Supplemental material

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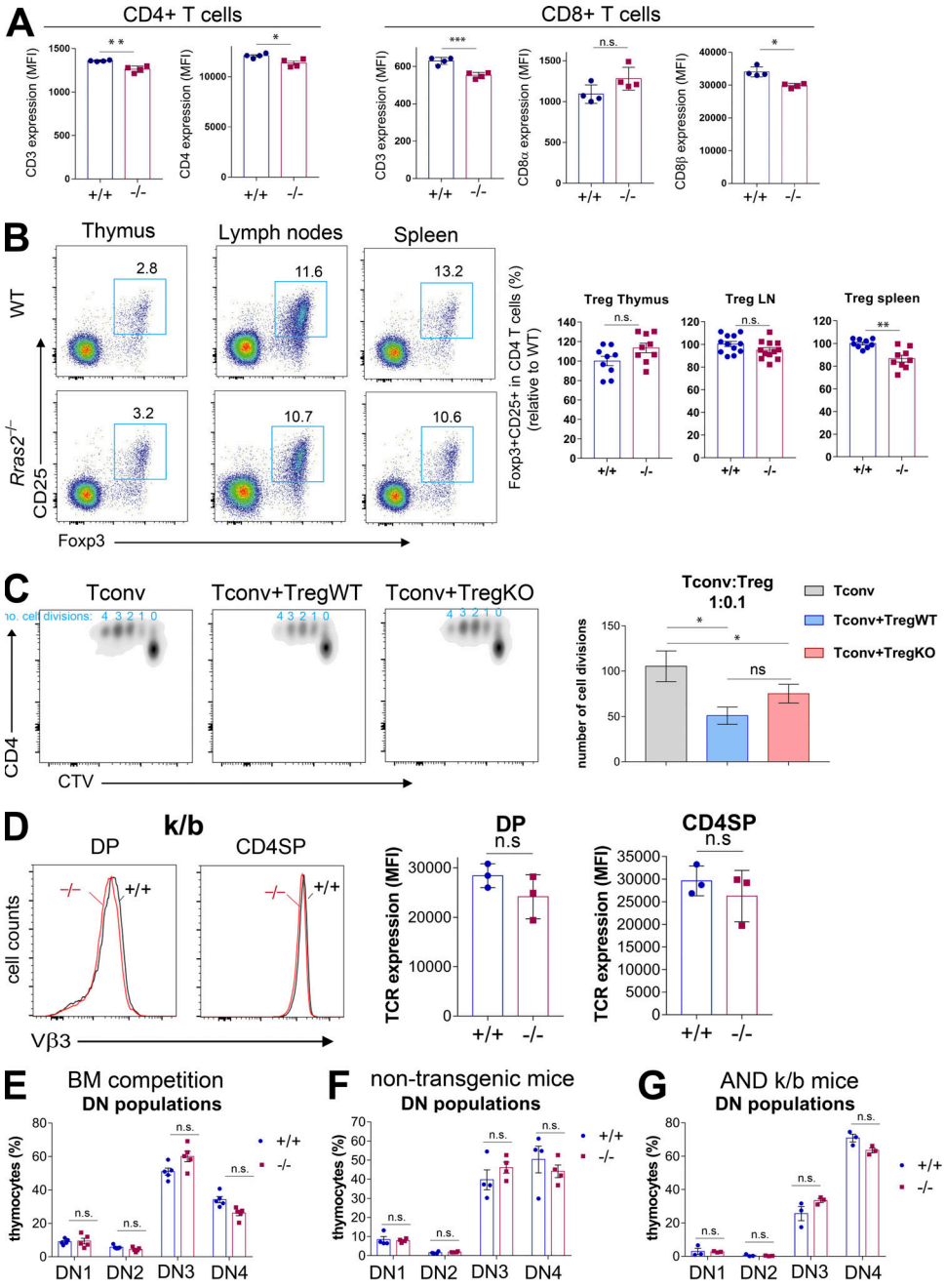

Figure S1. **Analysis of TCR and co-receptor expression in peripheral CD4 and CD8 T cells. (A)** Flow cytometry plots illustrate CD4 and CD8 populations in lymph nodes of WT and *Rras2*$^{-/-}$ mice. Bar plots show the expression of CD3 and CD4 in the CD4 T cells and CD3, CD8α and CD8β in the CD8 T cells of WT and *Rras2*$^{-/-}$ mice. Quantitative data are means ± SEM (n = 4). *, P < 0.05; **, P < 0.005; ***, P < 0.0005 (unpaired two-tailed Student's t test). This experiment was repeated three times. **(B)** Frequency of nT reg cells within thymic and peripheral CD4 T cells from WT and *Rras2*$^{-/-}$ mice. Two-color histograms show the gating strategy to quantify the percentage of Foxp3$^+$CD25$^+$ cells within the thymic CD4SP and peripheral CD4$^+$ T cell populations. Quantitative data are plotted to the right and normalized to the mean of the WT samples (taken as 100). The plots show means ± SEM (n = 8–12). n.s., P > 0.05; **, P < 0.005 (unpaired two-tailed Student's t test). This experiment was repeated three times. **(C)** T reg cell functional analysis in which conventional CD4$^+$ T cell proliferation in response to anti-CD3$^+$anti-CD28 stimulus was measured by CTV dilution after 3 d of incubation. CTV-labeled CD4 T cells were co-incubated at 