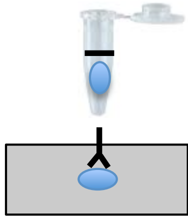
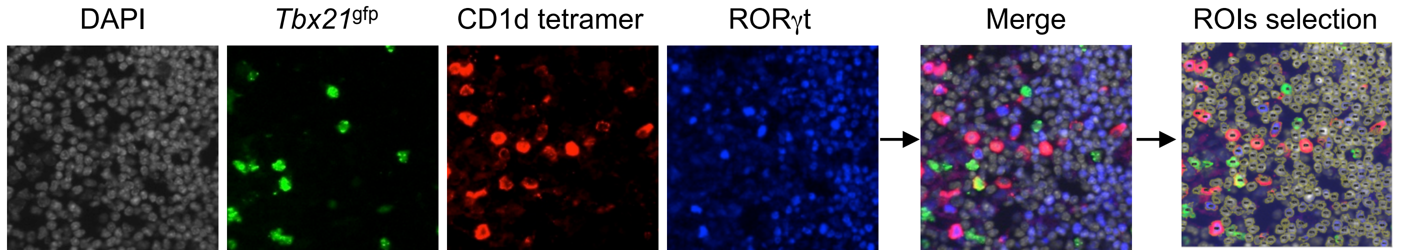
A

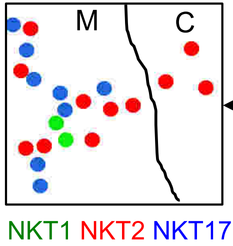


1. Live tissue incubation with PE conjugated CD1d tetramer
2. PFA fixation and freezing
3. Co-stain and signal amplification with anti PE antibody
4. IF and histocytometric analysis to determine localization

