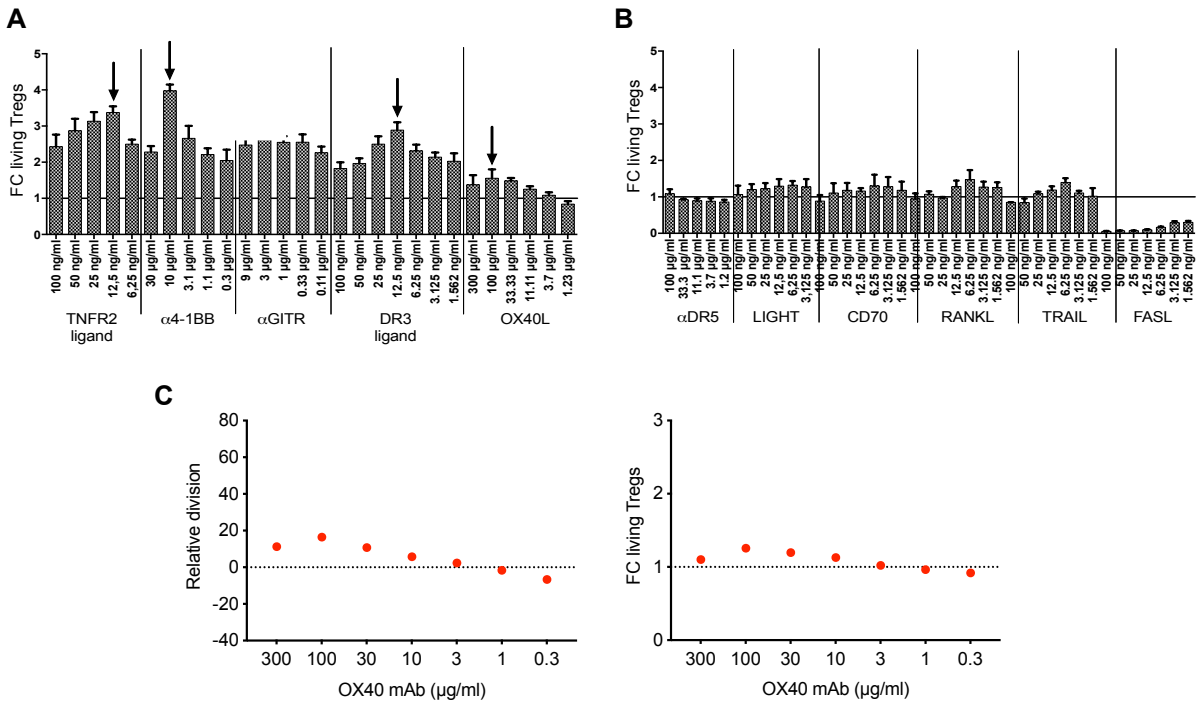

Figure S1. Titration of TNFRSF agonists on Treg expansion. Purified Tregs were stimulated for 3 days with anti-CD3/CD28 coated mAbs and different doses of TNFRSF agonists to measure the fold change (FC) of living cells or the relative division compared to control culture (horizontal line). The dose with the best effect, indicated with an arrow, was selected for the rest of the study. The mean (+SD) of three (A, B) or two (C) independent experiments is shown.