1:0.1 ratio with purified CD25$^+$CD4$^+$ T reg cells purified from either WT or *Rras2*$^{-/-}$ mice. As a control, CTV-labeled conventional CD4 T cells were co-cultured in combination with a 1:0.1 ratio of unlabeled conventional T cells. Bar plots to the right show a quantification of the number of total cell divisions as the mean ± SEM (n = 3). n.s., P > 0.05; *, P < 0.05 (unpaired two-tailed Student's t test). This experiment was repeated twice. **(D)** Flow cytometry analysis of AND transgenic Vβ3 expression in the indicated thymocyte populations in mice of mixed k/b background. An overlay plot of *Rras2*$^{+/+}$ (black line) and *Rras2*$^{-/-}$ (red line) is shown on the left, and quantification of Vβ3 expression is shown in the bar blots on the right. Quantitative data in all panels are means ± SEM (n = 3). Unpaired two-tailed Student's t test. This experiment was repeated three times. **(E-G)** Analysis of DN thymocyte distribution within the major four DN1–DN4 subpopulations (DN1: CD44$^+$CD25$^-$; DN2: CD44$^+$CD25$^+$; DN3: CD44$^-$CD25$^+$; DN4: CD44$^-$CD25$^-$) in experiments shown in Fig. 2, B–E (BM competition), Fig. 1, A–C (nontransgenic mice), and Fig. 1, D and E (AND k/b mice). Data are means ± SEM (n = 3–5). n.s., not significant (unpaired two-tailed Student's t test). Experiments in E–G were repeated twice. T conv cells, unlabeled conventional T cells.