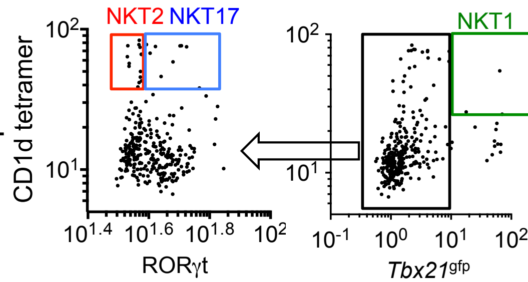
B



Plotting XY coordinates and quantification



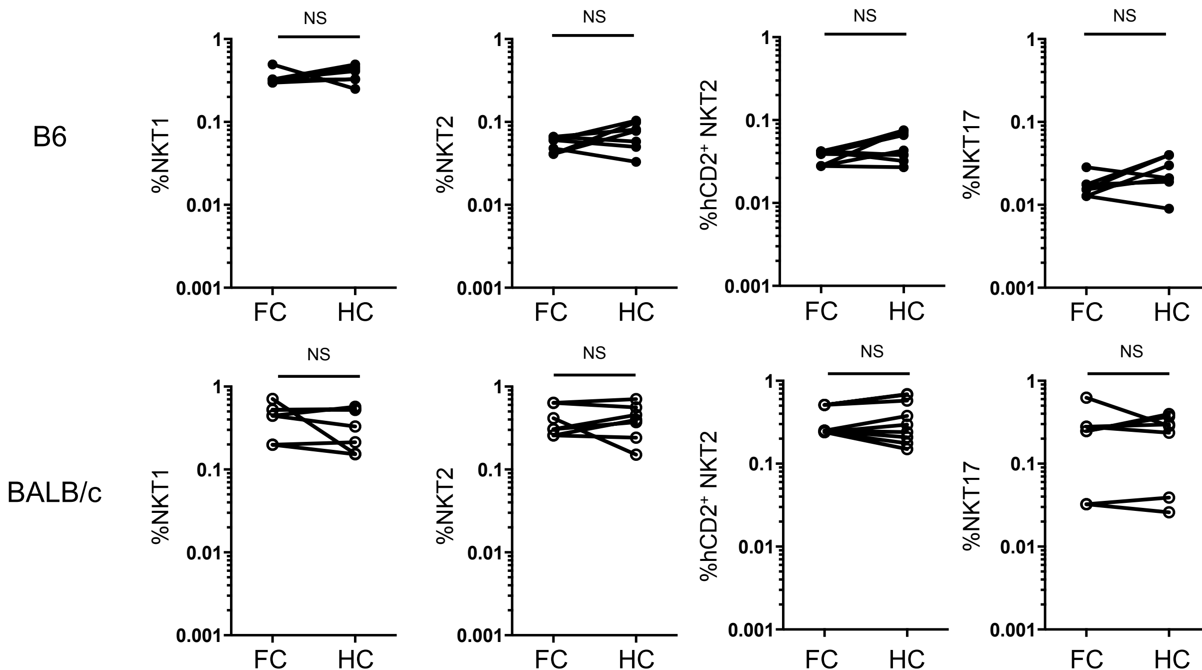
Fluorescence intensity plotting



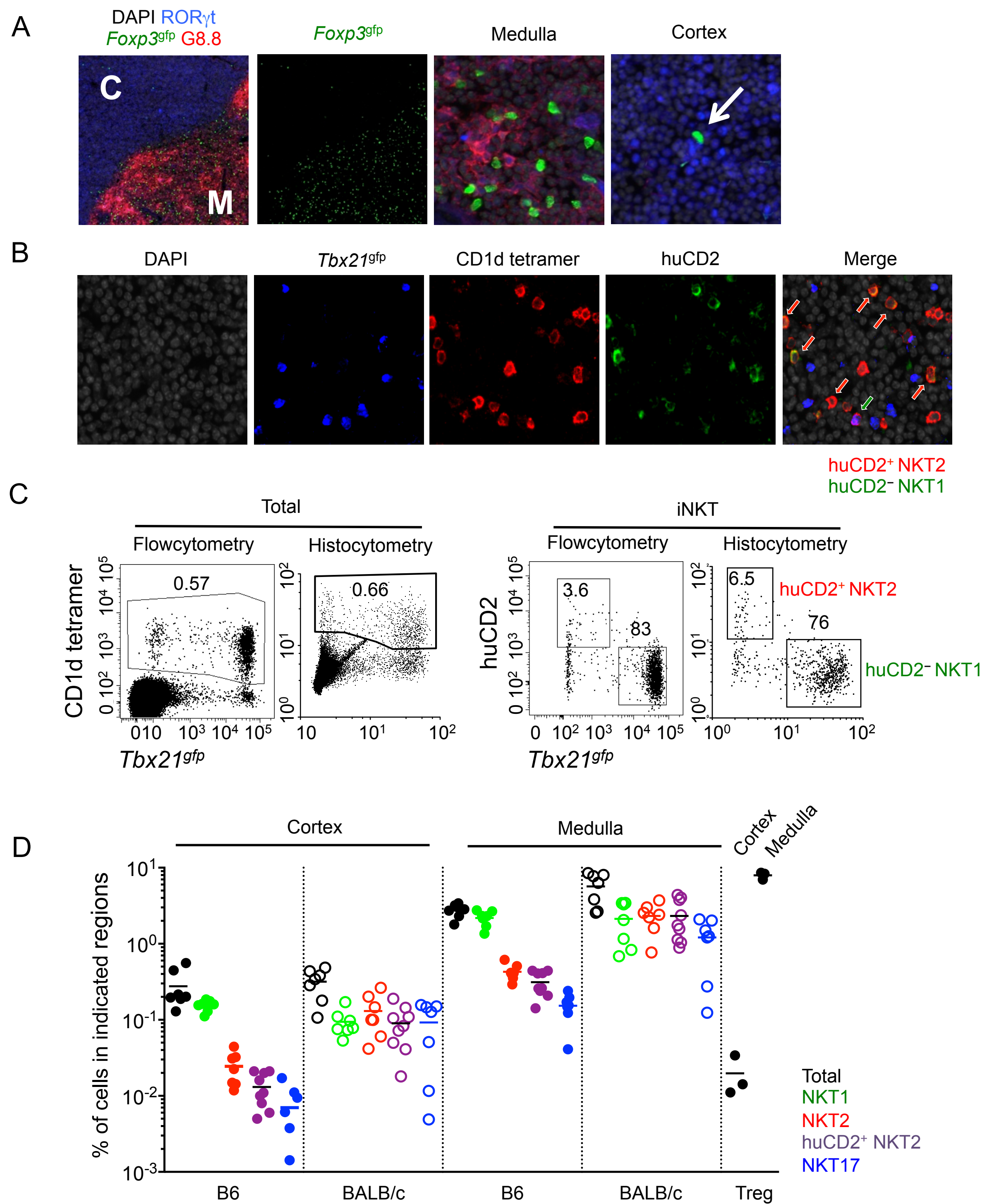
Apply ROI on single stained images and measure fluorescence intensity

