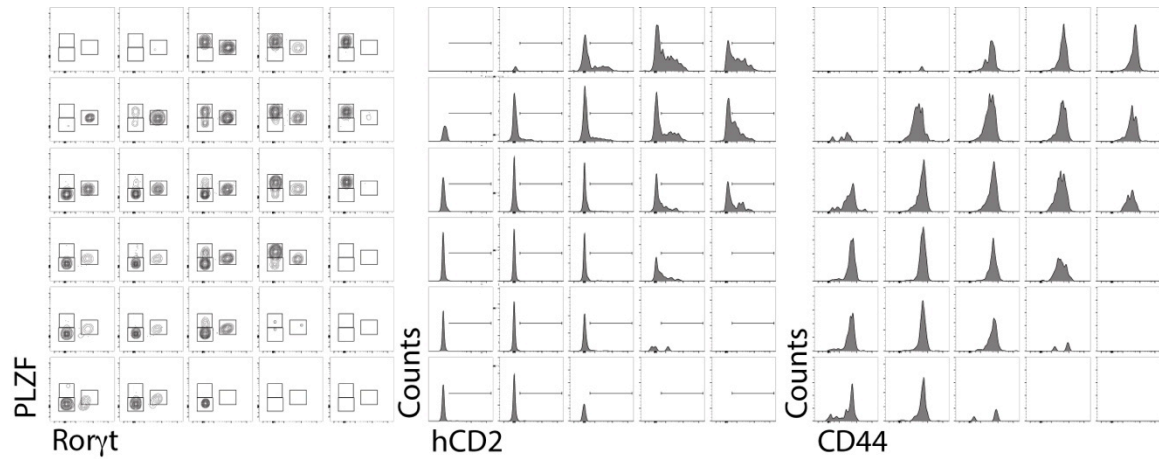


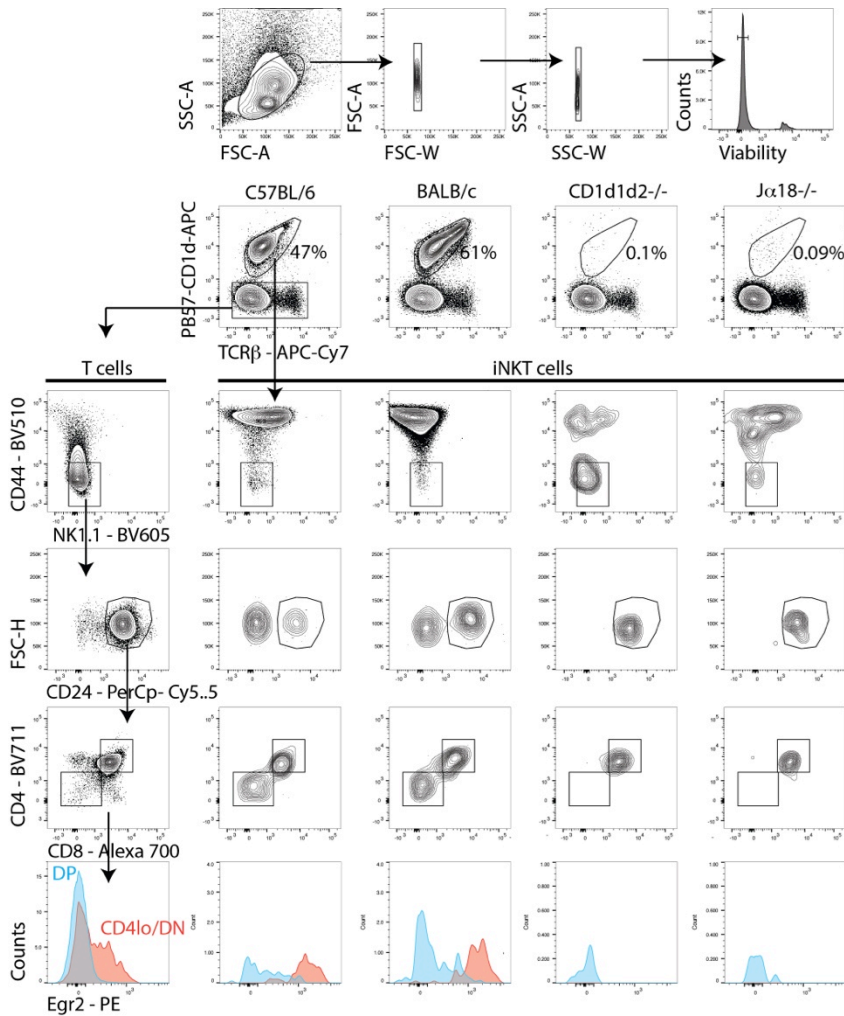
## Supplementary Material

### TCR signal strength controls thymic differentiation of iNKT cell subsets

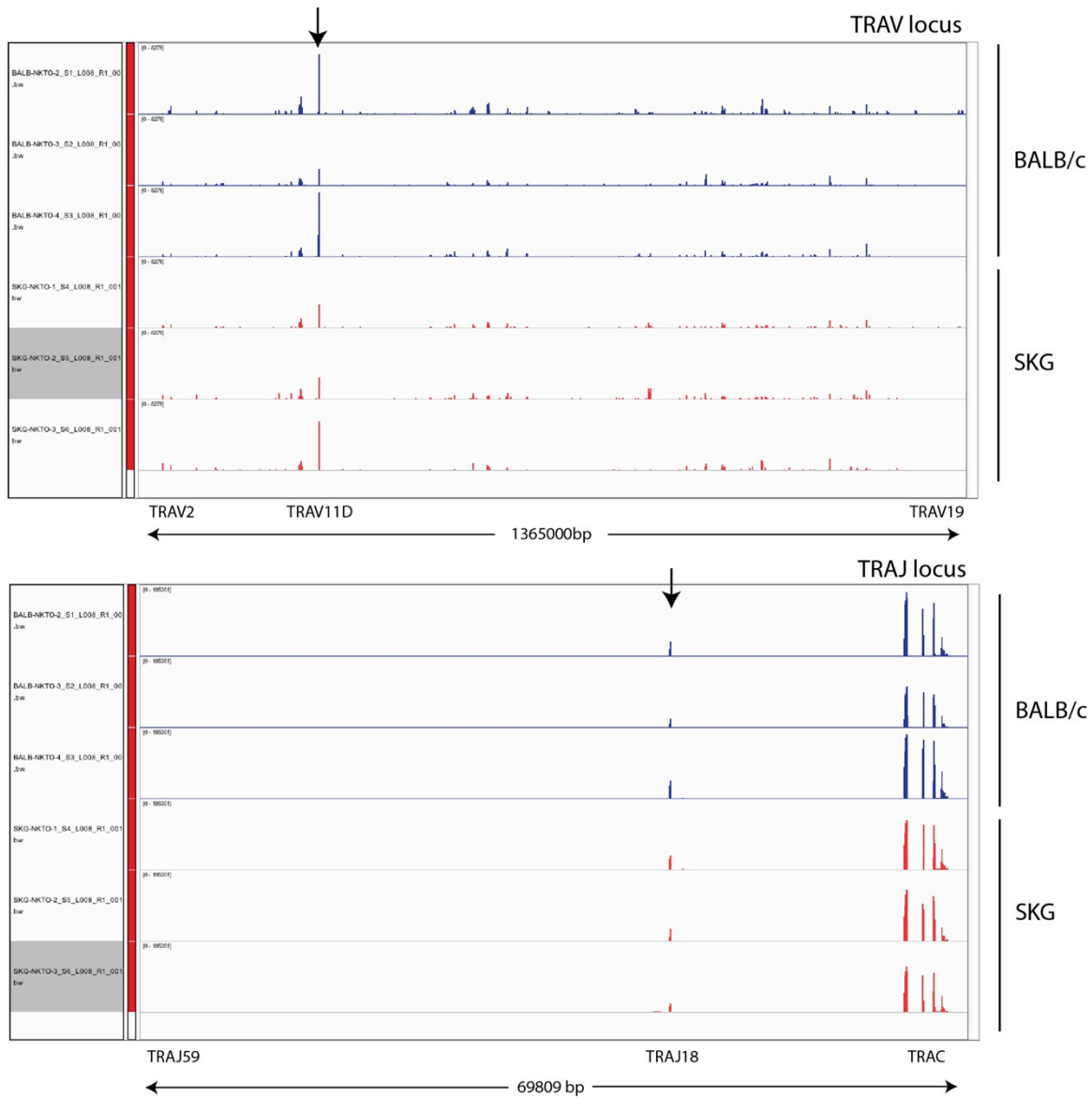
Tuttle et al.



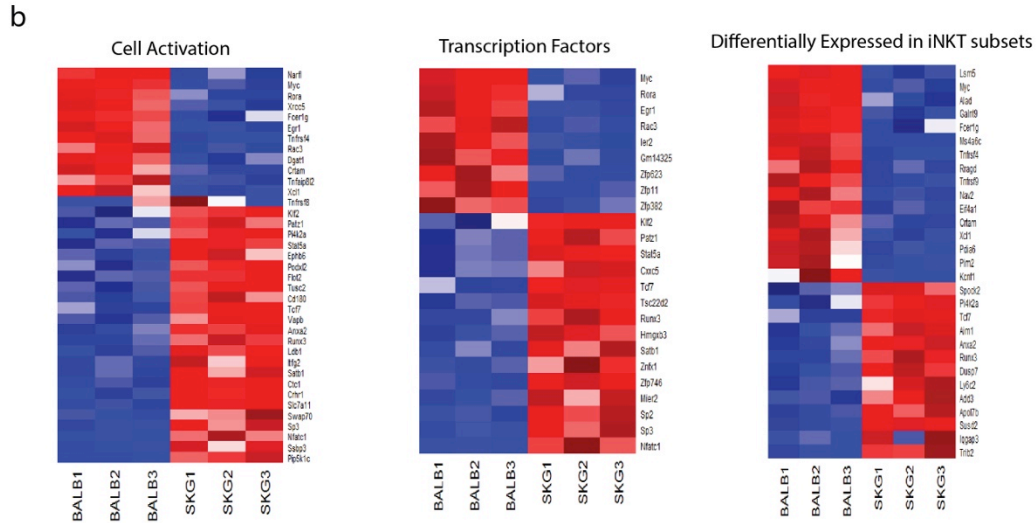
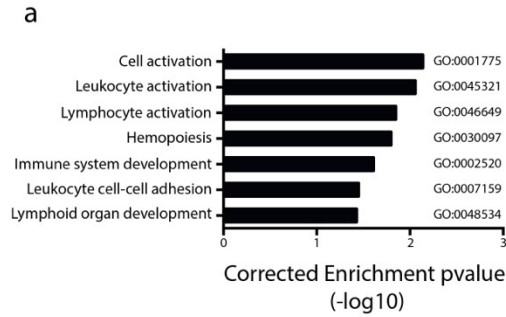
Supplementary Figure 1. Thymic iNKT subsets express TCRs of different avidities for the PBS57-CD1d tetramer. Cells from the thymus of BALB/c IL-4 reporter mice KN2 were stained with anti-TCR $\beta$  mAbs and PBS57-CD1d tetramers. iNKT cells were then electronically placed on a grid consisting of 30 gates (see Fig 1a). The proportion of NKT1 (PLZF<sup>lo</sup>, Ror $\gamma$ t<sup>-</sup>, Tbet<sup>+</sup>), NKT17 (PLZF<sup>int</sup>, Ror $\gamma$ t<sup>+</sup>, Tbet<sup>-</sup>), NKT2 (PLZF<sup>hi</sup>, Ror $\gamma$ t<sup>+</sup>, Tbet<sup>-</sup>) or hCD2<sup>+</sup> as well as the proportion of CD44<sup>hi</sup> cells in each gate was recorded.



