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Supplementary Information for

Inventories of naive and tolerant mouse CD4 T cell repertoires reveal a hierarchy of deleted and diverted T cell receptors

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Supplemental Material and Methods

Animals.

All analyses were performed in 6 to 10-week-old mice on C57BL/6 background. The gene (*Plp1*) encoding for PLP is located on the X-chromosome. We did not observe any differences in the number and composition of tetramer positive cells between PLP-sufficient females ($Plp1^{+/+}$) and males ($Plp1^{+/y}$) or between PLP–deficient females ($Plp1^{-/-}$) or males ($Plp1^{-/y}$). Therefore, female and male mice were included into the PLP-sufficient or - deficient groups and their genotype is referred to as $Plp1^{WT}$ or $Plp1^{KO}$, respectively. Mice were maintained under specific pathogen free conditions in individually ventilated cages.

Antibodies and flow cytometry.

Monoclonal antibodies to B220/CD45R (RA3-6B2), CCR7 (4B12), CD11b (M1/70), CD11c (N418), CD4 (RM4-5), CD25 (PC61), CD69 (H1.2F3), CD73 (TY/11.8), CD8α (53-6.7), Foxp3 (FJK-16s), F4/80 (BM8), FR4 (12A5), H-2K^b (AF6-88.5), TCR Vβ2 (B20.6), TCR Vβ3 (KJ25), TCR Vβ5.1/5.2 (MR9-4) and TCR Vβ6 (RR4-7) and TCR Vβ8.3 (F23.1) conjugated to different fluorochromes were purchased from BioLegend or BD Biosciences. Flow cytometric analyses were performed on a BD FACSCanto II or a BD FACSAria Fusion. Single-cell sorts were performed on a BD FACSAria Fusion sorter. The FACSDiva software was used for acquisition, recording and sorting, and FlowJo v9 and v10 software was used for data analysis.

Generation of I-A^b tetramers. Biotinylated monomers of the I-A^b beta chain with covalently linked peptides corresponding to $PLP_{9\text{-}20}$ (CLVGAPFASLVA) or $PLP_{237\text{-}247}$ (HLFIAAFVGAA) were produced in S2 insect cells together with the I-A^b alpha chain. I-A^b heterodimers were purified from culture supernatants and tetramerized with streptavidin (SA)-phycoerythrin (PE) or SA-allophycocyanin (APC). The resulting tetramers are referred to as Tet-1 ($PLP_{9\text{-}20}$:I-A^b) and Tet-3 ($PLP_{237\text{-}247}$:I-A^b).

Enrichment of Tet$^+$ cells. Single cell suspensions from thymus or pooled lymph nodes (axillary, mesenteric, inguinal and cervical) and spleen were co–stained for 1 h at RT with Tet-1 or Tet-3 conjugated to PE and APC. Tet–labelled cells were stained with anti-PE and anti-APC microbeads for enrichment on magnetized columns (Miltenyi Biotech). Bound cells were eluted in 100 µl and 5 µl were mixed with AccuCheck Counting Beads (Life technology) for cell quantification. The remaining cells were stained with antibodies to CD4 and the respective surface markers of interest as well as with a 'dump' cocktail of antibodies (F4/80, CD11b, CD11c and B220) to exclude non–T and autofluorescent cells in subsequent flow cytometric analyses.

Single cell TCRα sequencing. Single Tet$^+$ cells were deposited into 96-well plates and frozen at -80°C. Typical yields of sorted cells were in the order of 5 to 10% of the calculated 'theoretical' number of Tet$^+$ cells in a given organ or whole mouse. Reverse transcription was performed using the iScript Select™ cDNA Synthesis Kit (Bio-Rad) in a total volume of 4 µl iScript buffer containing 0.1% Triton X-100, 0.4 µl Enhancer, 0.2 µl iScript reverse transcriptase in the presence of 1.25 µM each TCRα constant region reverse primers (TRAC215r: GGTGAAGCTGTCTGGTTGCTC, TRAC222r: GATATCTTGGCAGGTGAAGCTTGTC and TRAC254r: ACTGGGGTAGGTGGCGTTG) for 5 min at 25°C followed by 30 min at 42°C for. Single stranded oligonucleotides were digested by adding 5 U exonuclease I and 1x reaction buffer (Thermo Fisher) in a volume of 5 µl/well and incubation at 37°C for 45 min. dGTP tailing was performed using the Terminal Deoxynucleotidyl Transferase Kit (Promega), containing 0.73 U/µl terminal transferase, 2.0 mM dGTP and 1 x terminal transferase buffer and incubation at 37°C for 45 min. Anchor PCR mix was added to reach a final PCR volume of 21 µl/well containing 215 µM of each dNTP, 4 %