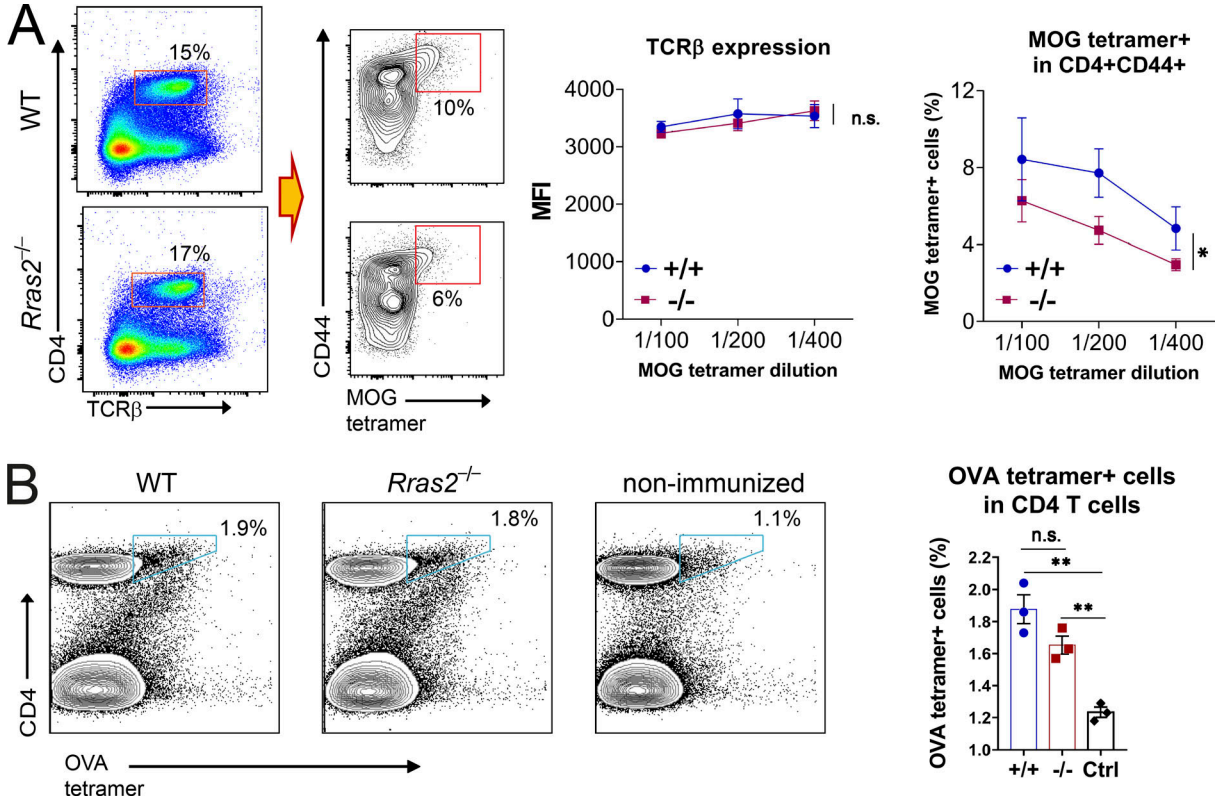

Figure S2. **Frequency of MOG tetramer⁺ CD4 T cells and T cell response to a foreign antigen. (A)** Detection of MOG tetramer⁺ CD4⁺ T cells in spleens of the mice used in Fig. 7 A, and sacrificed at day 30 after immunization, was evaluated in the CD4⁺TCRβ⁺CD44⁺ population. Line plots show the expression of TCRβ and the percentage of MOG tetramer⁺ cells versus the tetramer dilution used for staining. This experiment was repeated three times. **(B)** Detection of OVA tetramer⁺ CD4⁺ T cells in popliteal lymph nodes 7 d after footpad immunization with OVAp-II peptide plus LPS or with LPS alone (nonimmunized control). Bar plots are means ± SEM (n = 3). n.s., $P > 0.05$; **, $P < 0.005$ (unpaired two-tailed Student's t test). This experiment was repeated twice. n.s., not significant.