	CD1dt	RORgt	Tbet	X	Y
1	19.102	38.815	1.028	19	0
2	17.733	35.083	0.733	32	0
3	7.682	31.477	0.614	74	0
4	13.811	43.611	0.783	161	0
5	10.602	45.295	1.023	178	0

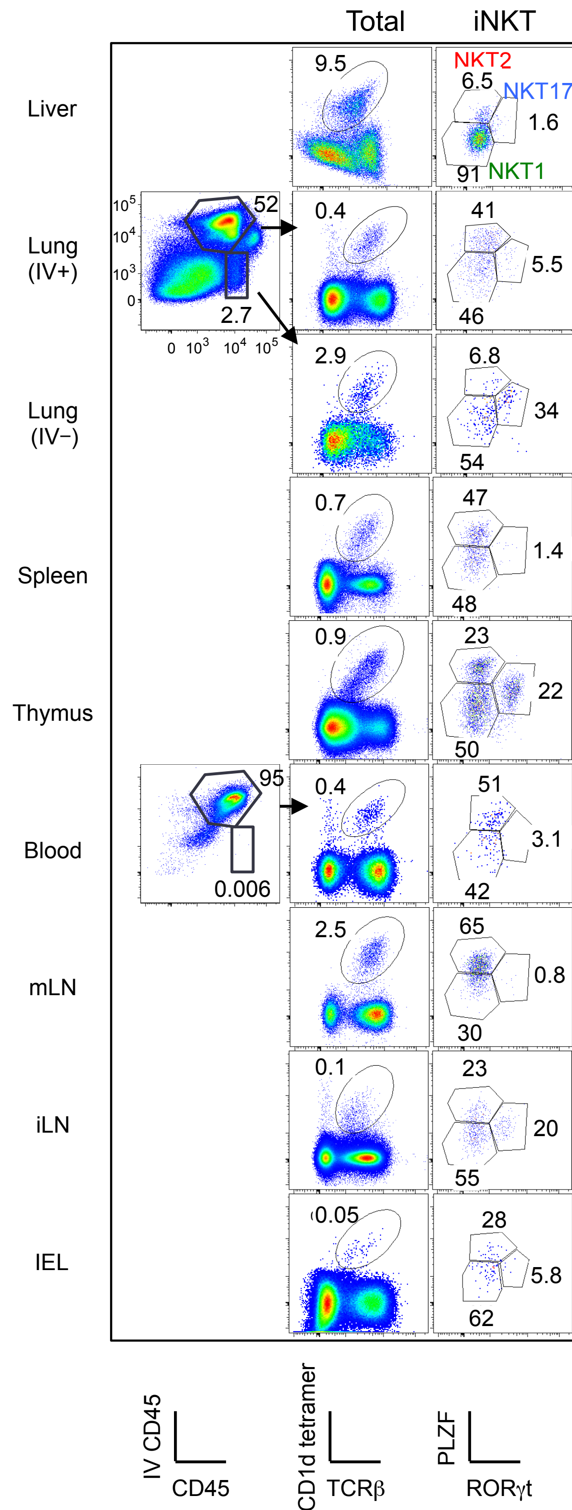
C



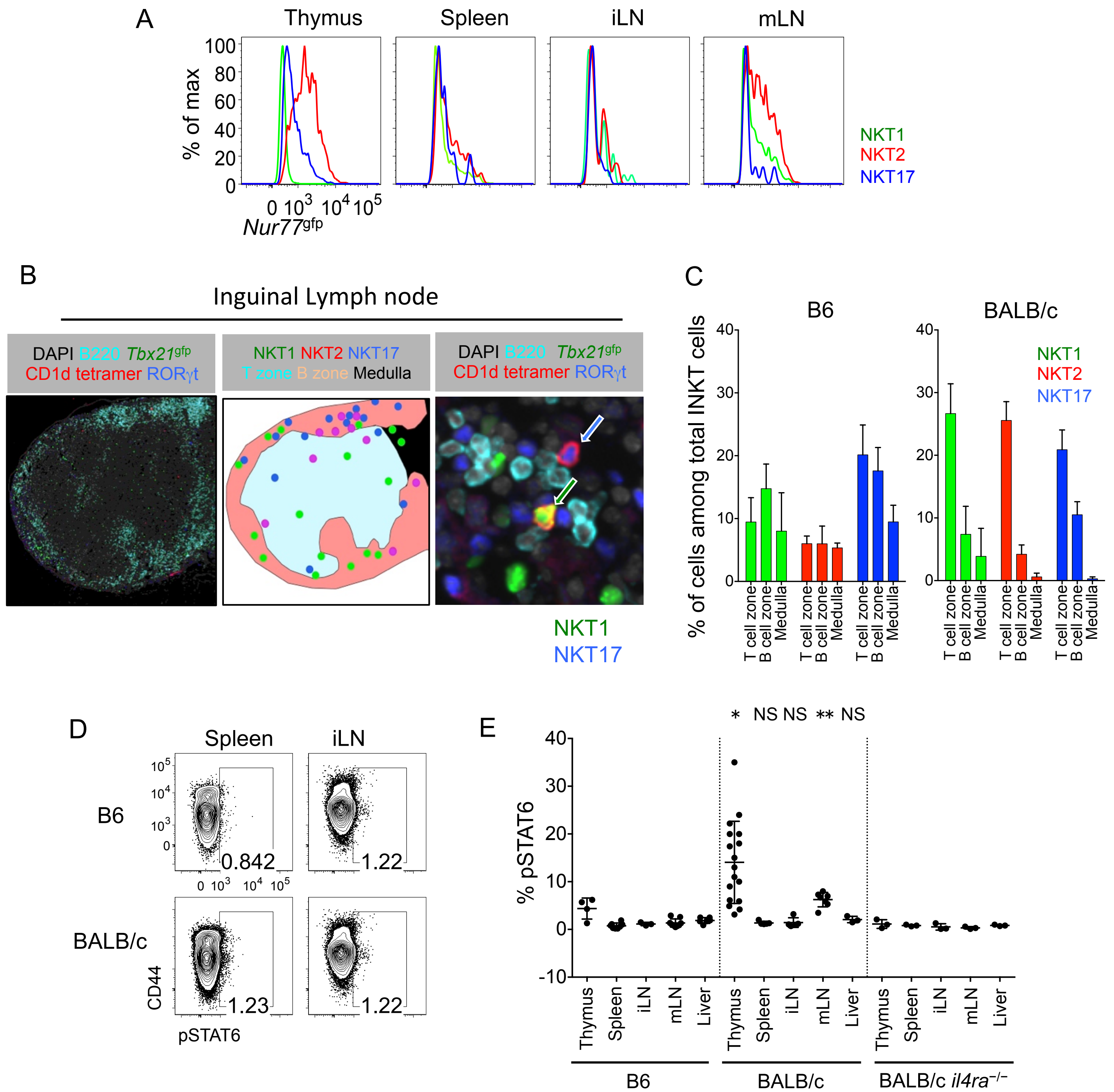
Supplementary Figure 1 related to Figure 1. Workflow pipeline to quantify localization of iNKT subsets. (A) Schematic representation showing CD1d tetramer immunofluorescence (IF) technique. PFA, paraformaldehyde. (B) Fluorescence intensities were quantified in each single cell identified by ImageJ and data were transferred into Prism software to gate cells. XY coordinates of gated cells were overlaid on an original image and the distribution of cells were analyzed. ROIs, region of interests. (C) Three to four different B6 and BALB/c mice were analyzed for their thymic frequencies of each subset of iNKT cells among total thymocytes either by flowcytometry (FC) or CD1d tetramer histocytometry (HC). NS, not significant (paired *t*-test).