DMSO, 0.25 µl Herculase II DNA polymerase (Agilent) and 540 mM each of the primers Anchor forward (ACAGCAGGTCAGTCAAGCAGTAGCAGCAGTTCGATAAGCGGCCGCCATGGACCCCCCCCC C-PTO-N) and TRAC A (GTCAAAGTCGGTGAACAGGC). This and the following nested PCRs were performed by incubation at 96°C for 3 min, followed by 24 cycles of 96°C for 15 sec, 60°C for 30 sec, 72°C for 40 sec and a final extension step at 72°C for 5 min. For the first nested PCR step, 1 µl of anchor PCR product was transferred to a new 96-well plate containing 20 µl 1x buffer including 190 µM of each dNTP, 4 % DMSO, 0.25 µl Herculase II and 475 mM each of the primers ACAGCAGGTCAGTCAAGCAGTA and GAGACCGAGGATCTTTTAACTG (Adaptor I and TRAC B). The second nested PCR step was performed under identical conditions after transfer of 1 µl nested PCR1 product into a new 96-well plate using the primers AGCAGTAGCAGCAGTTCGATAA and CAGGTTCTGGGTTCTGGATG (Adaptor II and TRAC C). PCR products were ligated into the plasmid pCR-Blunt (Thermo Fischer) for sequencing with the reverse M13 primer. TCRα chain sequences were aligned using the IMGT/V-QUEST data base (1). Typically, TCRα sequences were obtained for 30 to 50% of sorted single cells.

Re-expression and functional testing of TCRs. TCRα chains were sub-cloned into the lentiviral vector FUGW harboring a T2A peptide and a truncated human (hu)CD2 (pFUGW-T2A-huCD2). For virus production, HEK293FT cells were transiently transfected with the respective lentiviral vector, a PAX2 packaging plasmid and a VSVG envelope plasmid. The supernatant was collected and centrifuged at 14,000 rpm for 4 h to pellet viral particles. Pellets were resuspended in 3–4 ml of fresh DMEM and stored at −80°C. TCRs of interest were reconstituted by transduction of BW58 NFAT-GFP reporter hybridoma cells stably expressing the 'fixed' TCRβ chain with viral vectors encoding for the TCRα chains A, B, C and D (MOI 0.3). 7×10^4 hybridoma cells were stimulated with titrated amounts of peptide $PLP_{9\text{-}20}$ or anti-CD3 antibody in the presence of 5×10^4 congenically marked BM-derived DCs from *Plp1*^{KO} mice in 200 µl IMDM supplemented with 10 % FCS. After 20 h, cells were harvested and GFP expression was measured by flow cytometry.