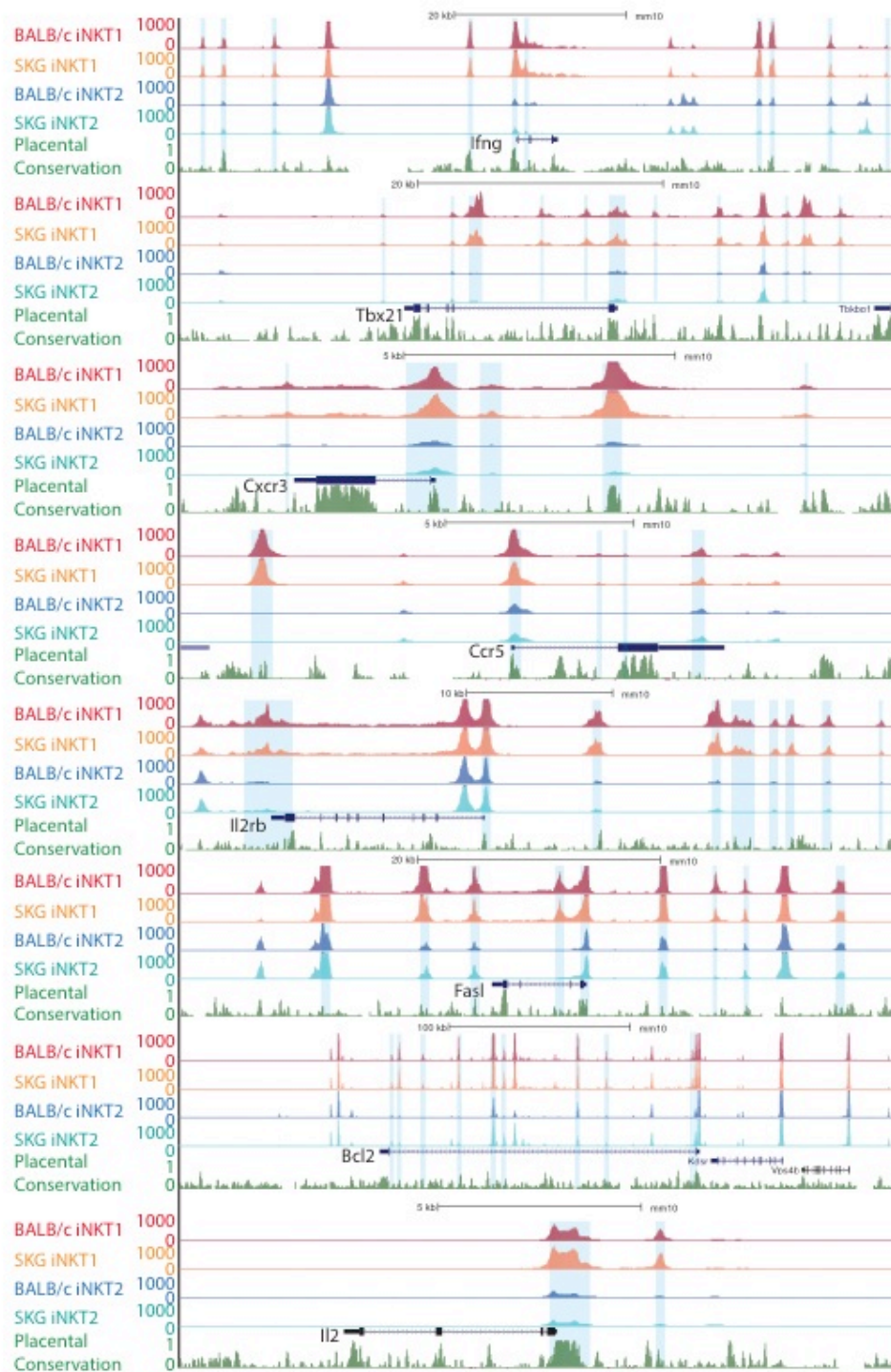
Supplementary Figure 2. Definition of stage 0 iNKT cells. Cells from the thymi of C57BL/6, BALB/c, CD1d1d2<sup>-/-</sup> and J $\alpha$ 18<sup>-/-</sup> mice were positively enriched for PBS57-CD1d tetramer<sup>+</sup> cells using magnetic beads and an autoMACS Pro Separator and stained for the indicated markers. Doublets were excluded and only viable cells were included in the analysis. iNKT cells or “conventional” T cells were gated and analyzed following the gating strategy shown. CD44<sup>-</sup> CD24<sup>+</sup> iNKT cells contain some CD4<sup>+</sup> CD8<sup>+</sup> DP thymocytes that likely correspond to non-specific staining, as exemplified by the presence of this population in the thymi of CD1d1d2<sup>-/-</sup> and J $\alpha$ 18<sup>-/-</sup> mice. These DP cells do not express Egr2 or CD69 (Fig 5a). PBS57-CD1d tetramer<sup>+</sup> TCR $\beta$ <sup>+</sup> CD44<sup>-</sup> CD24<sup>+</sup> cells that are CD4<sup>lo</sup> or CD4<sup>-</sup>CD8<sup>-</sup> (DN) are uniformly Egr2<sup>+</sup> (and CD69<sup>+</sup>) and can be detected only in the thymi of CD1d-sufficient mouse strains. Note that the levels of Egr2 expression detected in these cells is higher than what is detected in “conventional” T cells undergoing positive selection.



