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1 **TISSUE-SPECIFIC SHAPING OF THE TCR REPERTOIRE AND ANTIGEN SPECIFICITY**  
2 **OF INKT CELLS**

3

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20 **ABSTRACT**

21 Tissue homeostasis is critically dependent on the function of tissue-resident lymphocytes,  
22 including lipid-reactive invariant natural killer T (iNKT) cells. Yet, if and how the tissue  
23 environment shapes the antigen specificity of iNKT cells remains unknown. By analysing  
24 iNKT cells from lymphoid tissues of mice and humans we demonstrate that their T cell  
25 receptor (TCR) repertoire is highly diverse and is distinct for cells from various tissues  
26 resulting in differential lipid-antigen recognition. Within peripheral tissues iNKT cell recent  
27 thymic emigrants exhibit a different TCR repertoire than mature cells, suggesting that the  
28 iNKT population is shaped after arrival to the periphery. Consistent with this, iNKT cells from  
29 different organs show distinct basal activation, proliferation and clonal expansion. Moreover,  
30 the iNKT cell TCR repertoire changes following immunisation and is shaped by age and  
31 environmental changes. Thus, post-thymic modification of the TCR-repertoire underpins the  
32 distinct antigen specificity for iNKT cells in peripheral tissues

## 33 INTRODUCTION

34 Most anatomical compartments, including mucosal surfaces and solid organs, host large  
35 populations of tissue-resident lymphocytes which are uniquely placed to provide local  
36 networks for immune surveillance and defence against infection (Fan & Rudensky, 2016).

37 Within the families of tissue-resident lymphocytes, invariant Natural Killer T (iNKT) cells  
38 constitute the body's means to sense lipids, as antigens presented on CD1d (Salio *et al.*,  
39 2014). Accordingly, iNKT cells recognise through their T cell receptors (TCR) self-lipids as  
40 well as lipids from pathogenic bacteria, commensals, fungi or pollens; consequently  
41 contributing to anti-microbial, antitumor and autoimmune responses (Salio *et al.*, 2014).

42 Since iNKT cell activation can prevent or promote immunopathology in diverse disease  
43 contexts, the strict control of peripheral iNKT cell homeostasis is vital to regulate local  
44 immunity. Beyond the common features shared by all iNKT cells (including their CD1-  
45 restriction and innate-like properties), cells found in discrete tissues have distinct  
46 phenotypes and functions that critically modulate the outcome of immunity (Crosby &  
47 Kronenberg, 2018). This suggests that unique tissue-specific factors (including local lipid  
48 antigens, cytokines and/or hormones) may shape the population of iNKT cells resident in  
49 those tissues, ultimately regulating local immune responses. Consistent with this, alterations  
50 in CD1d-lipid presentation in the gut or the liver result in dysregulated homeostasis of local  
51 iNKT cells driving increased susceptibility to inflammation in these tissues (An *et al.*, 2014;  
52 Zeissig *et al.*, 2017). Nonetheless, how signals from the tissue environment shape the iNKT  
53 cell population to best fit their function in their tissues of residency remains unclear.

54

55 iNKT cells have been traditionally defined by the expression of an invariant TCR  $\alpha$ -chain  
56 ( $V\alpha 14$ - $J\alpha 18$  in mice or  $V\alpha 24$ - $J\alpha 18$  in humans) and their capacity to recognize the glycolipid  
57 antigen  $\alpha$ -galactosylceramide ( $\alpha$ GalCer) presented on CD1d. Despite this prototypical TCR  
58 repertoire gene usage, in recent years it has become apparent that there are variations  
59 within the iNKT cell repertoire that ultimately impact the antigen recognition capacity and  
60 consequently the functional outcomes during an immune response. In mice, although most



61 iNKT cells express the canonical V $\alpha$ 14-J $\alpha$ 18 TCR  $\alpha$ -chain, they can use different V $\beta$  chains  
62 and the combination of V $\beta$ -, J $\beta$ -, and CDR3 $\beta$ -encoded residues will ultimately determine the  
63 type of ligands that iNKT cells can bind (Cameron *et al.*, 2015; Mallevaey *et al.*, 2009;  
64 Matsuda *et al.*, 2001). Moreover, a population of  $\alpha$ GalCer-reactive NKT cells that express  
65 V $\alpha$ 10 TCR and has a distinct lipid-recognition capacity has been identified (Uldrich *et al.*,  
66 2011). In humans, while the majority of  $\alpha$ GalCer-binding iNKT cells express the prototypical  
67 V $\alpha$ 24V $\beta$ 11 TCR, populations of *atypical NKT cells* have been found in the blood, with cells  
68 expressing a range of TCR $\alpha$  and TCR $\beta$  chains that show differential recognition of lipid  
69 antigens (Le Nours *et al.*, 2016; Matulis *et al.*, 2010). Therefore, the so-called *invariant NKT*  
70 *cells* constitute a polyclonal population with a broader antigen recognition capacity than  
71 previously assumed. Since iNKT cells are tissue-resident cells an important question  
72 remains regarding whether the iNKT cell TCR repertoire (and consequently antigen  
73 specificity) is related to their anatomical location and/or shaped by the antigens that these  
74 cells encounter in peripheral tissues. Similarly, whether the iNKT cell population changes in  
75 response to environmental challenges including infection, vaccination, alterations in the diet  
76 or antibiotic use is unknown.

77

78 While the TCR repertoire is determined during thymic selection the relevance of post-thymic  
79 TCR shaping has been demonstrated for both conventional CD4<sup>+</sup> T cells and regulatory T  
80 cells (Tregs). Accordingly, the TCR repertoire of thymic and peripheral CD4<sup>+</sup> T cells (or that  
81 of recent thymic emigrants (RTE) and mature naïve T cells) are not identical, suggesting that  
82 certain clones are preferentially enriched and/or deleted in the periphery (Correia-Neves *et al.*,  
83 2001; Houston & Fink, 2009). Similarly, the TCR repertoire of natural Tregs is unique for  
84 individual tissues, is shaped by the local antigenic landscape and controls Treg-mediated  
85 tolerance to the tissues (Lathrop *et al.*, 2011; Lathrop *et al.*, 2008). In the case of iNKT cells,  
86 CCR7<sup>+</sup> iNKT cell precursors are known to emigrate from the thymus and home to peripheral  
87 tissues where they undergo further maturation (Wang & Hogquist, 2018). In this way

