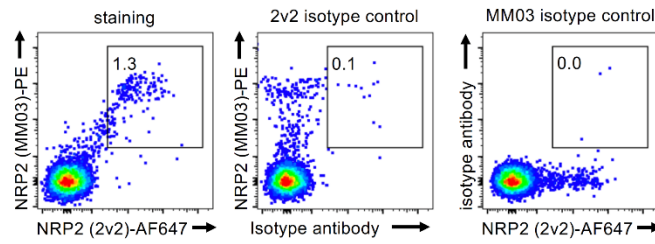
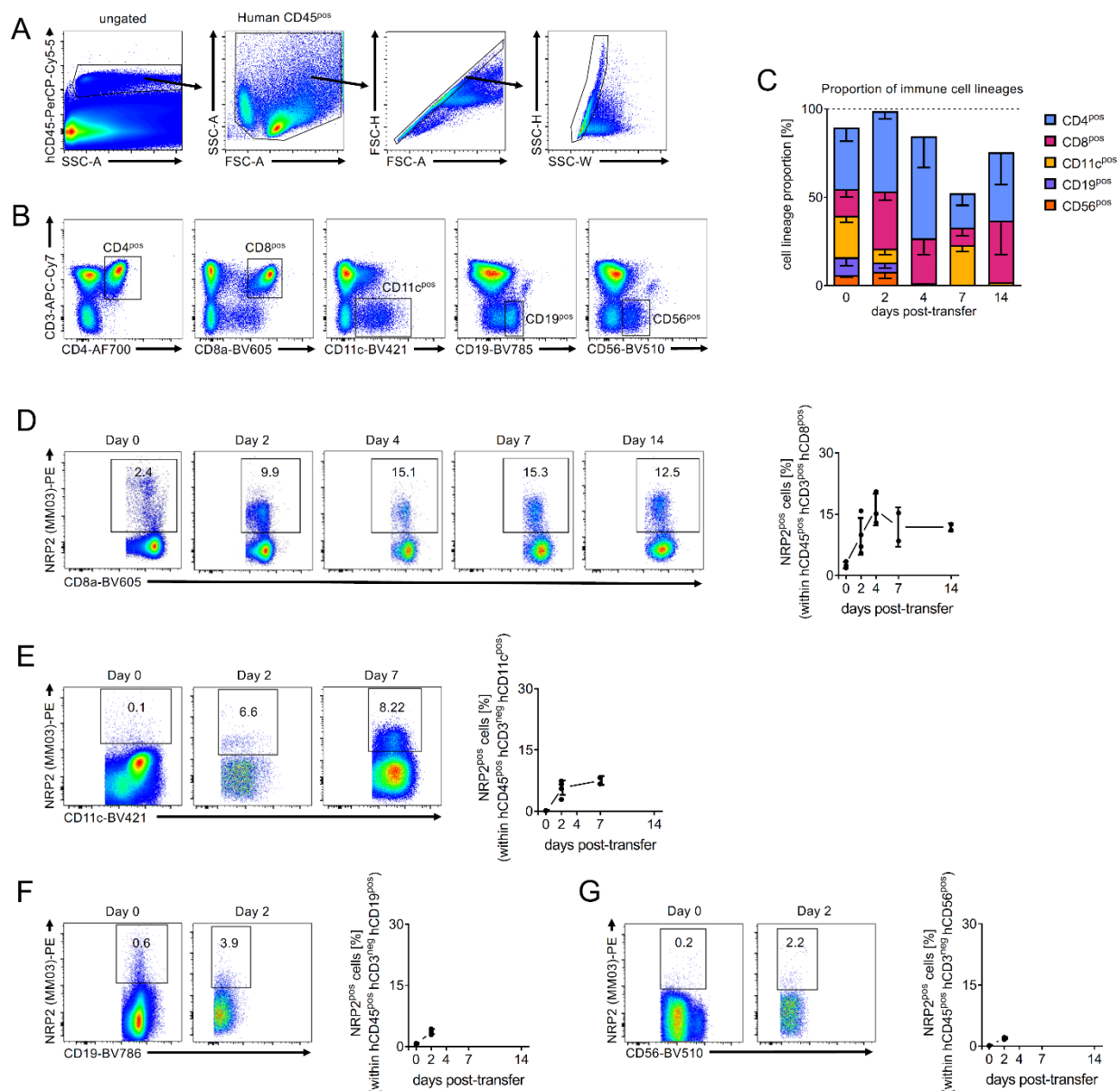