Supplementary Figure 3. Mapping of RNA-seq reads on the TRAV and TRAJ loci. Stage 0 iNKT cells from BALB/c and SKG mice were sorted and their RNA was extracted followed by library construction for Illumina sequencing. Three independent samples from BALB/c and SKG mice were prepared. Sequence reads were mapped to the mouse genome and individual tracks for each sample are shown for the whole TRAV (upper panel) and TRAJ (lower panel) loci. The results demonstrate a large enrichment for TRAV11D (encoding  $V\alpha 14$ ) and TRAJ18 (encoding  $J\alpha 18$ ) containing sequences in agreement with the TCR sequence expected from iNKT cells.

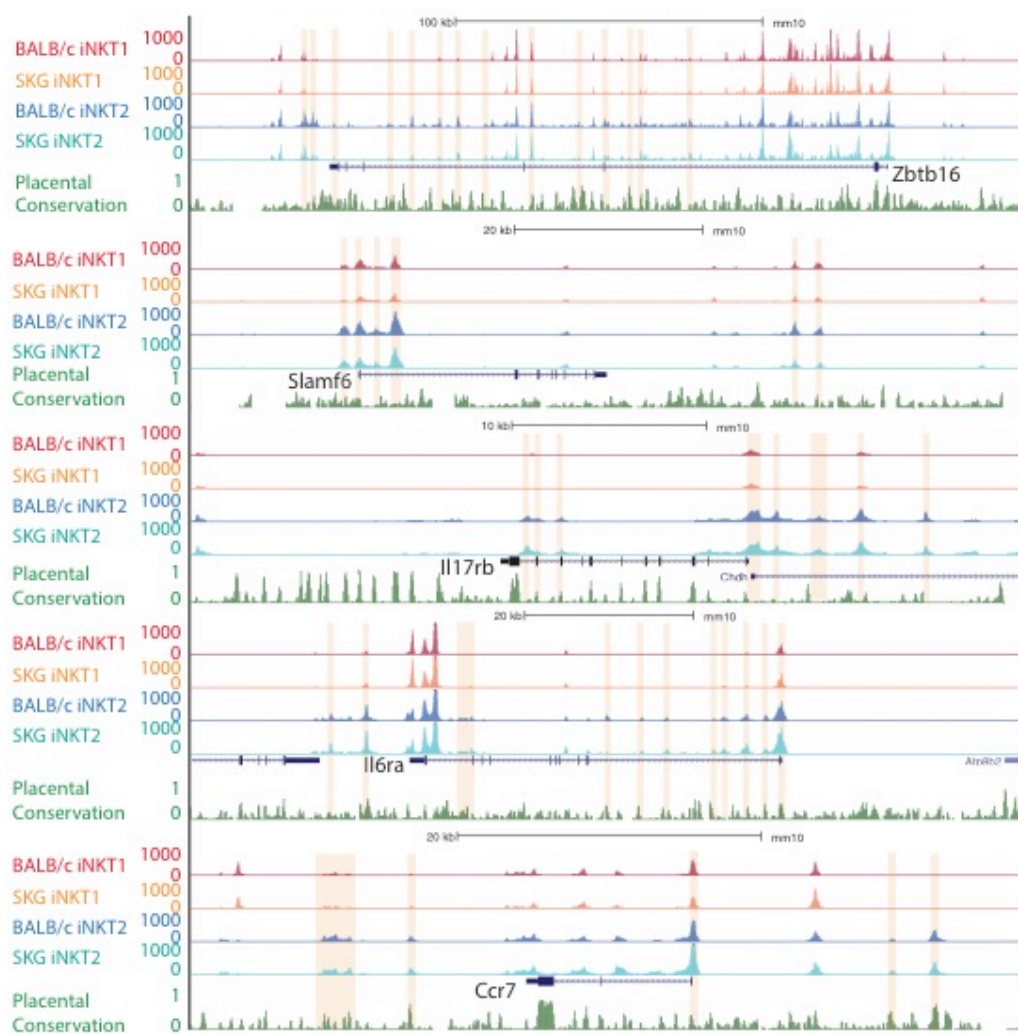


Supplementary Figure 4. Gene expression profile of thymic stage 0 iNKT cells from BALB/c and SKG mice. **(a)** Pathway-enrichment analysis of differentially expressed genes between stage 0 iNKT cells of BALB/c and SKG mice using gprofiler (<http://biit.cs.ut.ee/gprofiler/>) **(b)** Expression of genes encoding selected genes involved in cell activation (left), transcription factors (middle) and gene previously described as differentially expressed between iNKT cell subsets. The data are presented as row-wise z-scores (red, higher expression and blue, lower expression, relative to the other conditions). Data are from one experiment with three biological replicates per genotype.