88 peripheral signals presumably shape the iNKT cell population into a mature iNKT cell pool.  
89 However, whether they impact the antigen recognition capacity of iNKT cells in their tissues  
90 of residency remains unknown.  
91  
92  
93 Here, we have investigated the impact of the anatomical location in the peripheral iNKT cell  
94 population. By combining flow-cytometry analyses, lipid-loaded tetramers and RNAseq  
95 experiments we demonstrate that the iNKT cell TCR repertoire is highly polyclonal and it is  
96 different for iNKT cells resident in various tissues resulting in differential lipid antigen  
97 recognition. In line with this, the repertoire of iNKT RTE is distinct from that of mature iNKT  
98 cells suggesting that local signals shape the mature iNKT cell population. Accordingly, the  
99 basal activation, proliferation and clonal expansion of iNKT cells is dictated by anatomical  
100 location. Moreover, the repertoire and phenotype of human iNKT cells is also different for  
101 cells found in various anatomical locations. Thus, our data uncovers a novel mechanism of  
102 tissue-specific immunoregulation that underpins the antigen-specificity of iNKT cells in  
103 different sites.

## 104 RESULTS

### 105 Distinct TCR repertoire and clonal expansion for iNKT cells from various tissues

106 The majority of  $\alpha$ GalCer-reactive iNKT cells express an invariant TCR  $\alpha$ -chain (V $\alpha$ 14-J $\alpha$ 18),  
107 however the V $\beta$  chain usage is variable, with higher percentages of cells expressing V $\beta$ 7 or  
108 V $\beta$ 8 chains. Thus, to evaluate whether the TCR repertoire of iNKT cells is related to their  
109 anatomical location we examined the expression of V $\beta$ 7 and V $\beta$ 8.1/8.2 by iNKT cells from  
110 various lymphoid tissues of WT C57BL/6 mice. We identified iNKT cells from thymus,  
111 spleen, inguinal lymph node (iLN) and mesenteric lymph node (mLN) by PBS57-loaded  
112 CD1d tetramer (CD1d-tet-PBS57; being PBS57 an analogue of  $\alpha$ GalCer) and TCR $\beta$  co-  
113 staining (Figure 1; Figure 1- figure supplement 1A). Within the iNKT cell population we found  
114 that the percentage of V $\beta$  usage varied according to anatomical location, and particularly  
115 iNKT cells from LNs showed decreased percentage of cells using V $\beta$ 8.1/8.2 chains and  
116 increased percentages of cells using “other” V $\beta$ s (other than V $\beta$ 7 or V $\beta$ 8.1/8.2) in  
117 comparison with cells from thymus or spleen (Figure 1A). We also observed significant  
118 differences in TCRV $\beta$  usage for iNKT cells identified in non-lymphoid tissues including liver,  
119 lung and small intestinal lamina propria (SI-LP) (Figure 1- figure supplement 1A and 1B).  
120 Interestingly, we detected the same shift in TCRV $\beta$  usage after iNKT cells (enriched from  
121 spleen and thymus) were adoptively transferred into congenic mice. Twelve days after  
122 transfer, donor cells found in LNs showed a decreased frequency of TCRV $\beta$ 8.1/8.2 usage in  
123 comparison with cells found in the spleen, recapitulating the TCRV $\beta$  usage of endogenous  
124 iNKT cells (Figure 1- figure supplement 1C). Thus, all together this data indicates that the  
125 TCR $\beta$  repertoire of peripheral iNKT cells varies according to their anatomical location.

126

127 To get an unbiased overview of the properties and TCR repertoire of peripheral iNKT cells,  
128 we sort-purified iNKT cells from various lymphoid tissues (thymus, spleen, iLN, mLN), and  
129 performed whole transcriptome RNA sequencing (RNAseq). This method, not only enabled  
130 identification of the differentially expressed genes and pathways in iNKT cells from individual  
131 tissues, but also analyses of iNKT cell clonality and TCR repertoire (Brown *et al.*, 2015; Li *et*

132 *al.*, 2016). Thus, using our RNAseq data we took advantage of MiXCR software to identify  
133 the CDR3 sequences, V, D and J segments and the repertoires for iNKT cells isolated from  
134 individual tissues (Bolotin *et al.*, 2015; Bolotin *et al.*, 2013). With this approach, we identified  
135 a total of approximately 80,000 (productive) TCR sequences (Figure 1- figure supplement  
136 2A) obtained from 4 biological replicates per tissue. As expected, the majority of TRAV  
137 sequences (>96%) identified in all of the tissues corresponded to the canonical TRAV11-  
138 TRAJ18 rearrangement (V $\alpha$ 14-J $\alpha$ 18) and showed highly conserved CDR3 $\alpha$  sequences  
139 (Figure 1- figure supplement 2B). In agreement with our flow-cytometry data TRBV29 (V $\beta$ 7)  
140 and TRBV13 (V $\beta$ 8) segments represented the majority of TCR $\beta$  usage in all the tissues and  
141 the percentage of usage for each gene differed between tissues (Figure 1B-1C, Figure 1-  
142 figure supplement 2B). Specifically, the percentage of the most abundant TRBV13-2 gene  
143 (V $\beta$ 8.2) was decreased in samples from LNs in comparison with spleen or thymus and we  
144 also detected significant differences between tissues in the frequency of TRBV29 (V $\beta$ 7) and  
145 TRBV1 (V $\beta$ 2; Figure 1B). Moreover, we detected a highly diverse TRBJ usage and TRBV-  
146 TRBJ pairing with notable differences for iNKT cells from individual tissues (Figure 1C),  
147 which could suggest the existence of preferred TRBV-TRBJ pairing and/or the expansion (or  
148 deletion) of certain iNKT cell clones.

149

150 We found the CDR3 $\beta$  sequences to be highly polyclonal without conserved motives or  
151 specific residues associated with individual tissues (Figure 1D, Figure 1- figure supplement  
152 3). Comparative analyses of length and physicochemical properties for CDR3 $\beta$  amino acid  
153 (aa) sequences (including hydrophobicity (based on the Kyte–Doolittle scale (Kyte &  
154 Doolittle, 1982)), isoelectric point (pI, according to EMBOSS (Rice *et al.*, 2000)), frequency  
155 of polar (D, E, H, K, N, Q, R, S, T), aliphatic (A, I, L, V) or aromatic (F, H, W, Y) residues) did  
156 not find significant differences in sequences from iNKT cells isolated from various tissues  
157 (Figure 1- figure supplement 3). However, when we assessed the distribution of the iNKT  
158 cell TCR clonotype size (the proportion of CDR3 $\beta$  sequences which occur once, twice, etc.