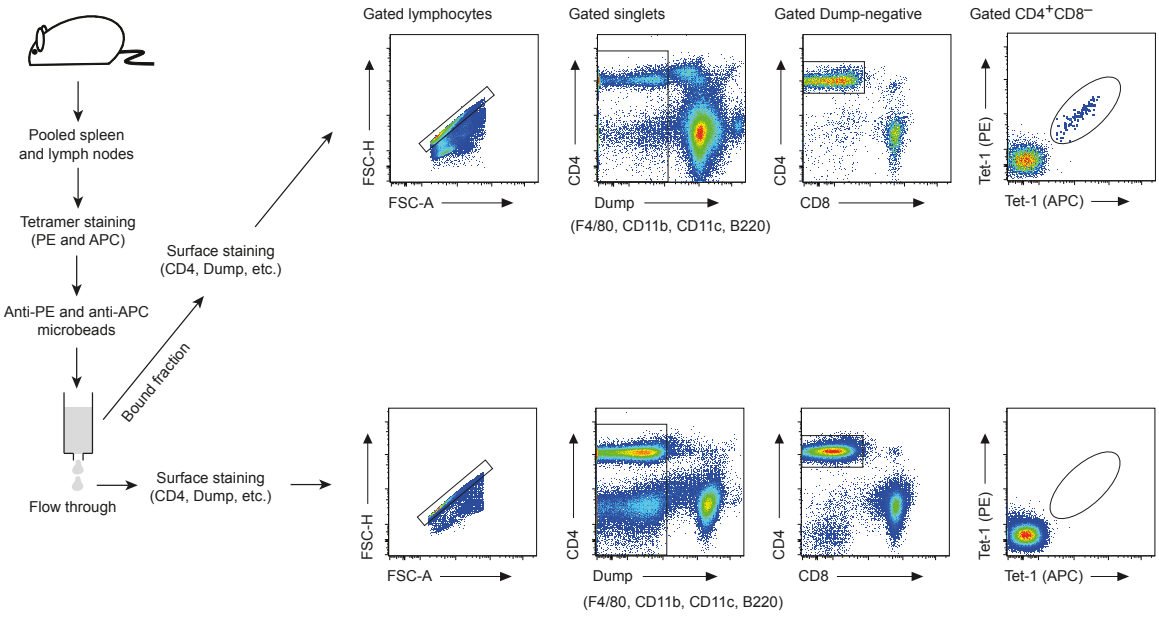

Fig. S1. Workflow and gating strategy for enrichment and analysis of tetramer stained cells. Single cell suspensions from pooled lymph nodes (axillary, mesenteric, inguinal and cervical) and spleen were co–stained with Tet-1 or Tet-3 conjugated to PE and APC. Tet–labelled cells were subsequently enriched using anti-PE and anti-APC microbeads and magnetized columns. Cells in the flow through as well as bound cells were stained with antibodies to CD4 and CD8 (and other surface markers of interest) as well as with a 'dump' cocktail of antibodies (F4/80, CD11b, CD11c and B220).

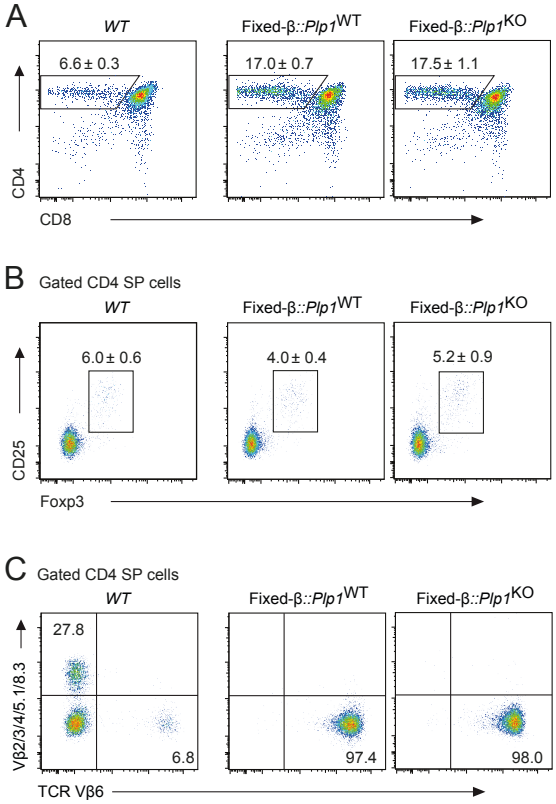

Fig. S2. Thymus phenotype of Fixed-β mice. (A) CD4 and CD8 expression on total thymocytes from mice of the indicated genotype. The mean number ± SEM of CD4 SP cells is indicated (n ≥ 3 each). (B) CD25 and Foxp3 in gated CD4 SP thymocytes from mice of the indicated genotype. The mean number ± SEM of $Foxp3^{+}CD25^{+}$ cells is indicated (n ≥ 3 each). (C) Expresison of the transgenic Vβ6-containing TCRβ chain (or endogenously expressed TCRβ6 rearrangements in the case of *WT* mice) versus a pool of 'endogenous' TCRβ rearrangements (Vβ2, Vβ3, Vβ4, Vβ5.1/2, Vβ8.3) on gated CD4 SP cells. Data are representative of n ≥ 3 each.