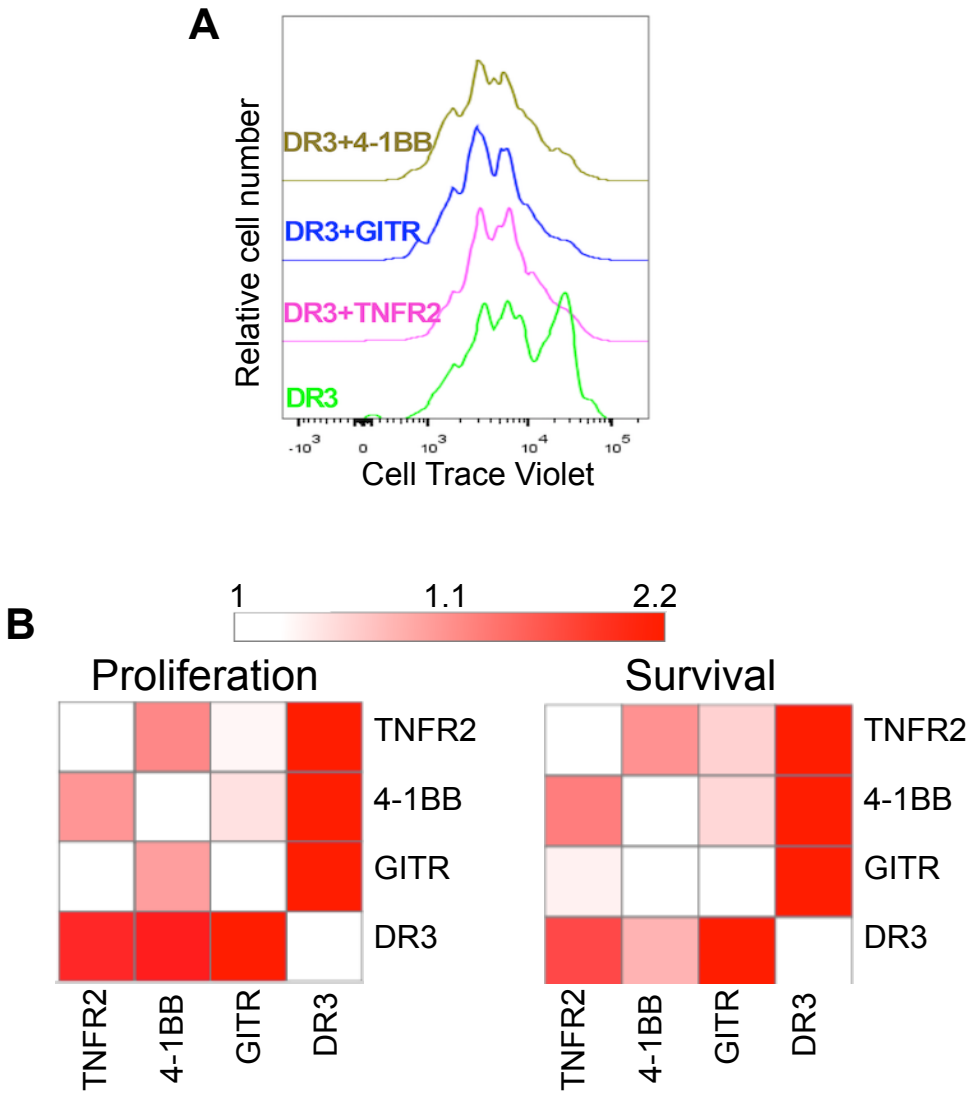

Figure S2. Additive effect of combining two TNFRSF agonists on Treg proliferation and survival. Tregs cultured as in figure 1B-D were co-stimulated by one or two TNFRSF agonists. (A) Representative cell proliferation profile of cells co-stimulated with DR3 agonist alone or combined with the indicated TNFRSF agonists. (B) Relative proliferation and FC living cells relative to the control culture with a single agonist. The mean of three independent experiments is shown.