159 in a specific tissue), we detected a higher clonal size for iNKT cells from iLN and mLN in  
160 comparison with splenic and thymic iNKT cells (Figure 1E-1G). Accordingly, while in spleen  
161 and thymus CDR3 $\beta$  sequences appearing only once in our data-set represented around  
162 65% of the total sequences, this percentage decreases to 50% in mLN and 40% in iLN  
163 (Figure 1E-F). We further assessed individual iNKT TCR repertoires for evidence of clonal  
164 expansion by using cumulative frequency curves to measure the 25 most prevalent  
165 clonotypes (Figure 1G). These analyses provided evidence for increase in clone sizes in  
166 iLN and mLN TCR repertoires in comparison with iNKT cells from spleen or thymus.

167

168 Thus, all together this data demonstrates that the TCRV $\beta$  repertoire and clonal expansion of  
169 iNKT cells varies according to their anatomical location.

170

### 171 **Peripheral shaping of the iNKT cell TCR repertoire**

172 Once selected in the thymus, the TCR repertoire of conventional T cells is further shaped in  
173 peripheral lymphoid organs possibly through differential modulation of the expansion and  
174 survival of individual T cells (Correia-Neves *et al.*, 2001; Houston & Fink, 2009). To analyse  
175 whether the TCR repertoire of iNKT cells is modulated after their arrival to peripheral organs,  
176 we compared the TCRV $\beta$  usage for iNKT cells just arriving to the tissues after thymic  
177 selection (iNKT RTE) with that of resident iNKT cells that have been in the tissues for  
178 several weeks (Figure 2, Figure 2 - figure supplement 1). To identify iNKT RTE we took  
179 advantage of Rag2<sup>GFP</sup> reporter mice (Boursalian *et al.*, 2004). In these animals GFP  
180 identifies RTE in peripheral tissues and the label is brightest in the youngest RTEs and  
181 decays over time until it can no longer be detected on T cells that have been in the periphery  
182 for more than 3 weeks (Boursalian *et al.*, 2004). We identified small populations of GFP<sup>+</sup>  
183 iNKT RTE in all the analysed tissues of Rag2<sup>GFP</sup> reporter mice (Figure 2A). In the thymus the  
184 majority of iNKT cells are mature tissue-resident cells (GFP<sup>-</sup>) but there is a population of  
185 GFP<sup>+</sup> iNKT cells that also includes cells in early developmental stages (Wang & Hogquist,  
186 2018). In agreement with this, GFP expression was higher in thymic iNKT RTE but

187 comparable in RTE from spleen or LNs (Figure 2A). We found that the TCRV $\beta$  usage was  
188 different when we compared RTE and resident (GFP<sup>-</sup>) iNKT cells within the peripheral  
189 tissues of Rag2<sup>GFP</sup> animals (Figure 2B) with a consistent increase in the percentage of  
190 TCRV $\beta$ 7<sup>+</sup> in the resident iNKT cell population compared to the recently arrived counterparts.

191

192 To further explore the peripheral shaping of the iNKT cell repertoire, we took advantage of a  
193 recently described strategy to identify iNKT cell precursors (CCR7<sup>+</sup>PD-1<sup>-</sup>Qa2<sup>low</sup> iNKT cells)  
194 which have been shown to emigrate from the thymus to the periphery where they terminally  
195 differentiate (Wang & Hogquist, 2018). Using this combination of markers, we detected  
196 populations of iNKT precursors in thymus and spleen (as previously described), but also in  
197 peripheral LNs (Figure 2 - figure supplement 1A). Notably, CCR7<sup>+</sup>PD-1<sup>-</sup>Qa2<sup>low</sup> and  
198 Rag2GFP<sup>+</sup> iNKT cell populations don't fully overlap and while Rag2GFP<sup>+</sup> NKT cells are  
199 enriched for CCR7<sup>+</sup> cells, not all CCR7<sup>+</sup> NKT cells are RTE (Figure 2 - figure supplement  
200 1B) (Wang & Hogquist, 2018). Nevertheless, when we compared the TCRV $\beta$  usage for  
201 CCR7<sup>+</sup>PD-1<sup>-</sup> iNKT cell precursors and "mature" iNKT cells (excluded from the CCR7<sup>+</sup>PD-1<sup>-</sup>  
202 gate) we observed consistent changes in V $\beta$  usage with an increase in the percentage of  
203 TCRV $\beta$ 7<sup>+</sup> cells in the mature iNKT cell population compared to the recently arrived  
204 counterparts (Figure 2 - figure supplement 1C). Thus, these results suggest that after arrival  
205 to the tissues iNKT cells are exposed to local signals that shape the newly arrived iNKT cell  
206 population into a mature iNKT cell pool with a distinct TCR repertoire.

207

208

### 209 **LN iNKT cells show increased proliferation which is associated with a distinct TCR $\beta$** 210 **repertoire**

211 To investigate the processes shaping the iNKT population in the different lymphoid tissues  
212 we studied the gene expression profile for iNKT cells isolated from thymus, spleen, mLN and  
213 iLN (Figure 3, Figure 3 - figure supplement 1A,B). Transcriptome analyses showed around  
214 200-400 genes that were differentially expressed amongst iNKT cells from various tissues

215 (e.g. mLN and splenic iNKT cells differed by ~200 genes; adjusted  $p$  value <0.01, fold  
216 change >1.5) (Figure 3A). Compared to spleen iNKT cells, cells from thymus, iLN and mLN  
217 showed differential expression of transcripts encoding transcriptional regulators (e.g. *Fos*,  
218 *Fosb*, *Egr1*, *Egr3*), nuclear factors (e.g. *Na4r1*, *Tcf7*), cytokine/chemokine receptors (e.g.  
219 *Ccr8*, *Ccr4*, *Il12r2b*) and molecules related to cytotoxicity (e.g. *Gzma*, *Klrg1*). Interestingly,  
220 iNKT cells from iLN and mLN (but not from thymus) showed upregulation of genes related to  
221 T cell activation/TCR signalling including *Na4r1* (Nur77), *Icos*, *Cd28*, *Fos*, *Fosb* as well as  
222 the chemokine receptors *Ccr4* and *Ccr8* which are upregulated after TCR-mediated  
223 activation (D'Ambrosio *et al.*, 1998; Sallusto *et al.*, 1999) (Figure 3B). Conversely, negative  
224 regulators of TCR signalling such as *Dok2* or *Ptpn22* were downregulated in LN cells vs  
225 iNKT cells from spleen or thymus (Figure 3B). Indeed, gene ontology enrichment analysis  
226 comparing the transcriptome of LN iNKT cells with cells from spleen and thymus,  
227 demonstrated significant enrichment in LN iNKT cells for genes encoding molecules related  
228 to positive regulation of T cell activation and positive regulation of cellular proliferation  
229 (Figure 3 - figure supplement 1A,B). Hence, these results suggest that the basal activation of  
230 iNKT cells is distinct for cells found in various lymphoid tissues.