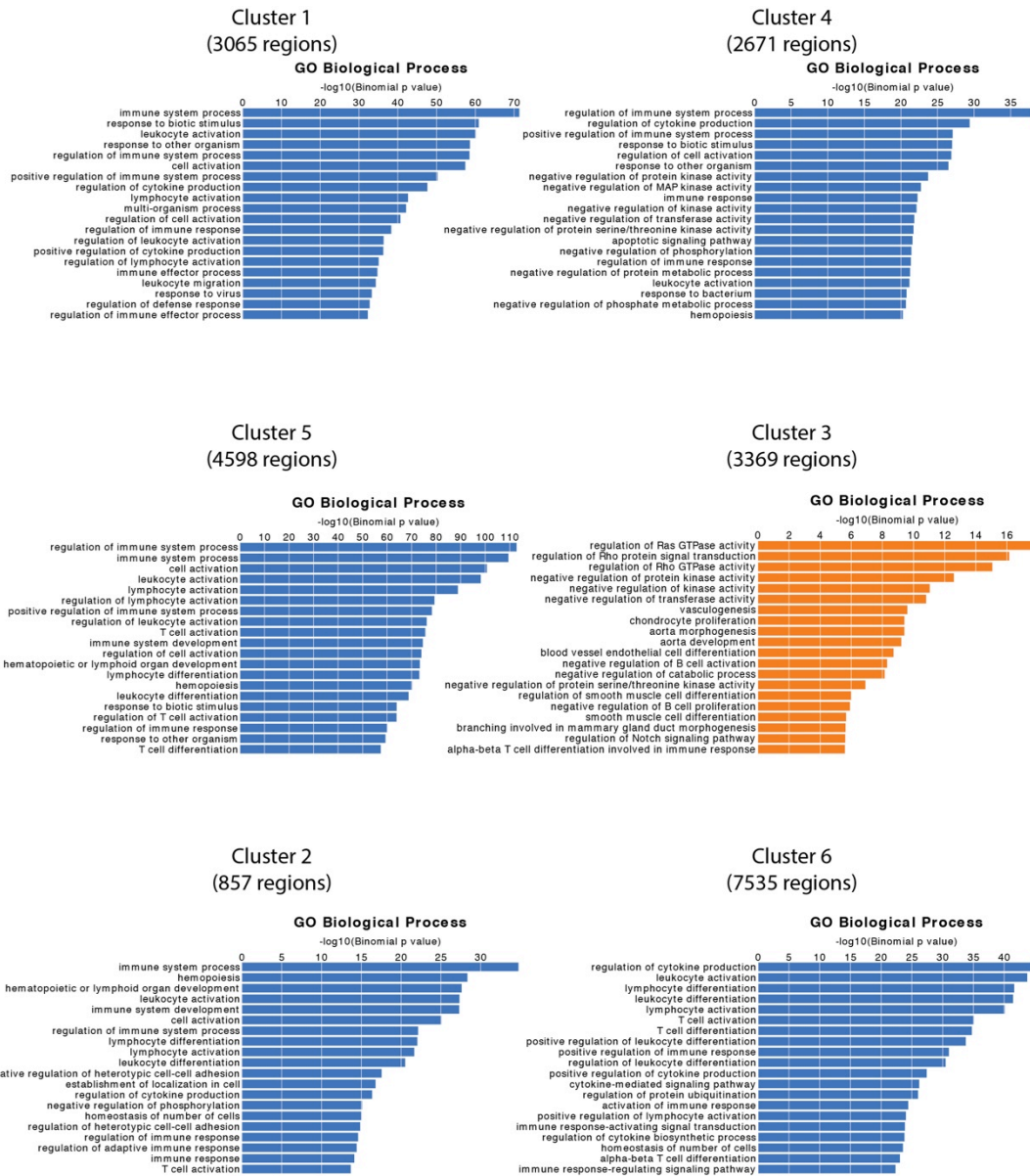


Supplementary Figure 5. Chromatin accessibility surrounding iNKT1 expressed genes. Mean ATAC sequencing coverage at the *Ifng*, *Tbx21*, *Cxcr3*, *Ccr5*, *IL2rb*, *FasL*, *Bcl2* and *Il2* loci for BALB/c and SKG iNKT1 and iNKT2 cells with a scale from 0-1000 for the ATAC-Seq tracks. Placental conservation (in green) is indicated. Regions highlighted in light blue are more accessible in iNKT1 than in iNKT2 cells.