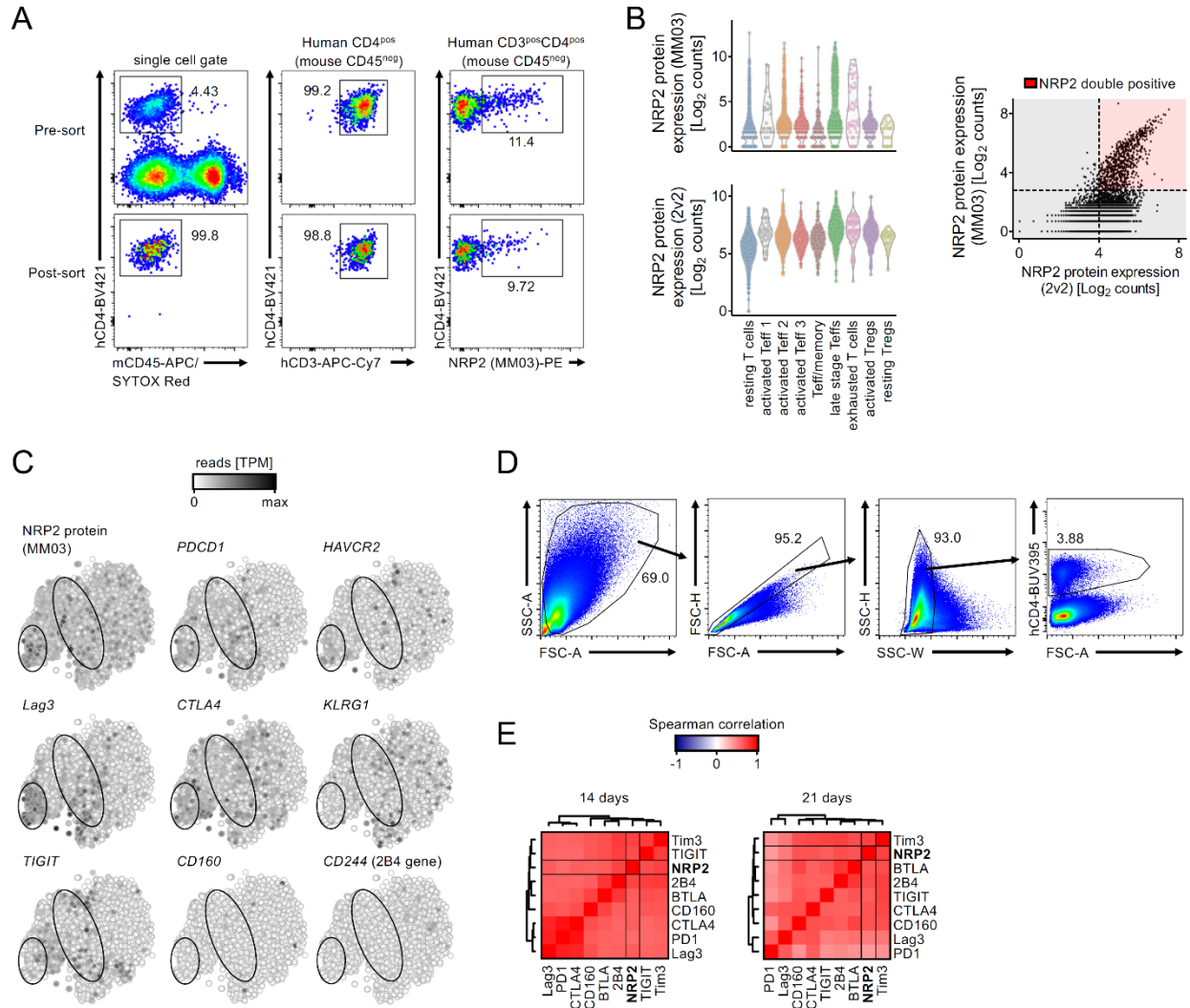
Supplemental Material



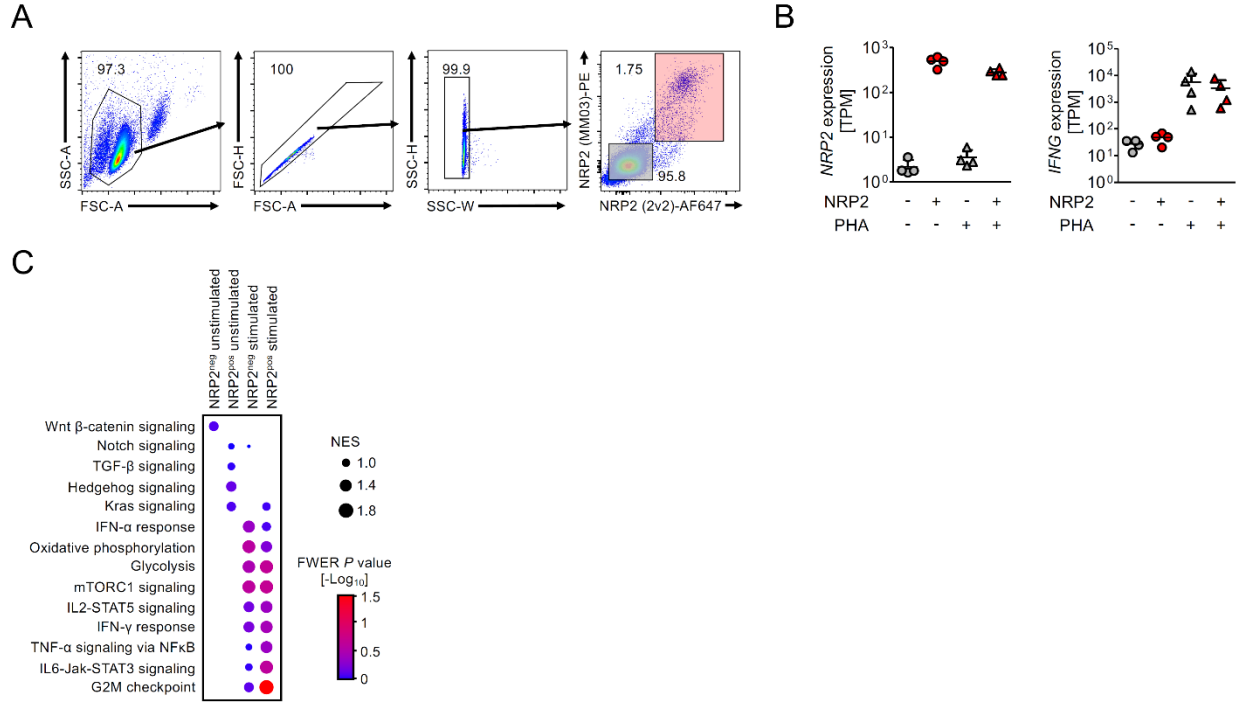
Supplemental Figure 1: Specificity of NRP2 flow cytometric staining was evaluated using freshly isolated PBMC and isotype control antibodies. Dot plots show CD4⁺ T cells (gated).