231

232 Amongst the significantly changed genes linked to T cell activation, the nuclear receptor  
233 transcription factor Nur77 (*Nr4a1*) is induced rapidly upon TCR stimulation and in a manner  
234 proportional to TCR signalling intensity (Moran *et al.*, 2011) and was found to be upregulated  
235 in LN iNKT cells in comparison with thymic and splenic iNKT cells. We confirmed this result  
236 by taking advantage of Nur77<sup>GFP</sup> reporter mice in which we observed that iNKT cells from  
237 mLN and iLN express high levels of GFP while cells from spleen and thymus are  
238 predominantly GFP<sup>-</sup> (Figure 3C). Furthermore, the activation markers ICOS and CD25 and  
239 the chemokine receptor CCR8 were also found to be expressed at higher levels in LN iNKT  
240 cells in comparison with cells from spleen (Figure 3C). As LN iNKT cells showed an  
241 enrichment of genes involved in positive regulation of cellular proliferation we analysed iNKT  
242 cell proliferation by measuring the expression of the proliferation marker Ki-67 and the

243 incorporation of EdU *in vivo* (Figure 3D-F). We detected a consistently higher expression of  
244 Ki-67 (Figure 3D-E) and higher EdU incorporation (48 h after EdU injection; Figure 3F) in LN  
245 iNKT cells vs. splenic or thymic cells, confirming a higher degree of local proliferation for  
246 iNKT cells in LNs. Thus, in homeostatic conditions LN iNKT cells show increased TCR  
247 signalling, activation and proliferation in comparison to splenic or thymic cells.

248

249 To investigate whether the increased proliferation of LN iNKT cells is related to their  
250 increased clonal expansion and contributes to their distinct TCR $\beta$  repertoire we analysed the  
251 TCRV $\beta$  usage of proliferating (EdU<sup>+</sup>) vs non-proliferating (EdU<sup>-</sup>) iNKT cells after EdU  
252 administration *in vivo* (Figure 3G, Figure 3 - figure supplement 1C). These experiments  
253 showed a bias in the TCRV $\beta$  repertoire, with an enrichment on V $\beta$ 7 and a decrease in  
254 V $\beta$ 8.1/8.2 usage in proliferating vs non-proliferating cells. In agreement with this data, when  
255 we compared the TRBV repertoire of sequences present only once in our RNAseq-derived  
256 dataset vs more abundant sequences (2 copies or more), we detected increased frequency  
257 of TRBV29 (V $\beta$ 7) and decreased TRBV13-2 gene usage (V $\beta$ 8.2) in abundant vs low-copy  
258 sequences (Figure 3H). Thus, this data suggests that LN iNKT cells have increased basal  
259 activation and proliferation that contribute to shape the local iNKT cell TCR repertoire.

260

### 261 **The anatomical location governs the basal activation and TCRV $\beta$ usage of all iNKT** 262 **cell subsets**

263 iNKT cells are a heterogeneous population that can be classified into several subsets based  
264 on the expression of signature transcription factors: NKT1 (T-bet<sup>+</sup>); NKT2 (PLZF<sup>hi</sup>); NKT17  
265 (ROR $\gamma$ t<sup>+</sup>)(Engel *et al.*, 2016; Lee *et al.*, 2013). Because iNKT cell subpopulations are  
266 present at different proportions in the various lymphoid organs we evaluated whether the  
267 changes in proliferation, basal activation and TCR repertoire could be due to different iNKT  
268 subsets present in those tissues. As previously reported, in C57BL/6 mice we found that  
269 NKT1 cells represent the majority of iNKT cells in thymus, spleen, mLN and iLN, but we also  
270 detected significant proportions of NKT2s and NKT17s in LNs (Figure 4A). Importantly, we



271 found that the increased expression of activation and proliferation markers in LN iNKT cells  
272 was evident for all iNKT cell subpopulations (Figure 4B). As such, the expression of Ki-67,  
273 Nur77, ICOS and CD25 was higher in NKT1, NKT2 and NKT17 cells from LNs vs their  
274 splenic counterparts, indicating that the tissue environment controls the basal activation and  
275 proliferation of iNKT cells regardless of the subset to which they belong. In the same line, we  
276 found that the anatomical location led to variation in TCRV $\beta$  usage within individual iNKT cell  
277 subsets (Figure 4C). For instance, within the NKT1 population we detected a lower  
278 frequency of V $\beta$ 8.1/8.2 in cells from the LNs vs spleen or thymus. Likewise, V $\beta$ 7 usage was  
279 significantly different in all subsets when comparing cells from different tissues. In line with  
280 these results, we also detected significant differences in TCRV $\beta$  usage associated with the  
281 tissue of residency when iNKT cells were subdivided on the basis of their expression of CD4  
282 and/or NK1.1 (Figure 4 - figure supplement 1). Thus, all together this data indicates that the  
283 anatomical location shapes the basal activation, proliferation and TCR repertoire of all iNKT  
284 cell subsets.