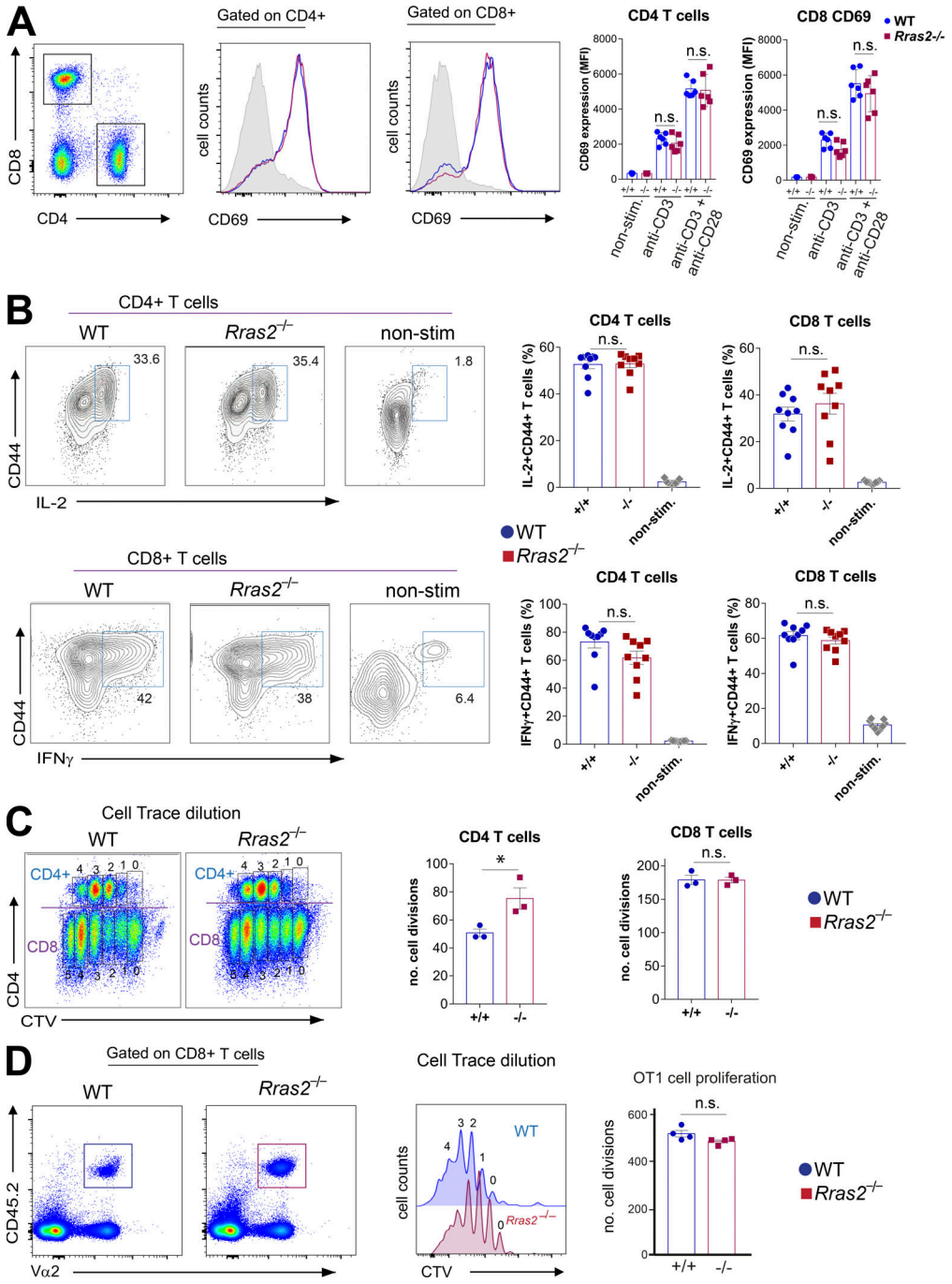

Figure S3. **T cells from *Rras2*$^{-/-}$ mice are not deficient in their response to TCR-triggering stimuli. (A)** Splenic T cells from WT and *Rras2*$^{-/-}$ mice were stimulated for 24 h with plate-bound anti-CD3 (20 µg/ml) alone or with soluble anti-CD28 (1 µg/ml). Histogram plots show the expression of CD69 in WT (blue) and *Rras2*$^{-/-}$ (red) CD4 and CD8 T cells after 24-h incubation with anti-CD3 or without stimulation (gray). Bar graphs show the quantification of CD69 expression after 24 h, represented as means ± SEM (n = 6). n.s., P > 0.05 (unpaired two-tailed Student's t test). **(B)** Splenic T cells from WT and *Rras2*$^{-/-}$ mice were stimulated for 24 h with plate-bound anti-CD3 (20 µg/ml) plus soluble anti-CD28 (1 µg/ml). Histogram plots show the expression of CD44 and intracellular IL-2 in CD4 T cells and of CD44 and intracellular IFNγ in CD8 T cells. Bar graphs show the quantification of IL-2 and IFNγ expression within the CD44$^+$ population of CD4$^+$ and CD8$^+$ T cells. Blue circles, WT mice; red squares, *Rras2*$^{-/-}$ mice. Gray symbols represent cytokine expression in nonstimulated WT T cells. Data are means ± SEM (n = 9). n.s., P > 0.05 (unpaired two-tailed Student's t test). **(C)** Splenic T cells from WT and *Rras2*$^{-/-}$ mice were labeled with CTV and stimulated for 6 