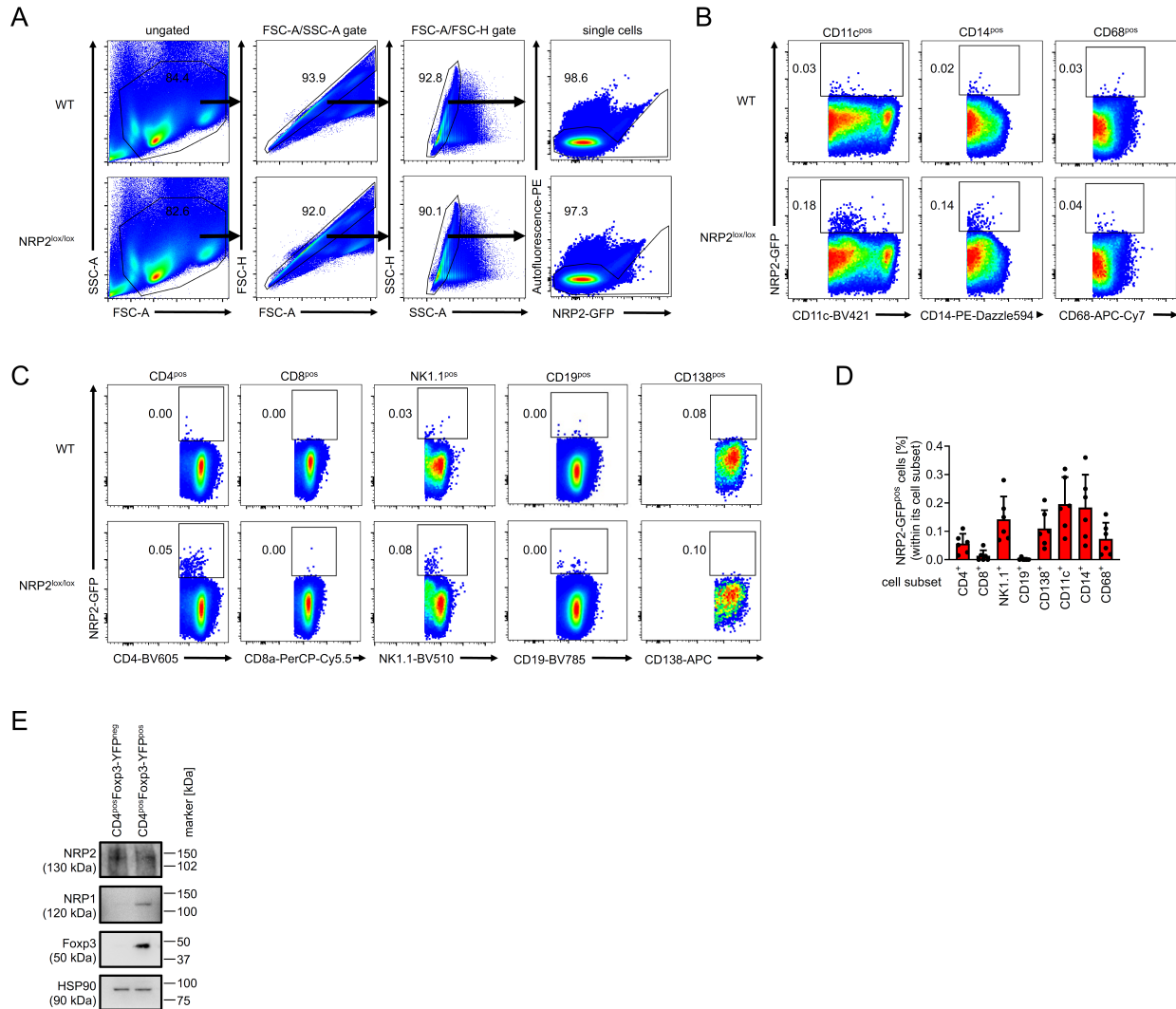
Supplemental Figure 2: Analysis of NRP2 expression on human immune cells within huSCID mice. (A) Gating strategy for the identification of human cells from huSCID mice (also depicted in Figure 1F-G). (B) Identification of human immune cell lineages isolated from splenocytes of huSCID mice. Dotplots are gated on human CD45^{pos} cells. (C) Bar graph represents the percent of each lineage and lineage persistence within human CD45^{pos} cells at different time-points post-transfer in SCID-beige mice. NRP2 expression on (D) human CD8⁺ T cells, (E) human CD11c⁺ myeloid cells, (F) human CD19⁺ B cells, and (G) human CD56⁺ NK cells for up to 14 days dependent on survival within the huSCID. Representative dot plots are gated on human CD45⁺ and each lineage marker⁺. Line graphs illustrate a summary of NRP2 expression in n=2-4 independent experiments per time-point.