285

### 286 **Differential lipid antigen recognition for iNKT cells in peripheral tissues**

287 Next, we investigated the functional relevance of the distinct TCR $\beta$  repertoire for iNKT cells  
288 found in different anatomical locations. Since the TCR $\beta$  usage in iNKT cells modulates lipid  
289 antigen recognition (Cameron *et al.*, 2015; Florence *et al.*, 2009) we investigated the antigen  
290 binding capacity for iNKT cells in individual tissues. To do this, we costained iNKT cells from  
291 various tissues (thymus, spleen, mLN, iLN, SI-LP, liver, lung) with CD1d-tet-PBS57 and  
292 CD1d-tetramers loaded with different glycolipid antigens including the  $\alpha$ GalCer analogue  
293 OCH,  $\alpha$ GlucosylCeramide (C26;  $\alpha$ GlcCer),  $\beta$ GlucosylCeramide (C24;  $\beta$ GlcCer) and  
294  $\beta$ GalactosylCeramide (C12;  $\beta$ GalCer). Within the CD1d-tet-PBS57<sup>+</sup> iNKT cell population we  
295 identified distinct populations of iNKT cells binding OCH and  $\alpha$ GlcCer in all analysed tissues  
296 (Figure 5A-5B, Figure 5 -figure supplement 1), but not  $\beta$ GlcCer or  $\beta$ GalCer (data not shown).  
297 Interestingly, the percentages of cells binding to the various lipid antigens was different  
298 amongst tissues (Figure 5A-5B, Figure 5 -figure supplement 1). For instance, while most

299 iNKT cells bind  $\alpha$ GlcCer and OCH in thymus, spleen, liver and lung, in iLN, mLN and SI-LP  
300 we identified clear populations of  $\alpha$ GlcCer<sup>-</sup> and OCH<sup>-</sup> iNKT cells (Figure 5A-5B, Figure 5 -  
301 figure supplement 1A-B). Also, the TCRV $\beta$  usage shapes antigen binding as the proportion  
302 of V $\beta$ 7<sup>+</sup> and V $\beta$ 8.1/8.2<sup>+</sup> iNKT cells were skewed when comparing OCH<sup>+</sup> vs OCH<sup>-</sup> iNKT cells  
303 or  $\alpha$ GlcCer<sup>+</sup> vs  $\alpha$ GlcCer<sup>-</sup> iNKT cells (Figure 5C, Figure 5 -figure supplement 1C).  
304 Consequently, we detected a strong correlation between the percentage of V $\beta$ 8<sup>+</sup> iNKT cells  
305 and the binding to OCH or  $\alpha$ GlcCer (Figure 5D, Figure 5 -figure supplement 1D). Thus, this  
306 data indicates that the distinct TCR $\beta$  repertoire found in iNKT cells from various anatomical  
307 locations relates to their differential capacity for lipid antigen recognition.

308

309 It is well established that in conventional T cells there are age-dependent changes in the  
310 TCR repertoire that lead to impaired immune responses (Yager *et al.*, 2008). Hence, we  
311 analysed the effect of age in iNKT cells by measuring the TCRV $\beta$  usage and lipid-binding  
312 capacity for iNKT cells in the tissues of 2, 6 and 11-week-old WT C57BL/6 mice (Figure 5E-  
313 5F). We detected significant changes in TCRV $\beta$  usage associated to the age of the mice.  
314 For instance, the percentage of V $\beta$ 8.1/8.2<sup>+</sup> iNKT cells decreases over time in iLN and mLN,  
315 whereas the percentage of cells expressing *other* V $\beta$ s in those tissues increases (Figure  
316 5E). The TCR changes over-time are more evident in LNs in comparison with the spleen and  
317 the differences in TCRV $\beta$  usage for LN iNKT cells are more prominent in adult mice (6w vs  
318 11w) than in younger animals (2w vs 6w). Importantly, the changes in the frequency of  
319 V $\beta$ 8.1/8.2<sup>+</sup> iNKT cells in older mice correlate with the capacity of the cells to bind  $\alpha$ GlcCer-  
320 loaded CD1d tetramers (Figure 5F). Hence, in the LNs both the frequency of V $\beta$ 8.1/8.2<sup>+</sup>  
321 iNKT cells and that of  $\alpha$ GlcCer<sup>+</sup> cells decrease as mice age. Thus, this data demonstrates a  
322 differential lipid antigen recognition capacity for iNKT cells from various tissues, which is  
323 shaped by age.

324

325 **The iNKT cell TCR repertoire changes in response to immunisation and**  
326 **environmental challenges**

327 We next explored the stability of the peripheral iNKT cell TCR repertoire in response to  
328 antigenic challenges and environmental changes. To analyse whether immunisation with  
329 lipid antigens induces lasting changes in the iNKT TCR repertoire we injected mice with the  
330 lipid antigen OCH and followed the changes in the iNKT cell population at 3 or 13 days after  
331 immunisation (Figure 6A-6D). Three days after OCH administration we detected strong  
332 proliferation of iNKT cells in spleen and LNs, resulting in an increase in Ki-67 expression and  
333 in the frequency of iNKT cells in comparison with control (PBS injected) mice (Figure 6A-  
334 6C). The vast majority (~80%) of highly proliferative iNKT cells (Ki-67<sup>hi</sup>) expressed  
335 TCRV $\beta$ 8.1/8.2. As a result, we detected a global change in the TCRV $\beta$  usage for the local  
336 iNKT cell populations in comparison with control mice with reduced frequency of TCRV $\beta$ 7<sup>+</sup>  
337 iNKT cells which are replaced by TCRV $\beta$ 8.1/8.2<sup>+</sup> cells (Figure 6D). Thirteen days after OCH  
338 administration Ki-67 expression and iNKT cell frequency returned to basal levels (Figure 6B-  
339 6C). However, the TCR repertoire of the iNKT cell population was still significantly different  
340 from control mice with increased frequency of TCRV $\beta$ 8.1/8.2<sup>+</sup> and reduced TCRV $\beta$ 7<sup>+</sup> iNKT  
341 cells (Figure 6D). In line with these results, we also detected significant changes in the TCR  
342 repertoire of iNKT cells after immunisation with  $\alpha$ GalCer (Figure 6- figure supplement 1). In  
343 response to this lipid, the frequency of TCRV $\beta$ 8.1/8.2<sup>+</sup> iNKT cells increased at the expense  
344 of V $\beta$ other<sup>+</sup> cells while the frequency of V $\beta$ 7<sup>+</sup> cells remained unchanged (Figure 6- figure  
345 supplement 1). Interestingly, after antigen stimulation (both *in vitro* and *in vivo*) V $\beta$ 7<sup>+</sup> iNKT  
346 cells showed reduced secretion of cytokines in comparison with V $\beta$ 8.1/8.2<sup>+</sup> or V $\beta$ other<sup>+</sup> cells  
347 (Figure 6- figure supplement 2). Thus, all together this data indicates that the TCRV $\beta$  usage  
348 of iNKT cells is associated with their distinct activation and proliferation in response to  
349 antigen stimulation. As a result, exposure to lipid antigens induces a restructure in the  
350 repertoire of the iNKT cell population that is maintained even after proliferation has ceased  
351 and the population has contracted.