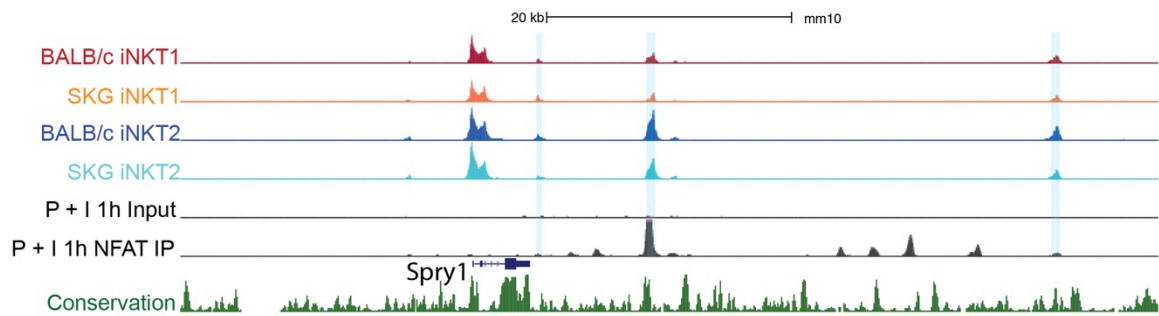
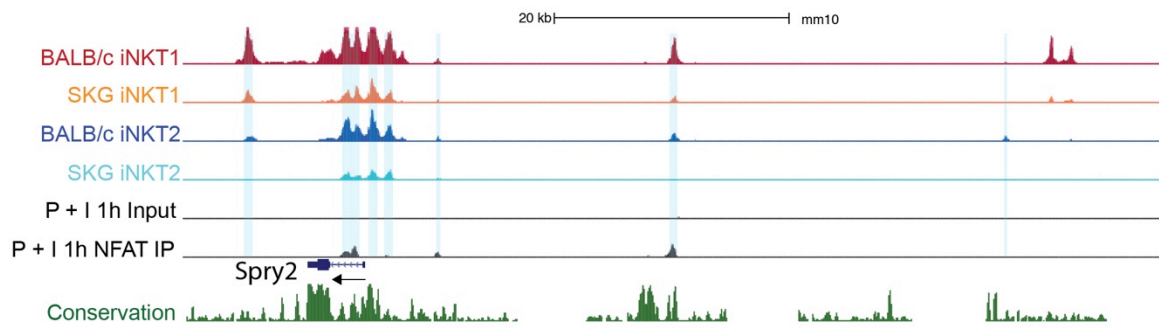
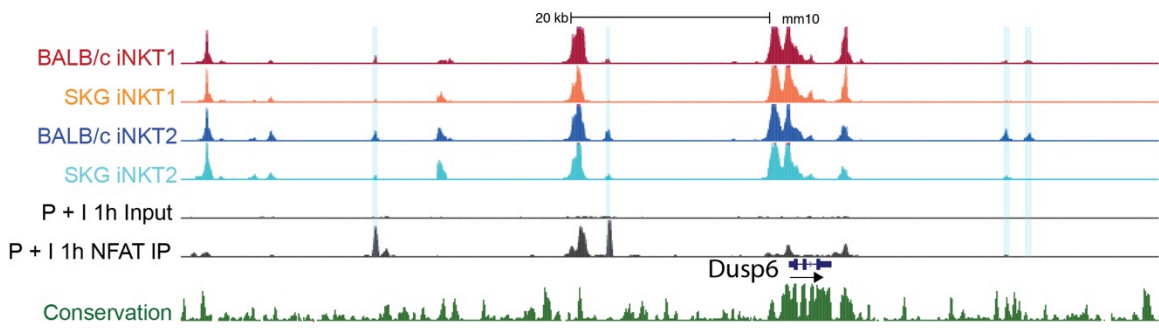
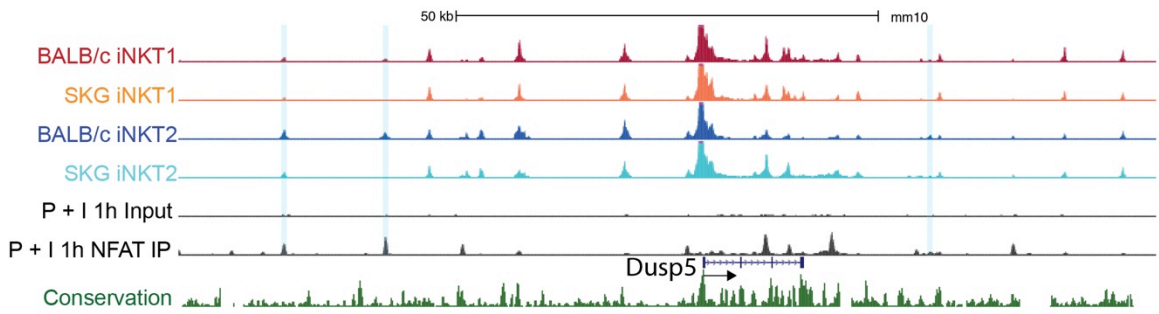
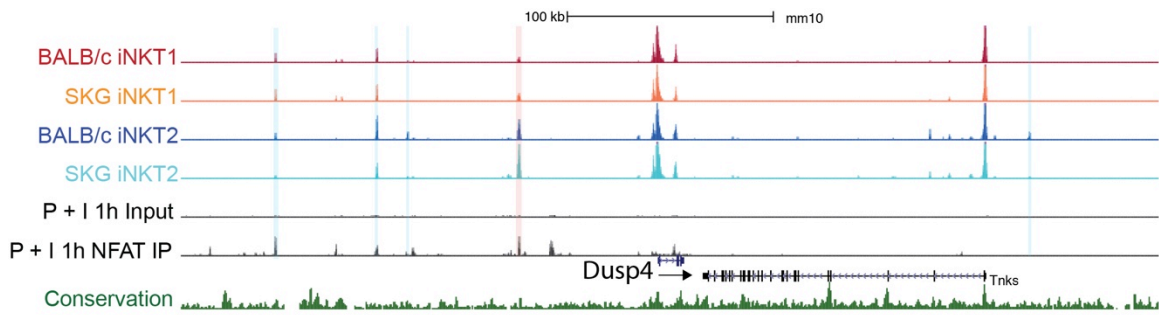




Supplementary Figure 6. Chromatin accessibility surrounding iNKT2 expressed genes. Mean ATAC sequencing coverage at the *Zbtb16*, *Slamf6*, *Il17rb*, *Il6ra* and *Ccr7* loci for BALB/c and SKG iNKT1 and iNKT2 cells with a scale from 0-1000 for the ATAC-Seq tracks. Placental conservation (in green) is indicated. Regions highlighted in light orange are more accessible in iNKT2 than in iNKT1 cells.