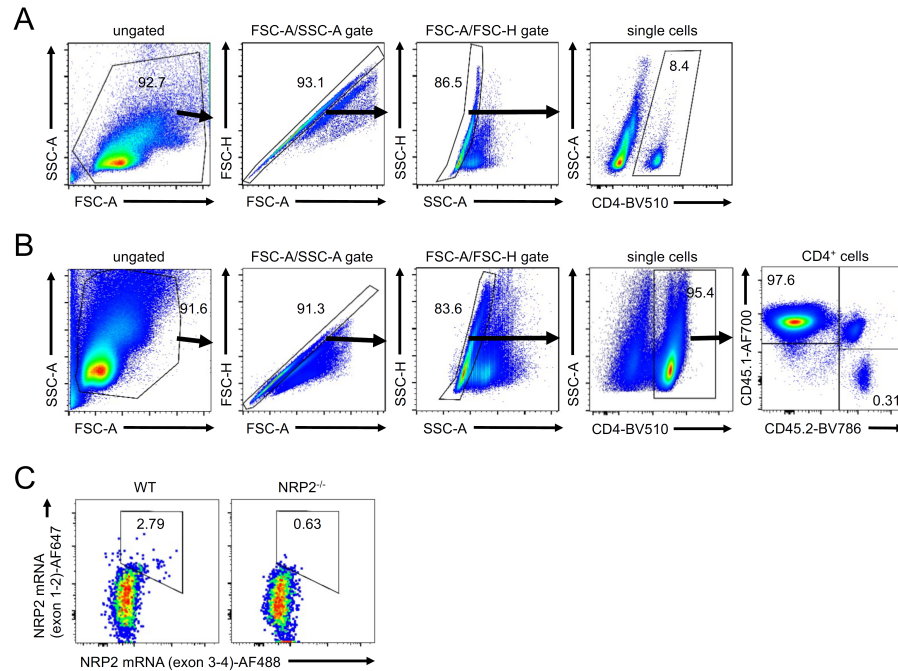
Supplemental Figure 3: Gating strategies and raw data used in Figure 2. (A) Sorting strategy and purity control for sorting of human CD3⁺CD4⁺ T cells from the huSCID model on day 7 post transfer (mCD45: mouse CD45; hCD3: human CD3; hCD4: human CD4). Single cell gating, and hCD4 cells in which mCD45 is gated out (CD45^{neg}) are shown. **(B)** Left: Violin blots of sequenced NRP2 protein expression on the cells identified in Panel A using CITE-seq (top: clone MM03; bottom: clone 2v2). Right: Scatter plot of scRNA-seq NRP2 antibody counts. Color highlighted areas define NRP2^{pos} and NRP2^{neg} cells for each antibody. Positivity using both antibodies is highlighted in red. **(C)** tSNE plots (grey-scale highlighted) of co-inhibitory receptor mRNA expression and NRP2 protein expression (using the MM03 clone). NRP2 enriched regions are highlighted by circles (see Figure 2A-B). **(D)** Gating strategy for flow cytometric analysis of human CD4⁺ T cells used in Figure 2E-J. **(E)** Flow cytometric analysis was performed on CD4⁺ T cells harvested from the huSCID model on day 7 (Figure 2E), day 14 and day 21 post humanization. Heatmaps were generated to illustrate the mean spearman's rank correlation coefficient between the expression of NRP2 and co-inhibitory receptors (n=4 independent experiments per time-point).



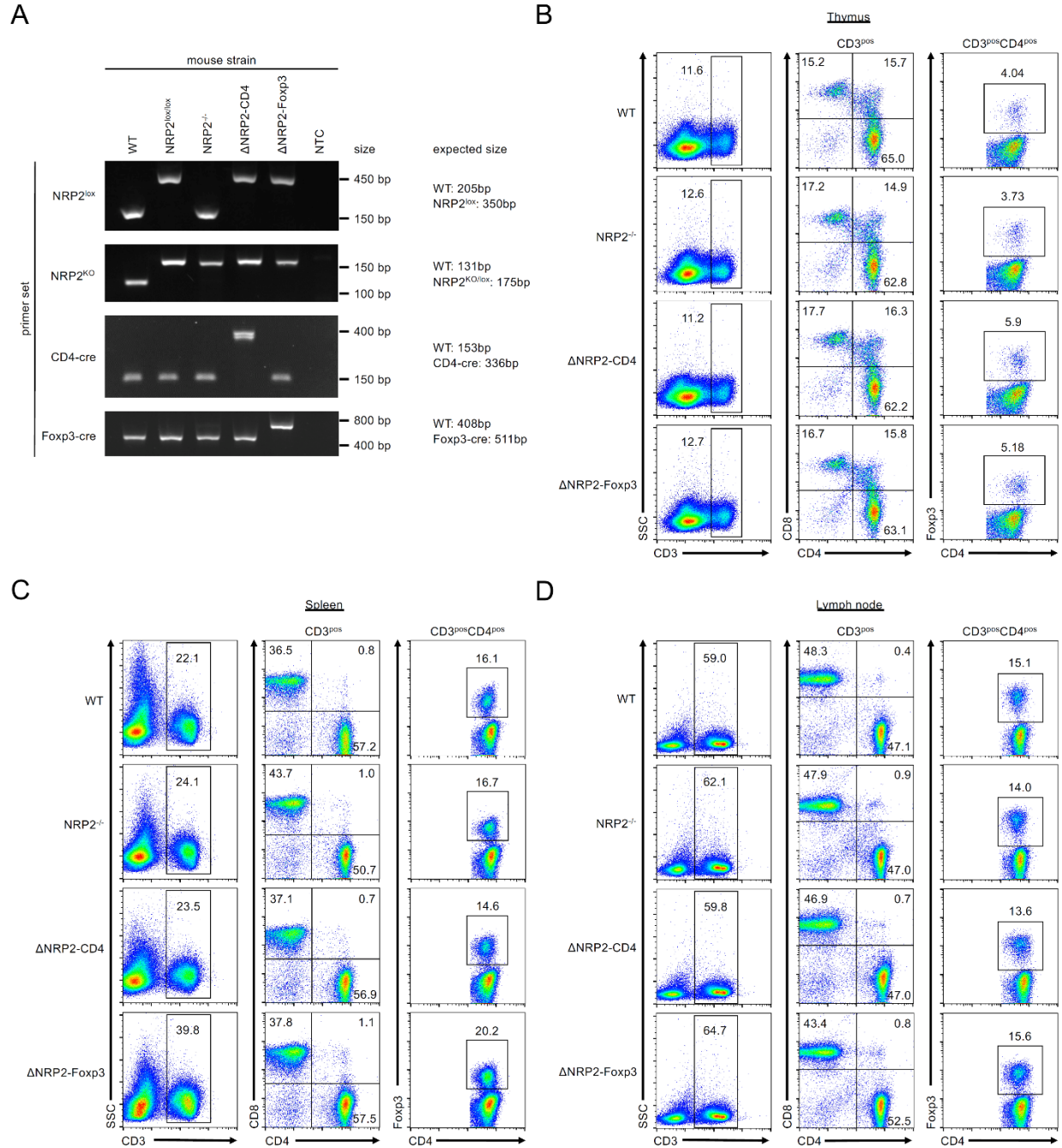
Supplemental Figure 4: Gating strategy and extended analysis of bulk RNA-seq data. (A) Sorting strategy for the purification of NRP2^{pos} and NRP2^{neg} cells. **(B)** NRP2 and IFNG mRNA expression in different conditions as quality controls for sorting and mitogen activation respectively. **(C)** Dot plot representing normalized enrichment scores (NES) and familywise error rate adjusted (FWER) P values for the gene set enrichment analysis. Signaling pathway activities of cells from four conditions NRP2^{pos} and NRP2^{neg} cells either unstimulated or PHA-stimulated (3 $\mu\text{g}/\text{ml}$ for 16 hours) was estimated using the Hallmark gene set database.