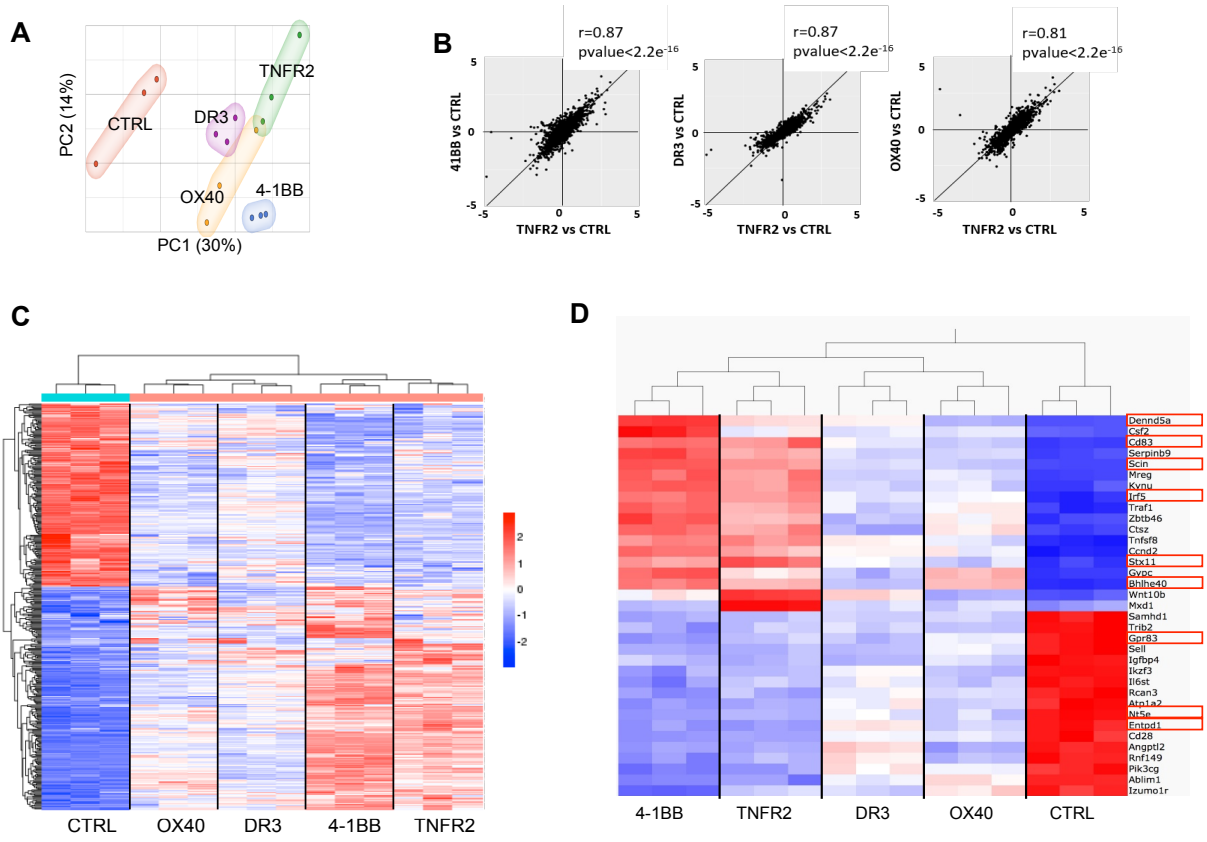

Figure S3. TNFRSF agonists induced a shared transcriptomic signature. RNA-sequencing was performed on Tregs stimulated with anti-CD3/CD28 mAbs and agonists of TNFRSF for 36 hr. (A) PCA analysis of the biological triplicates. (B) FC/FC plots (expressed in log2) of DEG compared to controls (FDR<0.05) to compare the effects of TNFR2 with the effects of the three other agonists (4-1BB, DR3 and OX40) on Tregs. (**C**) DEG (FDR<0.05) by comparing control Tregs with Tregs co-stimulated by the four agonists. (**D**) List of the top DEG in Tregs co-stimulated by TNFRSF agonists using the FDR to set the threshold. Genes belonging to the Treg signature are highlighted.