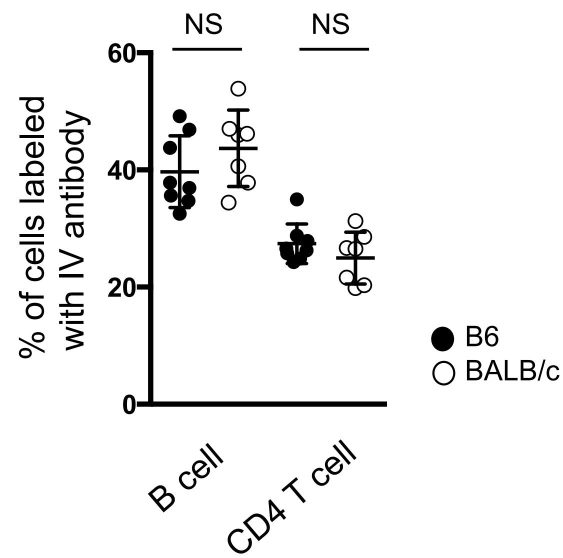
Supplementary Figure 2 related to Figure 2. Regulatory T cells and IL-4 producing NKT cells reside in thymic medulla. (A) Thymi of *Foxp3*gfp mice were stained with RORγt and G8.8 to analyze the localization of regulatory T cells (Tregs). An arrow indicates a Treg in the cortex. C, cortex; M, medulla. (B and C) Thymi of *Tbx21*gfp KN2+/– mice were stained with CD1d tetramer and huCD2 molecule (B) and analyzed by histocytometric algorithms (C). Numbers indicate frequency of cells in adjacent gates. (D) Compilation of cortical and medullary frequencies of total and each iNKT subset are shown for B6 (4 mice, 7 sections) and BLAB/c (4 mice, 8 secretions) iNKT cells and B6 regulatory T cells (Treg, 3 mice, 3 sections). Each dot represents an individual section and horizontal bars indicate mean values.



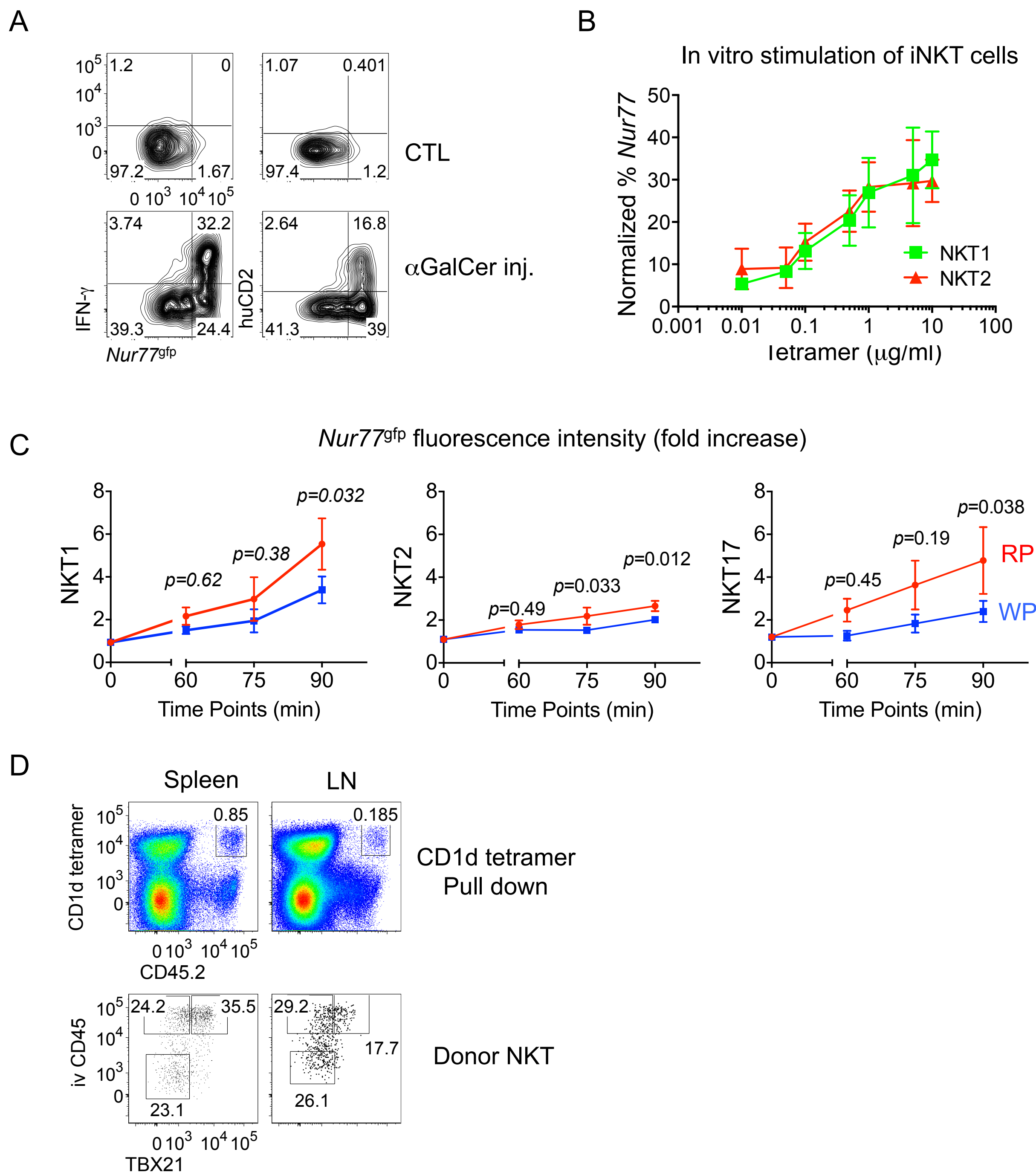
Supplementary Figure 3 related to Figure 3. Flowcytometry profiles of iNKT cells in multiple organs. Representative flowcytometry dot plots and gating strategies are shown in various organs. Numbers indicate frequency of cells in adjacent gates and all dot plots have the same axis scales. Representative data of at least five independent experiments are shown. IV, intravenous; mLN, mesenteric lymph node; iLN, inguinal lymph node; IEL, intraepithelial lymphocyte.



