DP CD69-**	47,261	11	36,783	221
	DP CD69+	70,310	33	54,529	806
	SP CD4	294,009	588	207,188	8,651
	SP CD8	100,893	95	78,583	2,099
	Treg	64,271	44	49,253	780
Exp3	**SP CD4**	28,872	6	23,967	111
	SP CD8	6,245	2	5,362	14
	P. CD4	112,178	99	88,190	1,760
	p. CD8	27,845	16	23,745	249

Table S10. Comparison of CDR3β length between different cell subsets for the top and bottom 1000 frequent sequences.

CDR3B length	p-value (top1000)	p-value (bottom 1000)
DP69- vs DP69+	< 2.2e-16	0.874
DP69- vs CD4	< 2.2e-16	0.5433
DP69- vs CD8	< 2.2e-16	0.693
DP69- vs Treg	< 2.2e-16	0.8581
DP69+ vs CD4	< 2.2e-16	0.4425
DP69+ vs CD8	2.73E-15	0.8131
DP69+ vs Treg	7.43E-05	0.9837

Table S11. Mean NT/AA ratio for CDR3βs unique to SP-CD4 and SP-CD8 cells and CDR3βs shared between these two subsets for the mice in Experiment 2.

Mean NT/AA	Shared in SP-CD4 and SP-CD8	Unique to SP-CD4	Unique to CD8
2autoA	1.820886	1.058942	1.022433
2autoB	2.086829	1.04837	1.029694
2autoC	2.928571	1.04837	1.029694
2alloA	2.008459	1.054977	1.041308
2alloB	1.81445	1.060285	1.033697
2alloC	2.291667	1.003684	1.006068

Table S12. Statistical significance of odds ratios of cross-reactivity and T1D-reactivity in shared vs unshared sequences for each cell subset in different experiments.

P-value	Cross-reactivity in shared vs unshared	Sharing in cross-reactives vs allo-non-crossreactives	T1D-reactivity in shared vs unshared
Exp1 Treg	0.000247	0.000173	0.149
Exp1 SP-CD8	2.28E-03	0.00441	3.68E-09
Exp1 SP-CD4	3.35E-21	2.86E-18	3.44E-10
Exp2 Treg	0.00379	0.00178	2.17E-05

Exp2 SP-CD8	5.33E-14	1.58E-11	1.97E-17
Exp2 SP-CD4	4.97E-57	1.89E-42	2.76E-34
Exp3 SP-CD4	0.0215	0.0144	0.00342
Exp3 P. CD4	0.000711	4.24E-05	1.53E-08

Table S13. The number of unique CDRαs, CDR3βs and paired CDR3α-CDR3βs, the fraction of cells with a β chain that have at least one paired α chain or two paired α chains and the fraction cells with an α chain that have a paired β chain for each of the five mice in Experiment 2, for which the single cell sequencing of SP-CD4 cells was done.

Mouse	# unique CDR3α	# unique CDR3β	# unique CDR3α-CDR3β pairs	Fraction of cells with a β chain with at least a paired α chain	Fraction of cells with a β chain that have two paired α chains	Fraction of cells with an α chain that have a paired β chain
2autoB	6,312	7,063	6,236	0.8729003	0.12528	0.9608629
2autoC	2,494	2,672	2,484	0.9166052	0.1136531	0.9829838
2alloA	5,709	6,373	5,682	0.8792943	0.1219437	0.9630508
2alloB	5,202	5,753	5,155	0.8842196	0.1221269	0.967167
2alloC	4,087	4,446	4,156	0.9104249	0.1349102	0.9808872