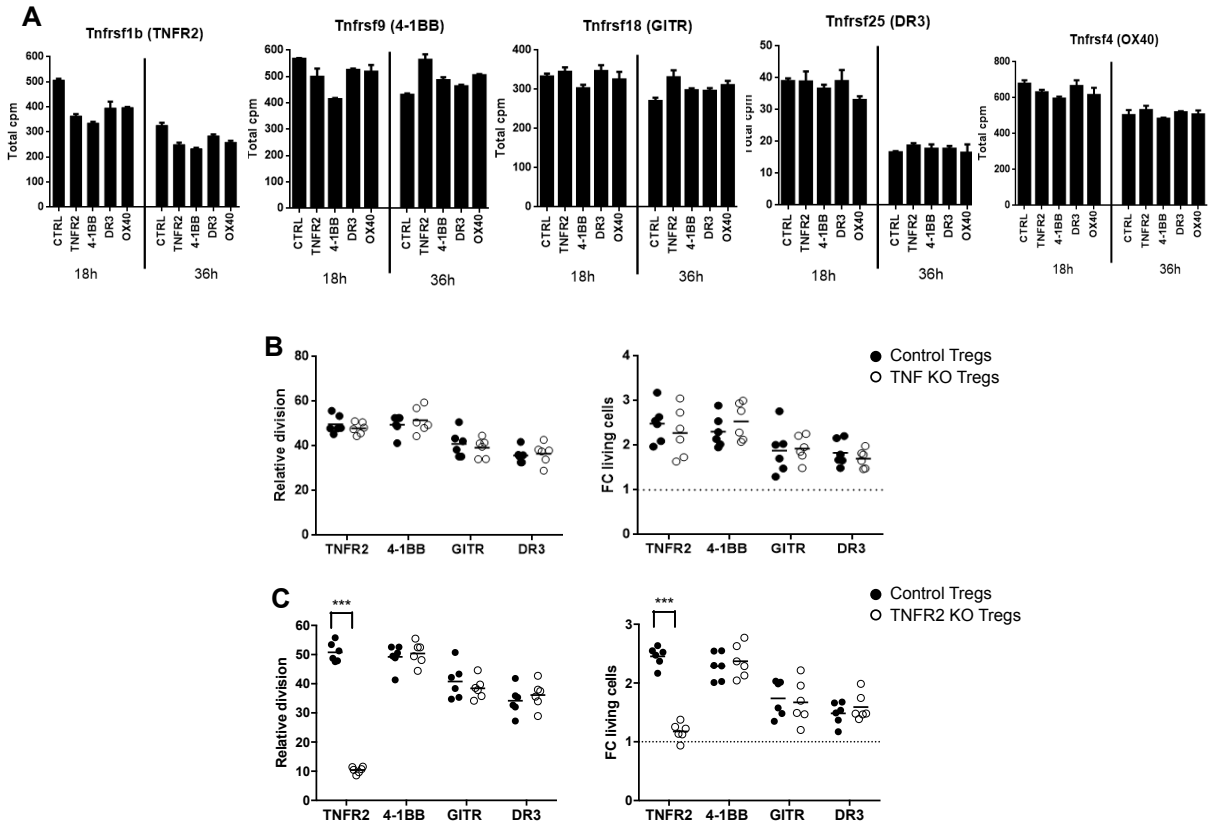

Figure S4. The effect of each TNFRSF agonist on Tregs is independent of autocrine TNF induction. (A) Counts per millions (cpm) of reads, obtained by RNA-sequencing, were used to quantify the expression of various TNFRSF in Tregs cultivated for 18 and 36 hr with coated anti-CD3/CD28 mAbs and different TNFRSF agonists (indicated below the x axis). (B, C) Effect of TNFRSF agonists on TNF-deficient (B) and TNFR2-deficient (C) Tregs compared to control WT Tregs, stimulated and analyzed as in figure 1. Each dot is the value from an individual mouse and the pool of three independent experiments is shown for e and f. Unpaired Mann-Whitney test was used.