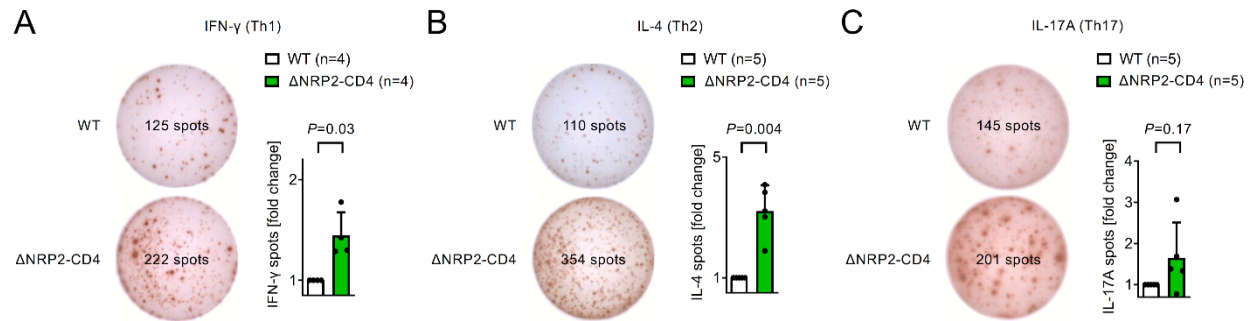
Supplemental Figure 5: NRP2 expression within murine splenocytes. (A) Gating strategy to identify NRP2-GFP expression in splenocytes. WT C57BL/6 mice were used to distinguish autofluorescence from specific NRP2-GFP signals in NRP2^{lox/lox} mice. (B-C) Representative dot plots and (D) a summary of n=6 independent experiments of NRP2-GFP expression within splenocyte subsets. (E) Western blot analysis of FACS-sorted CD4⁺Foxp3-YFP^{neg} (Treg) and CD4⁺Foxp3-YFP^{pos} (Teff) cells isolated from splenocytes of Foxp3-cre mice. Representative of n=4 independent experiments.