d with plate-bound anti-CD3 (20 µg/ml) plus soluble anti-CD28 (1 µg/ml). The number of cell divisions was calculated according to CTV dilution in both CD4$^+$ T cells and CD4$^-$ (CD8$^+$) T cells. Quantitative data in the bar plots are means ± SEM (n = 3). *, P < 0.05; n.s., P > 0.05; (unpaired two-tailed Student's t test). **(D)** CTV-stained CD8 T cells from WT and *Rras2*$^{-/-}$ CD45.2$^+$ OT1^{Tg} mice were inoculated into CD45.1$^+$ receptor mice that were subsequently immunized with OVA (200 µg/mice) plus LPS. Flow cytometry plots show the presence of transferred CD8 OT1 T cells (CD45.2$^+$ Vα2$^+$) from WT and *Rras2*$^{-/-}$ donor mice in the receptor mice 5 d after immunization. The histogram illustrates CTV-dilution in transferred T cells after 5 d, which is quantified according to the total number of cell divisions. Quantitative data are means ± SEM (n = 4). n.s., P > 0.05 (unpaired two-tailed Student's t test). All experiments in this figure were repeated twice. stim, stimulated.

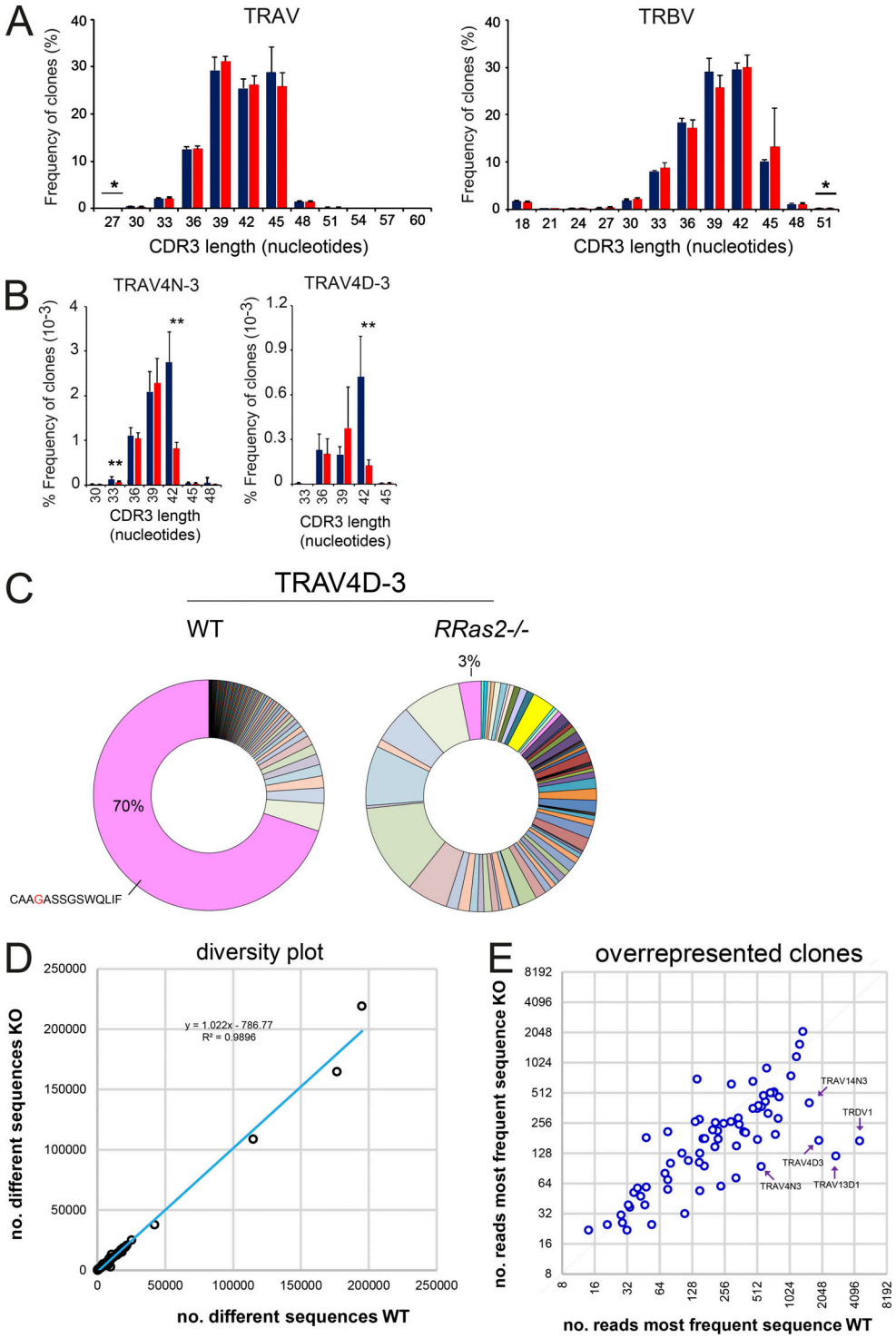

Figure S4. ***Rras2*$^{-/-}$mice have a biased TCR repertoire with altered CDR3α length in peripheral CD4^{+} cells. (A)** Differences in CDR3 amino acid length according to Vα usage. Bar plots show CDR3α and CDR3β length distribution in the total TCR repertoire. Data are means ± SD (n = 6 mice per group). *, P < 