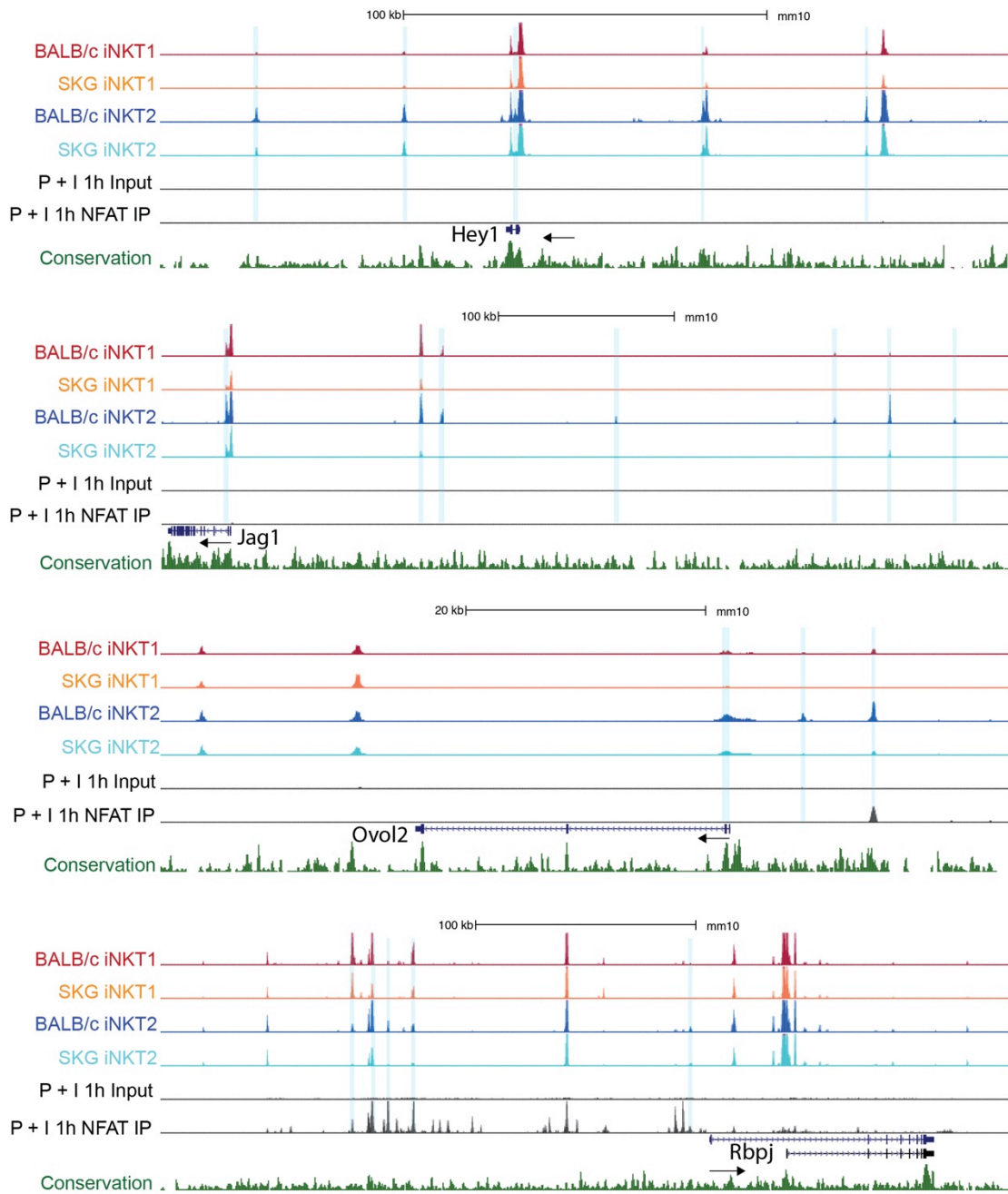


Supplementary Figure 7. Gene ontology (GO) terms enriched in peaks associated with each cluster defined in Fig 7f as determined through GREAT analysis. The number of regions in each cluster is indicated.





Supplementary Figure 8. ATAC-seq signal profiles of genes identified by GREAT with the GO biological process of negative regulation of kinase activity. The profiles for *Dusp4*, *Dusp5*, *Dusp6*, *Spry2* and *Spry1* are shown for BALB/c iNKT1 (red), SKG iNKT1 (orange), BALB/c iNKT2 (Blue) and SKG iNKT2 (cyan). Peaks that are less accessible due to the SKG mutation are highlighted in blue while peaks that are more accessible in SKG cells over BALB/c cells are highlighted in orange. A scale from 0-1000 for the ATAC-Seq tracks was used. Tracks from NFAT ChIP in CD8 T cells stimulated in the presence or absence of PMA/ionomycin for 1 hour (ref<sup>37</sup>) are also included.



Supplementary Figure 9. ATAC-seq signal profiles of genes identified by GREAT with the GO biological process of regulation of NOTCH signaling pathway. The profiles for *Hey1*, *Jag1*, *Ovo2* and *Rbpj* are shown for BALB/c iNKT1 (red), SKG iNKT1 (orange), BALB/c iNKT2 (Blue) and SKG iNKT2 (cyan). Peaks that are less accessible due to the SKG mutation are highlighted in blue while peaks that are more accessible in SKG cells over BALB/c cells are highlighted in orange. A scale from 0-1000 for the ATAC-Seq tracks was used. Tracks from NFAT ChIP in CD8 T cells stimulated in the presence or absence of PMA/ionomycin for 1 hour (ref<sup>37</sup>) are also included.