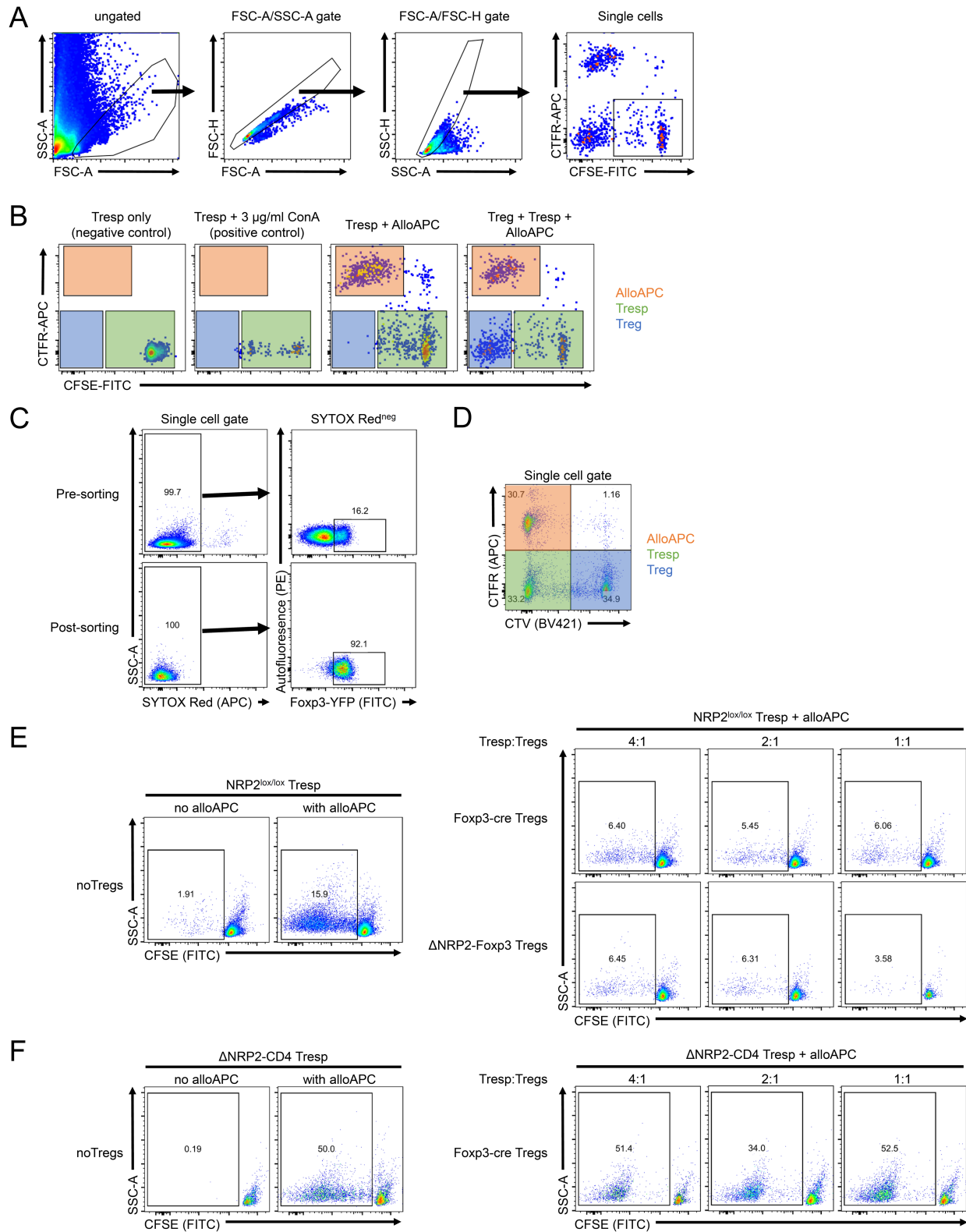
Supplemental Figure 6: Gating strategy and controls for PrimeFlow assays. (A) Gating strategy to identify CD4⁺ T cells depicted in Figure 4D-F. **(B)** Gating strategy to identify CD45.1^{pos} CD45.2^{neg} host and CD45.1^{pos} CD45.2^{pos} OT-II CD4⁺ T cells (depicted in Figure 4G-J). **(C)** Splenocytes from C57BL/6 WT and NRP2^{-/-} were stained in the same experiment to identify the gate for NRP2 mRNA positivity. Dot plots show the positive (left: WT splenocytes) and negative (right: NRP2 KO splenocytes) technical controls for the fluorescence in situ hybridization.



Supplemental Figure 7: Phenotyping of transgenic mice. (A) Genotyping of C57BL/6 WT, global $NRP2^{-/-}$ knockout, conditional $\Delta NRP2-CD4$ knockout and $\Delta NRP2-Foxp3$ knockout mice (DNA from tail biopsies). (B-D) Immunophenotyping of WT and transgenic mice by flow cytometry. Dot plots are representative of $n=3$ mice initially evaluated. $NRP2^{lox/lox}$ and Foxp3-cre mice were similar to WT mice (not shown). Frequencies of CD3, CD4, CD8 and Foxp3 positive cells in the (B) thymus (C) spleen and (D) inguinal lymph nodes.

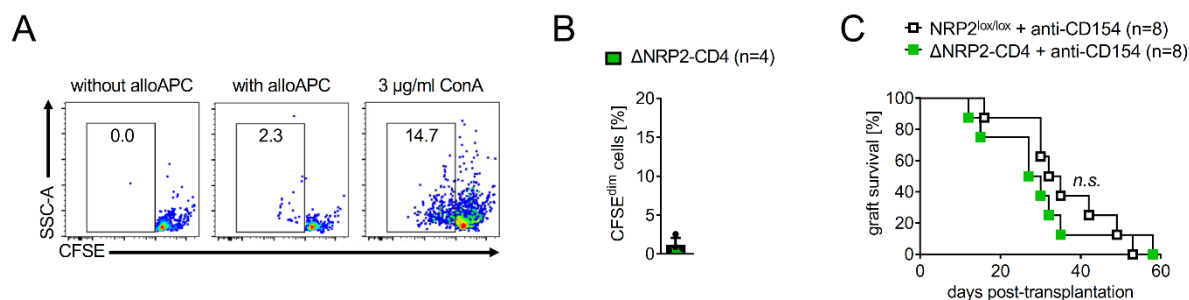


Supplemental Figure 8: Knockout of NRP2 in murine CD4⁺ T cells increases T helper cell differentiation *in vitro*. Naive CD4⁺ T cells from either WT or Δ NRP2-CD4 knockout mice were cultured in T helper cell (Th) polarizing conditions for 48 hours as described (78), rested for an additional 48 hours, and (A) Th1, (B) Th2 and (C) Th17 differentiation was evaluated using ELISPOT assays respectively. Representative images of ELISPOT wells (left) and bar graphs (from n=4-5 experiments, right) summarizing the fold change in IFN- γ , IL4 and IL-17A comparing knockout cells to WT cells (fold change of mean spots/well $\pm$ SD; One sample t-test).