352

353 Commensal-derived products are known to modulate the numbers and phenotype of iNKT

354 cells (An *et al.*, 2014; Saez de Guinoa *et al.*, 2018; Wingender *et al.*, 2012). Thus, we  
355 hypothesized that changes in commensal bacteria could also lead to a restructure of the  
356 iNKT cell TCR repertoire. To address this, we treated 5-week old mice with antibiotics in the  
357 drinking water for 6 weeks and analysed the TCRV $\beta$  usage in iNKT cells in those animals at  
358 11 weeks of age (vs. control mice; Figure 6E). Interestingly, we found that antibiotic  
359 treatment resulted in a small but significant increase of the frequency of iNKT cells in mLN  
360 and iLN (Figure 6 - figure supplement 3). Moreover, we detected changes in the TCRV $\beta$   
361 usage of the iNKT cell population with a significant increase in the percentage of V $\beta$ 8.1/8.2<sup>+</sup>  
362 iNKT cells in the gut-draining mLN. Added to this, we also detected changes in TCRV $\beta$   
363 usage for mLN iNKT cells when we compared cells from the tissues of 6-week old germ-free  
364 (GF) animals with those of conventional (specific pathogen free, SPF) mice (Figure 6F). It is  
365 worth noting that changes in TCRV $\beta$  usage in iNKT cells were not identical in GF or  
366 antibiotic-treated mice, possibly due to the incomplete depletion of commensal bacteria and  
367 to the drastic changes in the surviving commensal populations induced by antibiotic  
368 treatment (Hill *et al.*, 2010). Thus, these data suggest that modifications in the intestinal  
369 microbiota lead to changes on the TCR repertoire of iNKT cells.

370

### 371 **Different phenotype and TCR repertoire for iNKT cells from human tonsils and blood**

372 Finally, we explored whether the differences in repertoire and phenotype found in murine  
373 iNKT cells resident in various tissues were also present in humans. To do this, we compared  
374 the phenotype and the TCRV $\alpha$  and TCRV $\beta$  usage of PBS57-binding iNKT cells from human  
375 blood and tonsils (Figure 7). The percentage of CD1d-tet-PBS57<sup>+</sup> iNKT cells (from total  
376 CD3<sup>+</sup> cells) in the blood was approximately 0.1%, around 10 times higher than the  
377 percentages of iNKT cells found in tonsils (Figure 7A). Co-staining with CD4 and CD8  
378 revealed variable populations of cells, including CD4<sup>+</sup>, CD8<sup>+</sup> and double-negative (DN) iNKT  
379 cells (Figure 7B). The proportion of each of these populations was variable amongst donors,  
380 but we detected a consistently higher frequency of CD4<sup>+</sup> iNKT cells within the tonsil  
381 population (mean= 72.4%) compared to blood (mean= 46.6%). Moreover, the expression of

382 the activation markers CD25 and CD69 was also variable, but we detected an increase in  
383 the proportion of CD69<sup>+</sup> iNKT cells within the tonsils (Figure 7C), confirming a different  
384 phenotype for iNKT cells found in tonsils vs. blood.

385

386 While the majority of human iNKT cells express the prototypical V $\alpha$ 24V $\beta$ 11 TCR,  
387 populations of *atypical NKT cells* have been found in human blood, representing up to 10%  
388 of the iNKT cell population (Le Nours *et al.*, 2016). These cells retain the capacity to bind  
389 CD1d-tet-PBS57 but express a range of TCR $\alpha$  and  $\beta$  chains that result in differential  
390 recognition of lipid antigens. Thus, we analysed the usage of V $\alpha$ 24 and V $\beta$ 11 within the  
391 iNKT cell populations from tonsils and blood (Figure 7D-7E). As previously described, we  
392 found that the majority of CD1d-tet-PBS57<sup>+</sup>V $\alpha$ 24<sup>+</sup> iNKT cells express TCRV $\beta$ 11 in both  
393 blood and tonsils. Similarly, the majority of CD1d-tet-PBS57<sup>+</sup>V $\beta$ 11<sup>+</sup> iNKT cells expressed  
394 V $\alpha$ 24. However, we found a variable proportion (0-10%) of iNKT cells lacking expression of  
395 V $\alpha$ 24 or V $\beta$ 11 in blood and tonsils. These *atypical iNKT cell* populations were found at  
396 significantly higher frequency in the tonsils in comparison to blood. For instance, V $\alpha$ 24<sup>-</sup>V $\beta$ 11<sup>+</sup>  
397 iNKT cells represent up to 10% of the iNKT cell population in tonsils while they comprised  
398 between 0 and 3% of iNKT cells in blood (Figure 7D-7E). Thus, all together this data  
399 demonstrates that the TCR repertoire and phenotype of human iNKT cells are distinct for  
400 cells found in different anatomical sites.

401

402

## 403 **DISCUSSION**

404 Tissue-resident iNKT cells are known to have unique properties and functions related to the  
405 tissues in which they reside, yet the antigen specificity of these populations in individual  
406 tissues remains unknown. Here, we found that the basal activation, proliferation, TCR  
407 repertoire and antigen specificity of peripheral iNKT cells are modulated by their anatomical  
408 location. While the TCR of  $\alpha$ GalCer-reactive NKT cells has been described as “invariant”,  
409 recent studies have identified variability in TCR V $\alpha$  and V $\beta$  chains in both the mouse and

410 human repertoires resulting in differential capacity for lipid antigen recognition (Cameron *et*  
411 *al.*, 2015; Le Nours *et al.*, 2016; Matsuda *et al.*, 2001). In agreement with this data, we found  
412 that the V $\beta$  and J $\beta$  usage, V $\beta$ -J $\beta$  pairing and CDR3 $\beta$  in iNKT cells are highly variable and  
413 relate to their capacity for lipid recognition. Thus, the so-called *invariant* NKT cell population  
414 expresses a variable TCRV $\beta$  repertoire that differs according to their anatomical location and  
415 results in differential antigen recognition capacity for iNKT cells resident in individual tissues.  
416 Importantly, our data confirms that anatomical differences also apply to human iNKT cells.  
417 Thus, we found increased frequencies of *atypical* iNKT cells (V $\alpha$ 24<sup>-</sup> or V $\beta$ 11<sup>-</sup>) in tonsils vs  
418 blood while the frequency of CD4<sup>+</sup> iNKT cells and CD69<sup>+</sup> iNKT cells was also different in  
419 those anatomical locations. This data is particularly relevant, as the vast majority of studies  
420 related to human iNKT cells are focused on cells isolated from the blood that may not fully  
421 recapitulate the phenotype and specificity of iNKT cells residing in peripheral tissues.