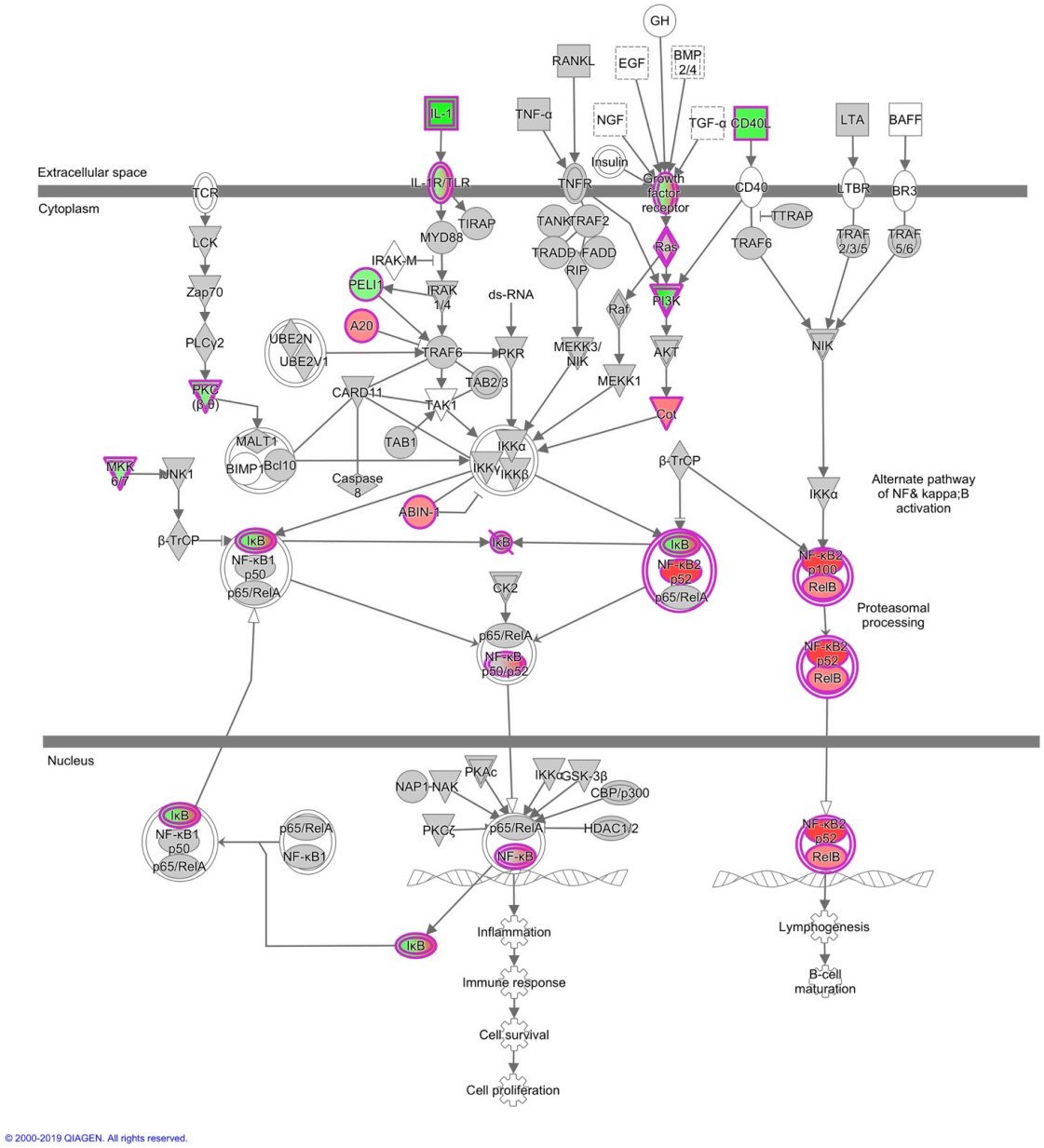

Figure S5. TNFR2 stimulation modulated the NF-κB pathway. Ingenuity Pathway Analysis was used to analyze DEG induced by TNFR2 agonist at 18 h. Genes up-regulated by the TNFR2 agonist are in red and genes down-regulated by the TNFR2 agonist are in green.