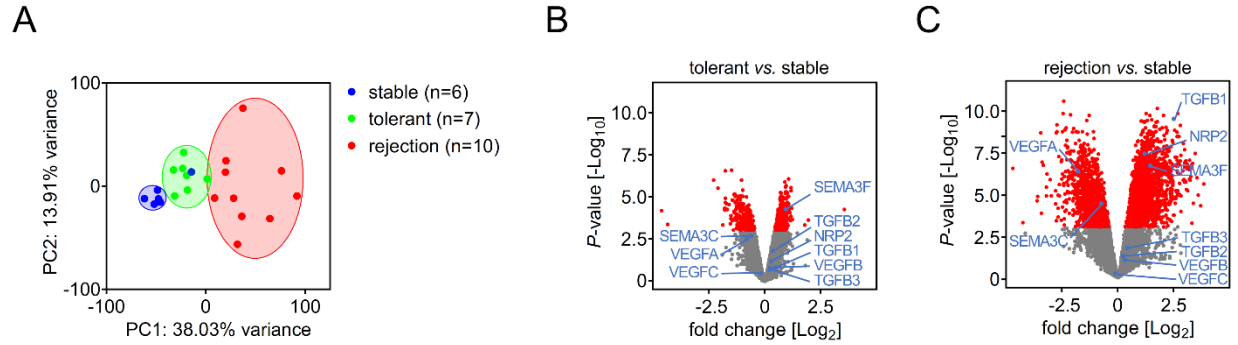


Supplemental Figure 9: Gating strategy and controls for Treg suppression assays. C57BL/6 NRP2^{lox/lox} and ΔNRP2-CD4 knockout mice received a fully MHC mismatched Balb/c skin transplant; Teff and Treg were harvested on day 14 and in vitro suppression assays were performed

following coculture with irradiated Balb/c APCs as in Figure 6G-I. **(A)** Gating strategy and **(B)** controls to identify a Tresp gate (highlighted in green). The same gating strategy and controls were used for Treg suppression assays in Figure 6G-H. **(C-F)** Validation in vitro suppression experiment using purified populations of Foxp3-YFP Tregs (complimentary to data shown in Figure 6G-I). C57BL/6 NRP2^{lox/lox}, ΔNRP2-CD4, Foxp3-cre and ΔNRP2-Foxp3 mice received a fully MHC mismatched Balb/c skin transplant. On day 14 post-transplant, CD4⁺Foxp3-YFP⁺ Tregs were FACS-sorted from Foxp3-cre and ΔNRP2-Foxp3 mice and stained with CellTrace Violet (CTV). CD4⁺CD25^{neg} Tresp were harvested from NRP2^{lox/lox} and ΔNRP2-CD4 recipients and stained with CFSE. Treg function was assessed in an in vitro suppression assay as depicted in Figure 6G-I using irradiated and CellTrace Far Red (CTFR)-stained Balb/c splenocytes (alloAPC; Tresp:alloAPC ratio 1:1). **(C)** Dotplots depict cell viability and Foxp3 positivity pre- and post-sorting. **(D)** Representative dotplot illustrates the gating strategy to identify CTV^{neg}CFSE^{pos}CTFR^{neg} Tresp cells for evaluating Tresp proliferation using CFSE dilution. **(E-F)** Proliferation of NRP2 WT (E) and NRP2 KO (F) Tresp without Tregs (left panels) or with increasing ratios of either NRP2 WT (right panel; top row) of KO (right panel; bottom row) Tregs. One validation experiment was performed.

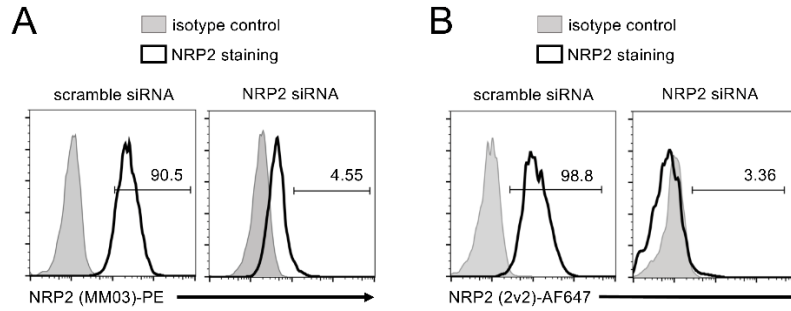


Supplemental Figure 10: Costimulatory blockade using anti-CD154 inhibits allogeneic priming of Tregs and abrogates NRP2 function. (A-B) Fully MHC mismatched Balb/c hearts were transplanted into NRP2^{lox/lox} wild type or ΔNRP2-CD4 knockout recipients treated with anti-CD154 on days 0 and 2 post transplantation. Allogeneic priming on day 14 post transplantation was evaluated by coculture of recipient CD4⁺ T cells with irradiated donor Balb/c splenocytes for 5 days; proliferation as assessed by CFSE dilution. Concavalin A (ConA, 3 µg/ml) was used as a positive control. **(A)** Representative dot plots and **(B)** bar graph of n=4 mice is depicted (frequency of alloreactive ΔNRP2-CD4 knockout CFSE^{dim} cells ± SD). **(C)** Kaplan-Meier graft survival curves (*n.s.* not significant).



Supplemental Figure 11: Expression of NRP2 ligands in tolerant renal transplant biopsies.

We analyzed a published database of renal transplant biopsies (GSE106675) that were taken from renal transplant recipients following tolerance induction using the Northwestern protocol (76-77). The database also included findings from surveillance biopsies from a control cohort of stable transplants recipients on conventional immunosuppression, and from patients with clinical rejection. **(A)** Principal component analysis of all samples. **(B-C)** Volcano plot analysis comparing stable patients on immunosuppression to **(B)** biopsies from tolerant patients off immunosuppression or **(C)** biopsies with evidence of rejection. Statistically significant ($P < 0.001$) regulated transcripts are highlighted in red, and NRP2 ligands are highlighted as blue dots.



Supplemental Figure 12: NRP2 antibody specificity. The specificity of the two anti-human NRP2 antibody clones used in this study was assessed in a knockdown approach using the NRP2-expressing U87MG glioblastoma cell line as previously described (45). **(A)** Binding of the MM03 anti-NRP2 clone, and **(B)** binding of the 2v2 anti-NRP2 clone to scramble siRNA (left panels) or NRP2 siRNA (right panels) transfected U87MG cells.