422

423 After selection in the thymus, peripheral CD4<sup>+</sup> T cell survival requires the expression of  
424 MHC. In the case of iNKT cells, while thymic development is CD1d-dependent, CD1d  
425 expression and the iNKT TCR have been proposed to be dispensable for the survival of  
426 peripheral iNKT cells (Matsuda *et al.*, 2002; Vahl *et al.*, 2013). However, CD1d-TCR signals  
427 are required for post-thymic maturation of iNKT cells in the periphery (e.g. NK1.1<sup>-</sup> to NK1.1<sup>+</sup>  
428 transition)(McNab *et al.*, 2005) and CD1d expression on hepatocytes and CD11c<sup>+</sup> cells  
429 regulates the phenotype and numbers of iNKT cells in liver and gut respectively (Saez de  
430 Guinoa *et al.*, 2018; Zeissig *et al.*, 2017). Thus, this suggests that while CD1d-TCR signals  
431 might not be required for iNKT cell survival, they can shape the peripheral iNKT cell  
432 population. Our data supports a model by which after selection in the thymus, the TCR  
433 repertoire of the iNKT cell population is subjected to further shaping in the periphery  
434 resulting in differential capacity for lipid recognition by iNKT cells resident in individual  
435 tissues. Another (but not exclusive) explanation for the tissue-skewed TCR repertoire could  
436 be that after thymus export the TCR of individual iNKT cells is linked to homing in specific  
437 tissues. However, it has been shown that the iNKT cell TCR specificity doesn't significantly

438 affect iNKT cell homing in the tissues. Accordingly, iNKT cells expressing a variety of  
439 TCRV $\beta$  chains and CDR3 $\beta$  sequences generated in retrogenic or transnuclear mice were  
440 found to home efficiently in a variety of tissues irrespectively of their TCR specificity or V $\beta$   
441 usage (Clancy-Thompson *et al.*, 2017; Cruz Tleugabulova *et al.*, 2016). In line with this, our  
442 own analyses of the iNKT cell CDR3 $\beta$  sequences did not reveal any obvious correlations  
443 between specific residues or their physicochemical properties and their tissue of origin.  
444 Thus, while our data doesn't preclude that after thymic egress certain iNKT cell clones may  
445 preferentially home in specific tissues, it supports a model in which local signals shape the  
446 TCR repertoire and specificity of the iNKT cell population after their arrival to the tissues.

447

448 Tissue-specific programming has been described for various tissue-resident immune cell  
449 populations including macrophages, innate lymphoid cells or Tregs whose properties are  
450 controlled by local tissue-derived signals (Miragaia *et al.*, 2019; Okabe & Medzhitov, 2014).  
451 In the case of iNKT cells, previous studies have shown that while cells from spleen and liver  
452 display relatively similar transcriptional programs, adipose tissue iNKT cells present a  
453 distinct transcriptional profile associated with a unique (PLZF-independent) developmental  
454 pathway (Lynch *et al.*, 2015). Our data shows a relatively similar transcriptome for iNKT cells  
455 isolated from thymus, spleen and LNs with only around 200-400 genes differentially  
456 expressed in these tissues (in contrast to the thousands of genes differentially expressed by  
457 adipose vs. splenic iNKT cells (Lynch *et al.*, 2015)). This suggests that while iNKT cells  
458 found in various lymphoid tissues likely share the same developmental program, local  
459 signals in the tissues in which they reside shape their phenotype and functions. Accordingly,  
460 iNKT cells isolated from LNs show differential expression of genes related to T cell  
461 activation, TCR signalling and proliferation indicating that in the LNs iNKT cells are  
462 recognising lipid antigens (e.g. endogenous or from commensals) that are not *seen* by iNKT  
463 cells residing in spleen or thymus in homeostatic conditions. Thus, it is feasible to speculate  
464 that the catalogue of (endogenous and exogenous) lipids presented by CD1d in specific  
465 tissues could contribute to shape the population of iNKT cells resident in those tissues.

466 Added to this, it is likely that other signals (such as cytokines or hormones) also contribute to  
467 modulate the phenotype and function of tissue-resident iNKT cells (Holzapfel *et al.*, 2014;  
468 Matsuda *et al.*, 2002).

469

470 The observation that the iNKT cell TCR repertoire is shaped by anatomical location  
471 resembles the unique repertoire observed for natural Tregs which is also shaped by  
472 environmental antigens possibly controlling Treg-mediated tolerance to the specific tissue  
473 environment (Lathrop *et al.*, 2011; Lathrop *et al.*, 2008). The tight control of CD1d-dependent  
474 lipid presentation and peripheral iNKT cell homeostasis are also critical to prevent local  
475 inflammation. Accordingly, dysregulation of intestinal iNKT cell homeostasis as a  
476 consequence of alteration in commensal lipids results in increased susceptibility to intestinal  
477 inflammation (An *et al.*, 2014; Olszak *et al.*, 2012; Wingender *et al.*, 2012). Also, CD1d-lipid  
478 presentation by hepatocytes controls peripheral induction of iNKT cell tolerance in the liver,  
479 protecting from hepatic inflammation (Zeissig *et al.*, 2017). Importantly, changes in the iNKT  
480 cell TCR repertoire have been also associated with autoimmune diseases as is the case in  
481 patients with diabetes (Tocheva *et al.*, 2017) and rheumatoid arthritis (Mansour *et al.*, 2015)  
482 and transgenic mice over-expressing an autoreactive NKT cell-TCR develop spontaneous  
483 colitis (Liao *et al.*, 2012). Thus, the local tissue-specific regulation of iNKT cell immunity may  
484 have important implications for the development and progression of autoimmune and  
485 inflammatory processes.

486

487 In summary, our study demonstrates that local signals shape the populations of tissue-  
488 resident lymphocytes and suggests that exposure to different immunisations, microbial  
489 infections or environmental changes can impact and shape the host's iNKT cell TCR  
490 repertoire. These findings may inform future development of novel therapies based on the  
491 manipulation of iNKT cells for vaccination or immunotherapy.