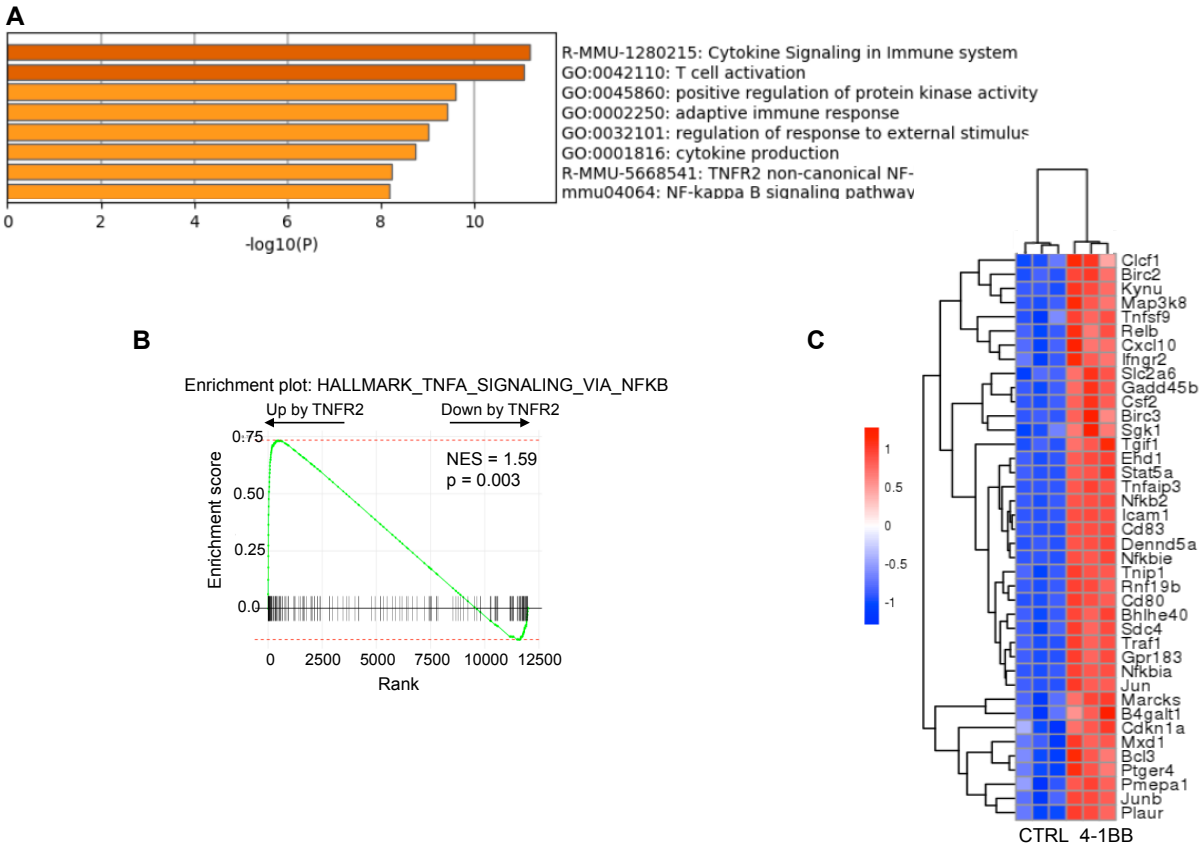

Figure S6. Up-regulation of genes of the NF-κB pathway in Tregs co-stimulated by 4-1BB agonist. Tregs were co-stimulated by the 4-1BB agonist for 18 h as in figure 2. (A) Most significantly represented pathways analyzed by gene ontology of DEG up-regulated by 4-1BB co-stimulation (FDR<0.05, FC>0.5). (B) GSEA plot showing significant enrichment of genes up-regulated by 4-1BB co-stimulation among the indicated "TNF signaling via NF-κB" pathway. (C) Heatmap representation of the leading edge subset genes extracted from the GSEA in B.