Supplemental Table 1. Antibodies used in these studies.

target (fluorochrome/enzyme/DNA-oligo)	clone	company
anti-Biotin (A0436 [DNA])	1D4-C5	Biolegend
anti-BrdU (AF488, AF647)	3D4	Biolegend
anti-human 2B4 (AF700)	C1.7	Biolegend
anti-human BTLA (PE-Cy5)	MIH26	Biolegend
anti-human CD11c (BV421)	3-HCL-3	Biolegend
anti-human CD160 (PE)	51-10C9	BD Biosciences
anti-human CD19 (BV785)	HIB19	Biolegend
anti-human CD279/PD-1 (BV421)	NAT105	Biolegend
anti-human CD3 (unconjugated, APC-Cy7)	HIT3a	Biolegend
anti-human CD38 (BV605)	HB-7	Biolegend
anti-human CD366/Tim3 (APC-Cy7)	F38-2E2	Biolegend
anti-human CD4 (BUV395)	RPA-T4	BD Biosciences
anti-human CD4 (BV421, APC, AF700)	OKT4	Biolegend
anti-human CD45 (PerCP-Cy5.5)	HI30	Biolegend
anti-human CD56 (BV510)	5.1H11	Biolegend
anti-human CD69 (BV421)	FN50	Biolegend
anti-human CD8a (BV605)	HIT8a	Biolegend
anti-human CTLA4 (BV605)	BNI3	Biolegend
anti-human HLA-DR (AF488)	L243	Biolegend
anti-human IFN γ (AF488)	4S.B3	Biolegend
anti-human Lag3 (BV510)	11C3C65	Biolegend
anti-human NRP2 (biotin, AF647)	2v2	aTyr Pharma
anti-human NRP2 (unconjugated, PE, APC)	MM03	Sino Biologicals
anti-human TIGIT (PE-Cy7)	A15153G	Biolegend
anti-human TruStain FcX	---	Biolegend
anti-mouse CD11c (BV421)	N418	Biolegend
anti-mouse CD138/Syndecan-1 (APC)	281-2	Biolegend
anti-mouse CD14 (PE-Dazzle594)	Sa14-2	Biolegend
anti-mouse CD19 (BV785)	6D5	Biolegend
anti-mouse CD197/CCR7 (BV605)	4B12	Biolegend
anti-mouse CD279/PD1 (BV785, PerCP-Cy5.5)	29F.1A12	Biolegend
anti-mouse CD3 (PE)	17A2	Biolegend
anti-mouse CD366/Tim3 (PE, APC-Fire750)	B8.2C12	Biolegend
anti-mouse CD4 (BV510, BV605, BV785, PerCP-Cy5.5, AF700)	GK1.5	Biolegend
anti-mouse CD44 (AF488, PerCP-Cy5.5)	IM7	Biolegend
anti-mouse CD45 (APC)	30-F11	Biolegend

anti-mouse CD45.1 (BV510, AF700)	A20	Biolegend
anti-mouse CD45.2 (BV786, PE-Dazzle594)	104	Biolegend
anti-mouse CD62L (BV421, PE)	MEL-14	Biolegend
anti-mouse CD68 (APC-Cy7)	FA-11	Biolegend
anti-mouse CD69 (PE, PE-Dazzle594)	H1.2F3	Biolegend
anti-mouse CD8a (PerCP-Cy5.5, AF647)	53-6.7	Biolegend
anti-mouse CXCR5 (BV605)	L138D7	Biolegend
anti-mouse Foxp3 (unconjugated, AF647)	FJK-16s	Thermo Fisher
anti-mouse HSP90	C45G5	Cell Signaling
anti-mouse IFN γ	AN-18	Biolegend
anti-mouse IFN γ (biotin)	R4-6A2	Biolegend
anti-mouse IgG (H+L) (HRP)	polyclonal	Vector Laboratories
anti-mouse IgG-Fc γ fragment (AF647)	polyclonal	Jackson Immuno
anti-mouse IL17A	eBio17CK15A5	Thermo Fisher
anti-mouse IL17A (biotin)	eBio17B7	Thermo Fisher
anti-mouse IL2	JES6-1A12	Biolegend
anti-mouse IL2 (biotin)	JES6-5H4	Biolegend
anti-mouse IL4	11B11	Biolegend
anti-mouse IL4 (biotin)	BVD6-24G2	Biolegend
anti-mouse NK-1.1 (BV510)	PK136	Biolegend
anti-mouse NRP1	D62C6	Cell Signaling
anti-mouse NRP2	D39A5	Cell Signaling
anti-mouse TruStain FcX	---	Biolegend
anti-phycoerythrin (A0911 [DNA])	PE001	Biolegend
Streptavidin (FITC)	polyclonal	Thermo Fisher

Supplemental Table 2. Primers used in these studies.

target	reverse	forward
NRP2-WT	5' CAG GTG ACT GGG GAT AGG GTA 3'	5' AGC TTT TGC CTC AGG ACC CA 3'
NRP2lox	5' CCT GAC TAC TCC CAG TCA TAG 3'	
NRP2-WT	5' TGA AAT CAG CAA AGA GGG AGC 3'	5' GGG AAA CCC TCG TGA TGT TGT 3'
NRP2 ^{ko}	5' ATC TTC CAT CAC GTC GAA CTC 3'	
CD4-WT	5' TAT GCT CTA AGG ACA AGA ATT GAC A 3'	5' GTT CTT TGT ATA TAT TGA ATG TTA GCC 3'
CD4-cre	5' CTT TGC AGA GGG CTA ACA GC 3'	
Foxp3-WT	5' GTG TGA CTG CAT GAC TAA CTT TGA 3'	5' CCC TTG ACC TCA AAA CCA AG 3'
Foxp3-cre		5' TGG CTG GAC CAA TGT GAA C 3'