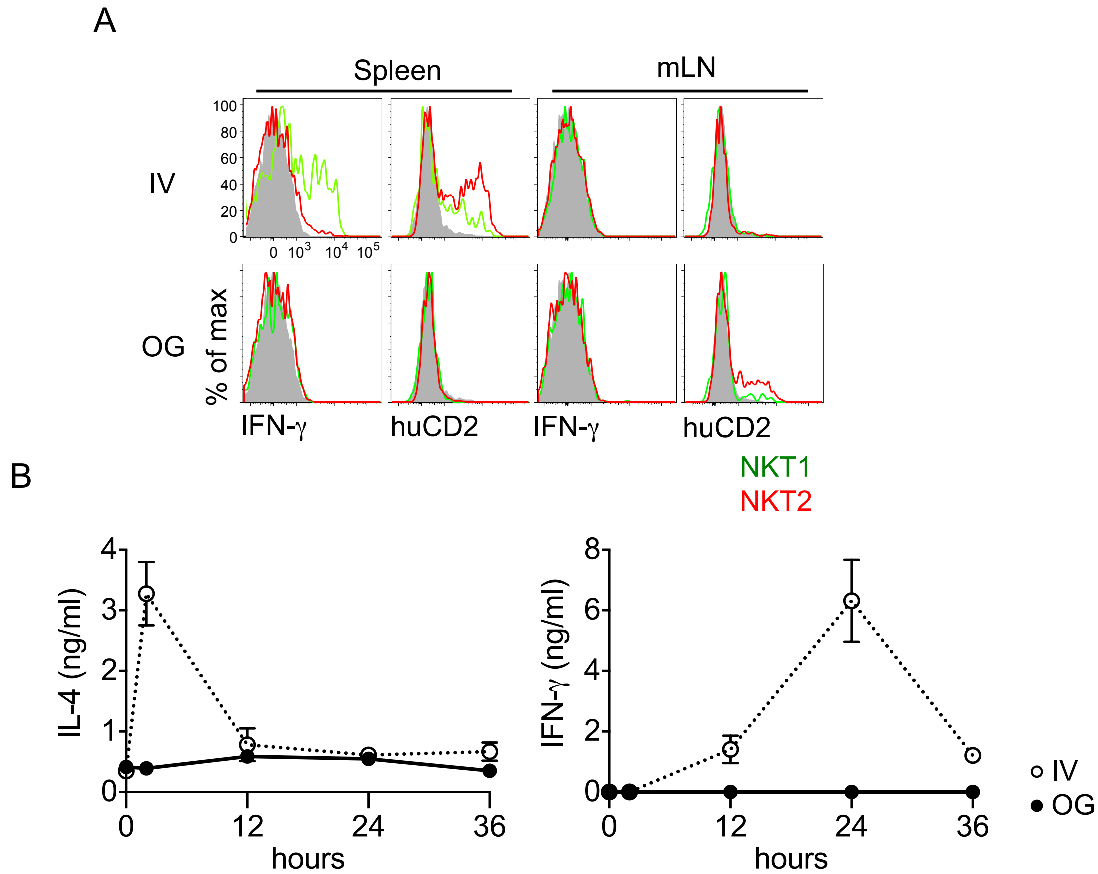
Supplementary Figure 4 related to Figure 4. mLN NKT2 cells condition lymphocytes to express pSTAT6. (A) *Nur77*gfp expression in each subset of iNKT cells is shown in indicated organs at steady state. Representative histograms of at least 3 independent experiments are shown. All histograms have the same axis scales. iLN, inguinal lymph node; mLN, mesenteric lymph node. (B) iLN of B6 *Tbx21*gfp mice was stained with indicated antibodies and CD1d tetramer. Arrows indicate NKT1 cells (green) and NKT17 cells (blue). (C) Average frequencies of each NKT subset among total iNKT cells in T cell zone, B cell zone and medulla of iLN were analyzed in B6 (n = 6) and BALB/c (n = 6) mice. Error bars indicate standard deviation. (D) pSTAT6 expressions in CD4 T cells from spleen and iLN of B6 and BALB/c mice are shown at steady state. Numbers indicated frequencies of cells at adjacent gates and dot plots have the same axis scales. (E) Frequencies of pSTAT6 positive CD4 T cells in indicated organs are shown in B6 (n = 3 ~ 8), BALB/c (n = 3 ~ 17) and BALB/c *il4ra*$^{-/-}$ (n = 3) mice. Each dot represents an individual mouse and horizontal bars indicate mean values. Error bars show standard deviation. *p<0.0001, **p<0.001 (one-way analysis of variance (ANOVA)). NS, not significant