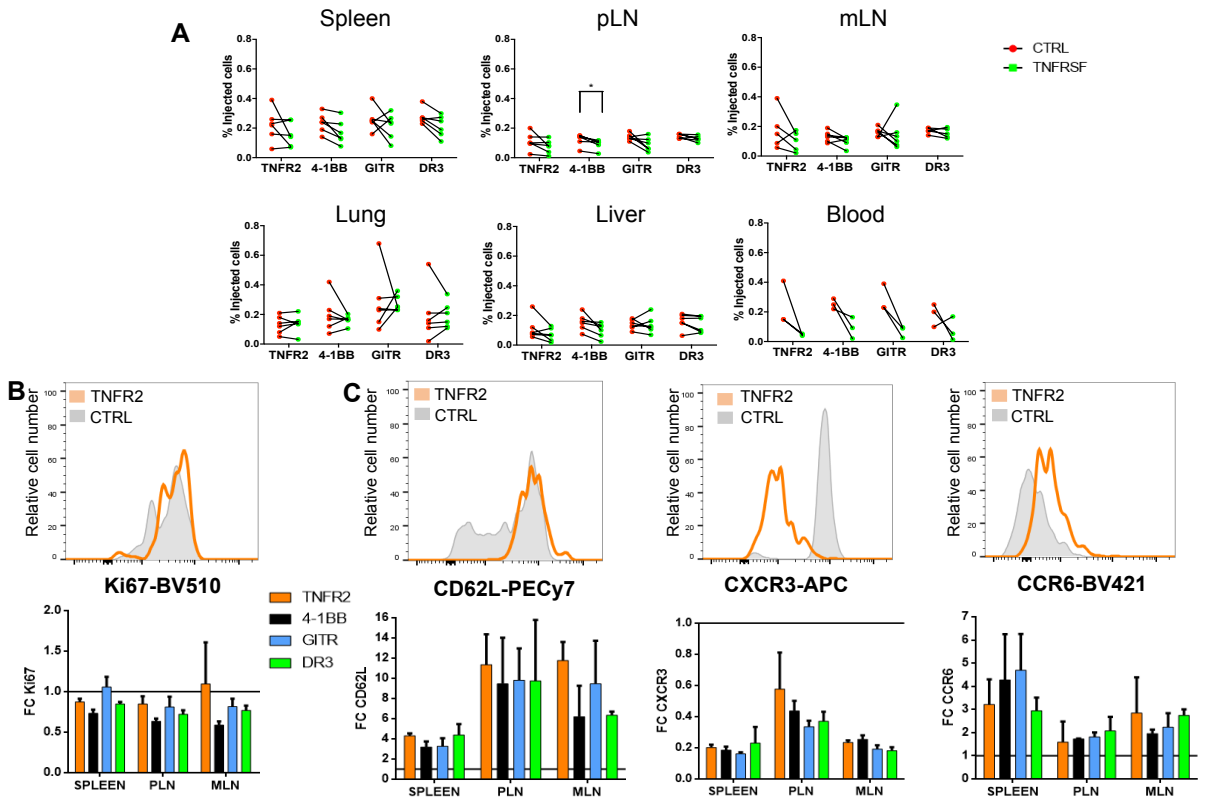

Figure S7. Early cell homeostasis of Tregs co-stimulated by TNFRSF agonists. Tregs, pre-activated with anti-CD3/CD28 mAbs alone (control Tregs) or combined with TNFRSF co-stimulation (co-stimulated Tregs), were co-transferred in equal numbers as in figure 4 to assess their in vivo homeostasis 12 h later. (A) Proportions of co-injected Tregs in indicated organs. Each symbol is a mouse and lines connect cells from the same mouse. Unpaired Mann-Whitney test was used. (B, C) Ki67, CD62L, CXCR3 and CCR6 expression among injected cells. Upper panels show representative histograms for control and TNFR2 co-stimulated Tregs in the spleen. Lower panels show the mean (+SD) of FC MFI expression of co-stimulated compared to control Tregs. Data were obtained from 2 independent experiments with 6 mice per group.