Gene	Regulation of Ras GTPase activity	regulation of Rho protein signal transduction	regulation of Rho GTPase activity	negative regulation of protein kinase activity	negative regulation of kinase activity	vasculogenesis	chondrocyte proliferation	aorta morphogenesis	aorta development	blood vessel endothelial cell differentiation	negative regulation of B cell activation	negative regulation of catabolic process	regulation of protein serine/threonine kinase activity	smooth muscle cell differentiation	branching involved in mammary gland morphogenesis	regulation of notch signaling pathway	alpha-beta T cell differentiation involved in immune response
Adcyap1	Als2	Als2	Adar	Adar	Adar	Ccm2	Ctgf	Eya1	Eya1	Ccm2	Btla	Adra2a	Apc	Ereg	Btla	Acvr1	Aak1
Agap1	Apoa1	Arhgap10	Arhgap10	Arhgap10	Arhgap10	Cited2	Ddr2	Hey1	Hey1	Hey1	Casp3	Cidea	Bmp7	Fgfr2	Casp3	Ctnnb1	Ar
Agf1	Arhgap15	Arhgap15	Apc	Apc	Apc	Ctmb1	Hmga2	Hey1	Kdr	Cdkn2a	Cnr1	Casp3	Prdm6	Prdm6	Cdkn2a	Ednrb	Bmp7
Als2	Arhgap24	Arhgap24	Bmp7	Bmp7	Bmp7	Epha2	Inh	Jag1	Jag1	Ctla4	Cpeb2	Cdkn2a	Shh	Shh	Ctcf4	Gata6	Gadd45g
Arap2	Arhgap24	Chn1	Casp3	Casp3	Casp3	Fbxw7		Mylk	Mylk	Tmem100	Fcgr2b	Fhit	Dusp16	Tmsb4x	Fcgr2b	Hes1	Gata3
Arhgap10	Arhgef18	Dic1	Cdkn2a	Cdkn2a	Cdkn2a	Fgf1		Pdgfrb	Pdgfrb	Hmgb3	Fyn	Dusp4	Tshz3	Il10	Itga8	Esr1	Ilng
Arhgap15	Arhgef26	Dock4	Dusp21	Dusp21	Cln8	Fgfr2		Prox1	Prox1	Id2	Grin2a	Dusp5	Zeb1	Prdm1	Mef2c	Phb2	Ilrf4
Arhgap24	Arhgef28	EfnA5	Dusp16	Dusp16	Dusp16	Gata4		Rbpbj	Rbpbj	Il10	Hdac4	Dusp6			Mki2	Slc12a2	Lef1
Asap1	Chn1	Epha1	Dusp4	Dusp4	Dusp4	Hdac7		Sox4	Sox4	Ndfip1	Ier3	Fbxw7			Npnt	Tbx3	Ptger4
Asa2	Cuif3	Epha2	Dusp5	Dusp5	Dusp4	Hey1		Srf	Srf	Prdm1	Il10	Hmgcr			Pdgfrb	Tsp2c	Stat6
Chn1	Dic1	Epha3	Dusp6	Dusp6	Dusp5	Hhex				Samsn1	Il3	Kat2b			Smad6	Tram1	
Dgki	Dock4	Ephb3	Epha1	Epha1	Dusp6	Kdr				Lrrprc	Lats2	Lats2			Srf	Rbpbj	
Dic1	EfnA5	Fzd10	Fbxw7	Fbxw7	Fbxw7	Ntrk2				Mad2l1	Lax1	Lax1			Tbx18	Slc35c1	
Dock4	Epha1	Gpr65	Gadd45a	Gadd45a	Fbxw7	Ntrk2				Mdm4	Prkca	Prkca			Wnt7b	Trp63	
EfnA5	Epha2	Igfb1bp1	Gadd45g	Gadd45g	Fbxw7	Ntrk2				Npc1	Prkcd	Prkcd					
Epha1	Epha3	Jun	Gnb2l1	Gnb2l1	Gadd45g	Ptprj				Pde3b	Ptpr6	Ptpr6					
Epha2	Ephb3	Ntrk2	Hmgcr	Hmgcr	Gnb2l1	Rasa1				Prkaa1	Ptprj	Ptprj					
Epha3	Farp1	Ophn1	Ibtk	Ibtk	Hmgcr	Rasip1				Ibtk	Hmgcr	Rasip1					
Ephb3	Fgd5	Prex2	Igf1r	Igf1r	Ibtk	Sgpl1				Rasip1	Spred2	Spred2					
Frd10	Fzd10	Ralbp1	Igfb1bp1	Igfb1bp1	Igf1r	Shh				Shh	Igfb1bp1	Igfb1bp1					
Gd2	Gpr65	Rasgrp1	Igfb1bp1	Igfb1bp1	Igf1r	Sox17				Smas3	Spry2	Spry2					
Gnas	Igfb1bp1	Rasip1	Kat2b	Kat2b	Igfb1bp1	T				Tbc1d14	Uchl1	Uchl1					
Gpr65	Jun	Sod1	Lats2	Lats2	Kat2b	Tgfb2				Timp3	Timp3	Timp3					
Igfb1bp1	Lpar1	Srgap1	Lax1	Lax1	Lats2	Tgfb2				Wac							
Jun	Ntrk2	Tbc1d7	Pkia	Pkia	Lax1	Tmem100											
Ntrk2	Ophn1	Tiam1	Pkib	Pkib	Wars2												
Ntrk3	Plekhg1		Prkag2	Prkag2	Pkib	Wnt7a											
Ophn1	Plekhg3		Prkca	Prkca	Prkag2	Wnt7b											
Prex2	Plekhg6		Prkcd	Prkcd	Prkca	Wt1											
Ralbp1	Prex2		Prkrip1	Prkrip1	Prkcd	Zfp3611											
Ralgapa2	Ralbp1		Ptpn6	Ptpn6	Prkrip1	Zmiz1											
Rap1a	Rasgrf2		Ptprj	Ptprj	Ptpn6												
Rap1gap	Rasgrp1		Sfrp5	Sfrp5	Ptprj												
Rasa1	Rasip1		Socs5	Socs5	Sfrp5												
Rasa2	Sod1		Spred2	Spred2	Socs5												
Rasgrp1	Srgap1		Spry1	Spry1	Spred2												
Rasgrp3	Srgap1		Spry2	Spry2	Spry1												
Rasip1	Tbc1d7		Trnb1	Trnb1	Spry2												
S1pr1	Tiam1		Trnb2	Trnb2	Trnb1												
Sigsm2	Tiam2		Ubash3b	Ubash3b	Trnb2												
Sipa11	Trio		Uchl1	Uchl1	Ubash3b												
Sod1	Vav3		Zfyve28	Zfyve28	Uchl1												
Spry1					Zfyve28												
Spry2																	
Srgap1																	
Tbc1d1																	
Tbc1d14																	
Tbc1d2																	
Tbc1d22a																	
Tbc1d23																	
Tbc1d4																	
Tbc1d7																	
Tbck																	
Tiam1																	
Usp6n1																	

Supplementary Table I. List of genes associated with each GO biological terms for cluster 3.