0.05 (unpaired two-tailed Student's t test). **(B)** Comparison of CDR3 amino acid length for CD4 T cell clones using two specific Vα regions (TRAV4N-3 and TRAV4D-3) between WT (blue) and *Rras2*$^{-/-}$ (red) mice. Quantitative data are means ± SD (n = 6). **, P < 0.005 (unpaired two-tailed Student's t test). **(C)** Frequency of specific CDR3α sequences for CD4 T cell clones bearing the TRAV4D-3 Vα region represented by pie charts. **(D)** Representation of the number of different TCRα amino acid sequences in all mice (n = 6 per genotype) classified according to their Vα region in WT (x axis) versus *Rras2*$^{-/-}$ CD4^{+} T cells (y axis). Data were fitted to a line plot with an R^2 of 0.9896. An average of 563 ± 825 (mean ± SD) different sequences per Vα type in WT cells and an average of 560 ± 814 sequences in *Rras2*$^{-/-}$ cells were detected. n.s., P > 0.05 (two-tailed paired t test). **(E)** Representation of the number of reads of the most frequent sequence within each Vα family for the sum of all mice per genotype (n = 6). Sequences with a number of reads <100 in WT and *Rras2*$^{-/-}$ CD4^{+} T cells were discarded. A line has been hand drawn to indicate a diagonal of equal expression. Data in this figure result from the aggregation of two experiments (three mice of each genotype per group and per experiment) performed independently.