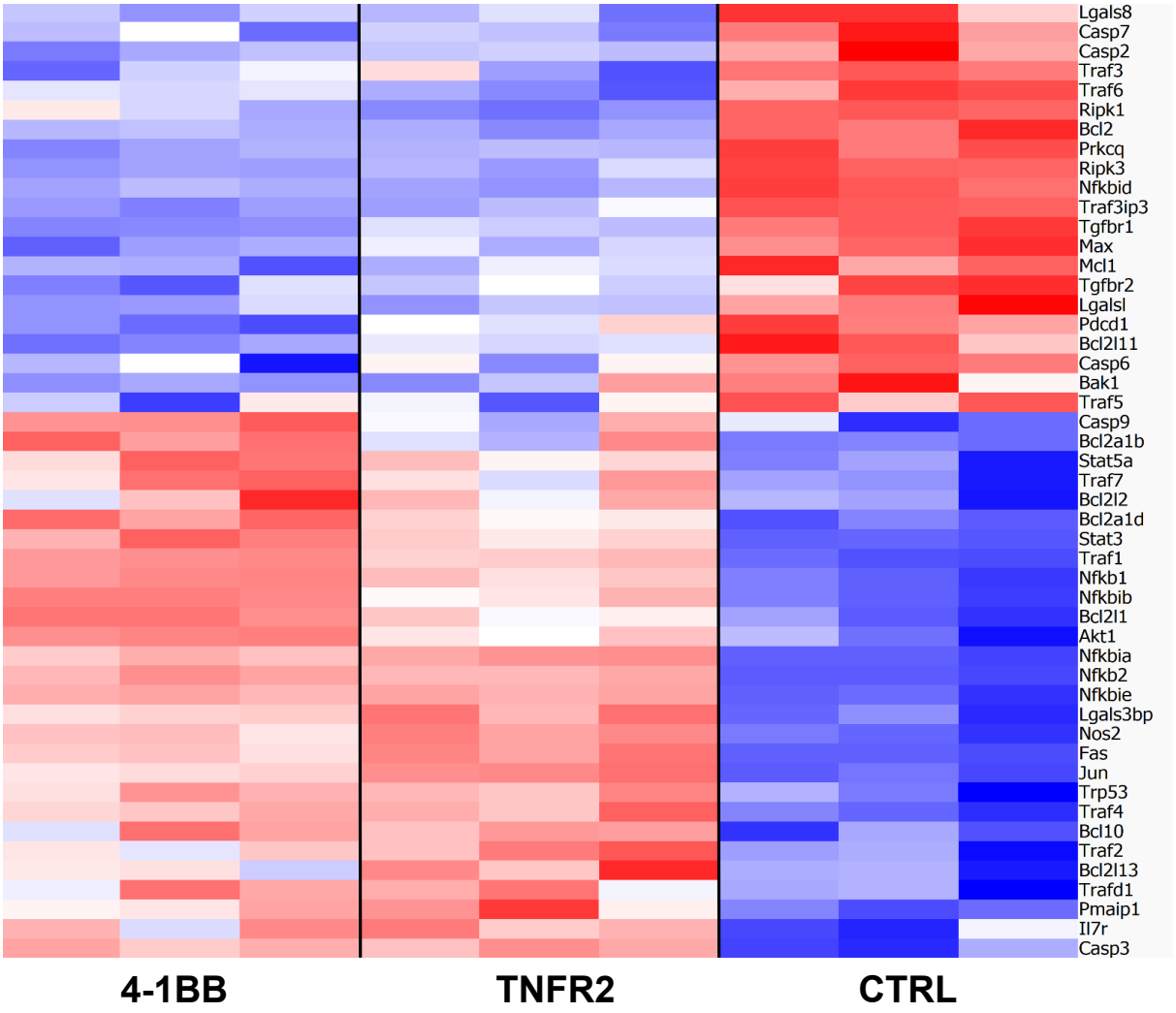

Figure S8. Differential expression of genes involved in T cell survival and apoptosis. DEG (FDR<0.05) between control and Tregs co-stimulated by TNFR2 or 4-1BB for 36 h among genes involved in T cell survival and apoptosis, listed in the Materials and Methods section.