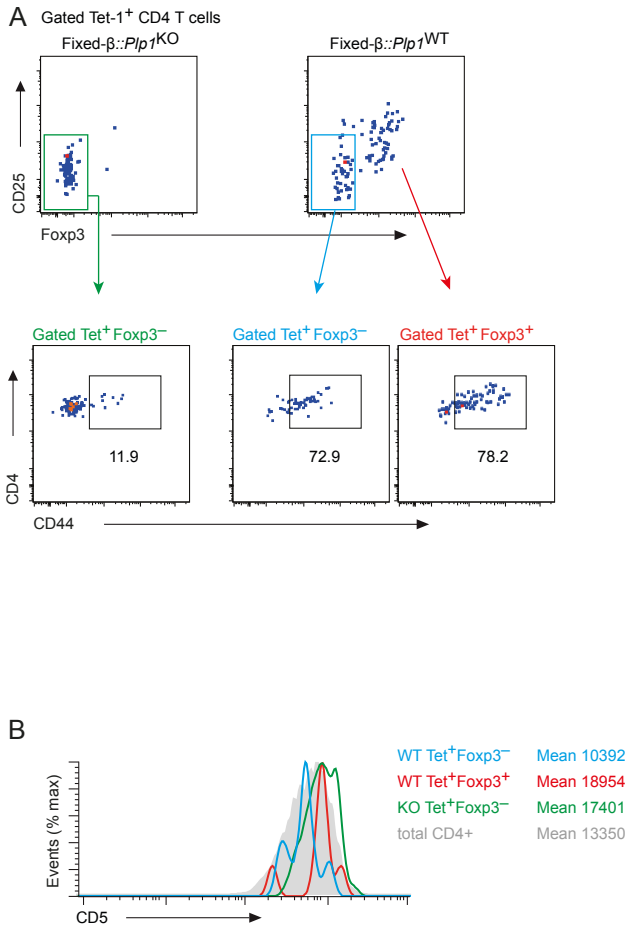

Fig. S3. CD44 and CD5 on PLP$_{11\text{-}19}$-specific peripheral CD4 T cells in *Plp1*^{KO} or *Plp1*^{WT} mice expressing a fixed TCRβ chain. (A) CD44 versus CD4 expression on tetramer-positive peripheral CD4 T cells from pooled spleen and lymph node cells (see also Fig. 3A), gated for Foxp3^{GFP} and CD25 expression as indicated (pooled cells from n ≥ 5 each in two independent experiments). (B) CD5 expression on tetramer-positive peripheral CD4 T cells from pooled spleen and lymph node cells, gated as in (A). The mean fluorescence is indicated (pooled cells from n ≥ 5 each in two independent experiments).

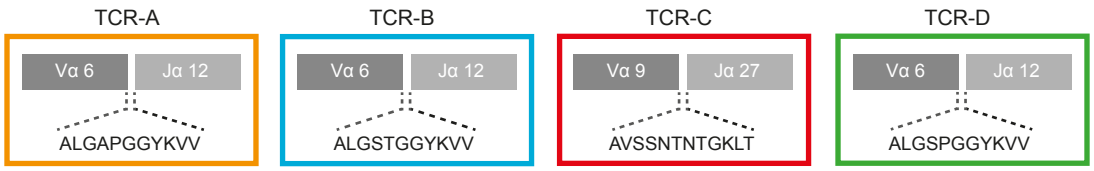

Fig. S4. TCRα chains of the of TCRs A, B, C and D. The four 'public' TCRs A, B, C and D contain the indicated Vα- and Jα-elements. The amino acid sequence of the respective CDR3 regions is depicted underneath.

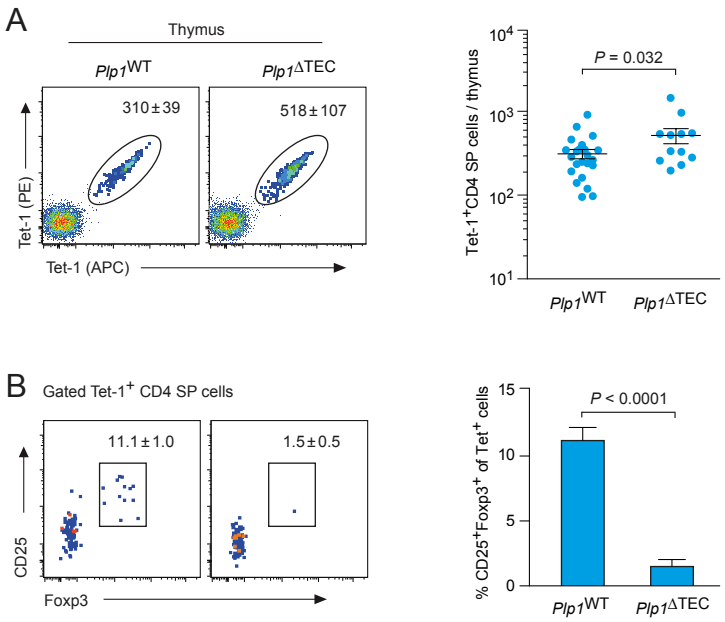

Fig. S5. Number and phenotype of PLP$_{11\text{-}19}$-specific CD4 SP thymocytes in *Plp1*^{WT} or *Plp1*$^{\Delta TEC}$ mice expressing a fixed TCRβ chain. (A) Representative flow cytometric analysis of thymocytes after enrichment of PLP$_{11\text{-}19}$ specific cells. Dot plots are gated on Dump$^-$CD8$^-$CD4$^+$ cells. The calculated mean number ± SEM of tetramer (PE/APC) double-positive cells per thymus is indicated (n ≥ 12 each). The graph on the right shows a summary. Each data point represents an individual mouse. (B) Representative flow cytometric analysis of Foxp3^{GFP} and CD25 expression in tetramer (PE/APC) double-positive CD4 SP cells. Numbers indicate the mean frequency ± SEM of Foxp3$^+$CD25$^+$ cells (n ≥ 12 each).

References

1. E. Alamyar, P. Duroux, M. P. Lefranc, V. Giudicelli, IMGT((R)) tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS. *Methods Mol. Biol.* **882**, 569-604 (2012).