Supplementary Figure 5 related to Figure 5. Intravital labeling of splenocytes in B6 and BALB/c mice. B6 and BALB/c mice were intravenously (IV) injected with anti-CD45 antibody and sacrificed 3 minutes later. Splenic B cells and CD4 T cells were analyzed antibody labeled frequencies in B6 (n = 8) and BALB/c (n = 7) mice. NS, not significant (unpaired t-test).



Supplementary Figure 6 related to Figure 6. iNKT cell localization determines cellular response to αGalCer. (A) BALB/c *Nur77*gfpKN2+/− mice were intravenously (IV) injected with αGalCer and analyzed for *Nur77*gfp, surface human CD2 (huCD2) and intracellular IFN-γ expressions after 3 hours. Representative data of at least 3 independent experiments are shown. Numbers indicate frequencies of cells in each quadrant and dot plots have the same axis scales. (B) Splenocytes from BALB/c *Nur77*gfp mice were *in vitro* culture with CD1d tetramer for 4 hours and measured *Nur77*gfp expression in NKT1 (green) and NKT2 (red) cells. Frequencies of GFP expression were normalized at 0.1μg/ml tetramer concentrations in three independent experiments. Error bars indicate standard deviation. (C) BALB/c *Nur77*gfp mice were IV injected with αGalCer and analyzed *Nur77*gfp expression in each subset at indicated time points and compared *Nur77*gfp fold increased between cells in red pulp (RP, IV antibody labeled) or white pulp (WP, IV antibody unlabeled). Error bars indicate standard deviation. P values indicate unpaired *t*-tests. (D) Vα14 TCR transgenic NKT cells from spleen and lymph node (LN) were transferred into αGalCer immunized host mice. Donor iNKT cells in spleen were analyzed after CD1d tetramer pull down. Numbers indicated frequency of cells in adjacent gates and dot plots have the same axis scales.



Supplementary Figure 7 related to Figure 7. Oral gavage of αGalCer stimulates NKT2 cells in mLN to produce IL-4. (A) BALB/c KN2$^{+/-}$ mice were administrated with αGalCer either by intravenous (IV) injection or oral gavage (OG) and analyzed NKT1 (green) and NKT2 (red) cells in spleen and mesenteric lymph node (mLN) for their expression of human CD2 (huCD2) and intracellular IFN-γ at 3 and 24 hours later respectively. Representative histograms of 3 independent experiments are shown. All histograms have the same axis scales. (B) BALB/c mice were measured their serum concentrations of IL-4 and IFN-γ by ELISA after either IV injection or oral gavage of αGalCer at indicated time points (n = 5). Error bars indicate standard deviation.