A

```
                <5'UTR
4   agacagaacctgtagtctgtaagcacaagtgtgtctgggcacaag 48
7   agacagaacctgtagtctgtaagcacaagtgtgtctgggcacaag 51

                                                5'UTR><L1
49  acacccttcagggctggagagaagacaaccAgcgattggacaggggtcatggagaggaacctgggagctgtgctggggat 128
52  acacccttcagggctggagagaagacaaccAgcgattggacaggggtcatggagaggaacctgggagctgtgctggggat 131

                    L1><L2   L2><FR1
129 tctgtgggtgcagatttgctgggtgagcggagataaggtgaagcaaagtccctcagcgctgagtctccaagagggaacca 208
132 tctgtgggtgcagatttgctgggtgagcggagataaggtgaagcaaagtccctcagcgctgagtctccaagagggaacca 211

                Forward D3/N3 primer
                              FR1><CDR1      CDR1><FR2
209 attctgctctgagatgcaatttttctatcgctgcgacaactgtgcagtggttcctacagaatcccaggggcagcctcatg 288
212 attctgctctgagatgcaatttttctatcgctgcgacaactgtgcagtggttcctacagaatcccaggggcagcctcatg 291

            FR2><CDR2      CDR2><FR3
289 aatctttttttacctggtgccaggaacaaaggagaatgggaggttaaagtcagcatttgattctaaggagagctacagcac 368
292 aatctttttttacctggtgccaggaacaaaggagaatgggaggttaaagtcagcatttgattctaaggagagctacagcac 371
                                                      <JP
                                              FR3><CDR3    V>JP><J
369 cctgcacattagggatgcccagctggaggactcaggcacttacttctgtgctgctg                          424
372 cctgcacattagggatgcccagctggaggactcaggcacttacttctgtgctgctg                          427

 18                                                        tgcatcttctggcagctggcaac 40

                                                                  Reverse D3/N3 primer
    CDR3><FR4                          FR4><C
41  tcatctttggatctggaacccaactgacagttatgcctg                                        79
 0                                       acatccagaacccagaacctgctgtgtaccagttAAAAGat 40
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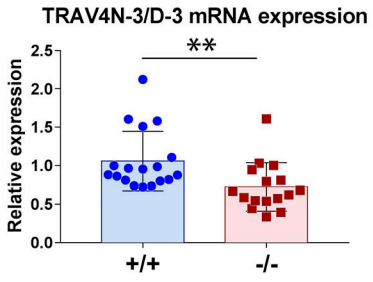
B



C

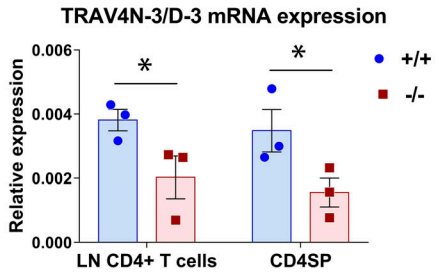


Figure S5. **qPCR analysis of TRAV4N-3/D-3 mRNA expression in peripheral CD4⁺ T cells and mature CD4SP thymocytes of *Rras*$^{-/-}$ versus WT mice. (A)** Nucleotide sequence of mRNA encoding TRAV4D-3 and TRAV4N-3 Vα sequences bearing the GASS CDR3α 5′ untranslated (UTR), leader sequence (L1, L2), framework FR1–FR4 sequences, and the beginning of the constant domain (C) are indicated. The coding sequence is in blue type except for the nucleotides encoding for the three CDRs, which are in red. Primers used for qPCR anneal to the boxed sequences. **(B)** RT-qPCR using the above forward and reverse primers to quantify the expression of mRNA for TRAV4D-3 and TRAV4N-3 sequences in the samples used for RNaseq (Fig. 8). Expression was normalized to expression of GAPDH, and final ratios were normalized to the mean of WT expression (set as 1.0). Each mRNA sample was run in triplicate (n = 6 mice per group). Data are means ± SEM. **, P < 0.005 (unpaired two-tailed Welch's t test). **(C)** RT-qPCR as in B using mRNA samples extracted from FACS-sorted lymph node CD4⁺ T cells and CD4SP thymocytes. Data are expressed normalized to expression of GAPDH. The mean of triplicate PCR runs per mRNA sample (n = 3 mice per group) ± SEM is shown. *, P < 0.05 (unpaired two-tailed Welch's t test). Experiments in B and C were repeated twice.

Datasets 1–3 are provided online as separate Excel files. Dataset 1 shows TRA sequences per mouse. Dataset 2 shows TRB sequences per mouse. Dataset 3 shows an analysis of TRA sequence diversity within Vα families.