492

493



## 494 MATERIALS AND METHODS

## 495 Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
<i>strain, strain background (Mus musculus)</i>	Rag2-GFP: Tg(Rag2-EGFP)1Mnz	PMID: 10458165	MGI:3784416	
<i>strain, strain background (Mus musculus)</i>	Nur77-GFP: C57BL/6- Tg(Nr4a1- EGFP/cre)820Khog/J	PMID: 21606508	MGI:5007644	
<i>strain, strain background (Mus musculus)</i>	CD1d-KO: Del(3Cd1d2- Cd1d1)1SbpJ	PMID: 14632651	MGI:5582477	
antibody	PBS57-loaded CD1d- tetramer (mouse)	NIH Tetramer Core Facility	<a href="https://tetramer.verkes.emory.edu">https://tetramer.verkes.emory.edu</a>	(1: 1000)
antibody	anti-mouse B220 (rat monoclonal)	BioLegend	103224	(1:200)
antibody	anti-mouse CD8 $\alpha$ (rat monoclonal)	BioLegend	100714	(1:200)
antibody	anti-mouse CD11b (rat monoclonal)	BioLegend	101226	(1:200)
antibody	anti-mouse CD11c (armenian hamster monoclonal)	BioLegend	117323	(1:200)
antibody	anti-mouse PLZF (armenian hamster monoclonal)	BioLegend	145807	(1:200)
antibody	anti-mouse ROR $\gamma$ t (mouse monoclonal)	BD Biosciences	564722	(1:200)
antibody	anti-mouse T-bet (mouse monoclonal)	BioLegend	644823	(1:200)
antibody	anti-mouse TCR $\beta$ (armenian hamster monoclonal)	BioLegend	109233	(1:200)
antibody	anti-mouse V $\beta$ 7 (rat monoclonal)	BioLegend	118306	(1:200)
antibody	anti-mouse V $\beta$ 8.1/8.2 (rat monoclonal)	eBiosciences	46-5813-80	(1:200)
antibody	anti-mouse CCR7 (rat monoclonal)	BioLegend	120105	(1:100)
antibody	anti-mouse PD1 (rat monoclonal)	BioLegend	135219	(1:200)
antibody	anti-mouse Qa2 (mouse monoclonal)	BD Biosciences	743312	(1:200)
antibody	anti-mouse CD25 (rat monoclonal)	BioLegend	102015	(1:200)
antibody	anti-mouse Ki-67 (rat monoclonal)	BioLegend	652425	(1:200)
antibody	anti-mouse ICOS (rat monoclonal)	BioLegend	117405	(1:200)
antibody	anti-mouse CD4 (rat monoclonal)	BioLegend	100433	(1:200)
antibody	anti-mouse NK1.1 (mouse monoclonal)	eBiosciences	11-5941-85	(1:200)

antibody	anti-mouse CD27 (armenian hamster monoclonal)	BioLegend	124215	(1:200)
antibody	anti-mouse CCR6 (armenian hamster monoclonal)	BioLegend	129809	(1:200)
antibody	anti-mouse CD45.1 (mouse monoclonal)	BioLegend	110731	(1:200)
antibody	anti-mouse CD45.2 (mouse monoclonal)	BioLegend	109805	(1:200)
antibody	anti-mouse CCR8 (rat monoclonal)	BioLegend	150320	(1:200)
antibody	anti-mouse IL-4 (rat monoclonal)	BioLegend	504111	(1:200)
antibody	anti-mouse IFN- $\gamma$ (rat monoclonal)	BioLegend	505810	(1:200)
antibody	PBS57-loaded CD1d-tetramer (human)	NIH Tetramer Core Facility	<a href="https://tetramer.yerkes.emory.edu">https://tetramer.yerkes.emory.edu</a>	(1:500)
antibody	anti-human CD3 (mouse monoclonal)	BioLegend	300418	(1:200)
antibody	anti-human CD4 (rat monoclonal)	BioLegend	357415	(1:200)
antibody	anti-human CD8a (mouse monoclonal)	BioLegend	300913	(1:200)
antibody	anti-human CD25 (mouse monoclonal)	BioLegend	302613	(1:200)
antibody	anti-human CD69 (mouse monoclonal)	BioLegend	310921	(1:200)
antibody	anti-human V $\alpha$ 24 (mouse monoclonal)	BioLegend	360003	(1:200)
antibody	anti-human V $\beta$ 11 (human monoclonal)	Miltenyi Biotech	130-108-799	(1:200)
antibody	anti-human CD19 (mouse monoclonal)	BioLegend	302223	(1:200)
antibody	anti-human CD14 (mouse monoclonal)	BioLegend	325615	(1:200)
commercial assay or kit	Zombie (fixable viability dye)	BioLegend	423105	
commercial assay or kit	Dynabeads Biotin binder	Invitrogen	11047	
commercial assay or kit	Click-iT Plus EdU Flow-cytometry assay kit	Invitrogen	C10418	
chemical compound, drug	$\alpha$ GalCer ( $\alpha$ -Galactosylceramide, KRN7000)	Enzo Life Sciences	ALX-306-027	
chemical compound, drug	OCH	Enzo Life Sciences	ALX-306-029	
software, algorithm	MiXCR	Bolotin et al., 2013	<a href="https://mixcr.readthedocs.io/en/master/">https://mixcr.readthedocs.io/en/master/</a>	
software, algorithm	Weblogo	Crooks et al., 2004	<a href="https://weblogo.berkeley.edu">https://weblogo.berkeley.edu</a>	
software, algorithm	Brepertoire	Margreitter et al., 2018	<a href="http://mabra.biomed.kcl.ac.uk/BRepertoire_5/?">http://mabra.biomed.kcl.ac.uk/BRepertoire_5/?</a>	

496

497

498

499 **Mice**

500 CD1d-KO (on C57BL/6 background), WT C57BL/6, congenic CD45.1 WT C57BL/6 and  
501 Nur77<sup>GFP</sup> mice were bred under specific pathogen-free (SPF) conditions at the Francis Crick  
502 Institute. Nur77<sup>GFP</sup> mice were provided by Adrian Hayday (Francis Crick Institute). Rag2<sup>GFP</sup>  
503 mice were bred at the University of Birmingham. Tissues from germ-free mice were obtained  
504 from the Wellcome Trust Sanger Institute (Cambridge, UK). All animal experiments were  
505 approved by the Francis Crick Institute and the King's College London's Animal Welfare and  
506 Ethical Review Body and the United Kingdom Home Office.

507

508 **Human tissues**

509 Human tissues used in this study were collected with ethical approval from UK Research  
510 Ethics Committees administered through the Integrated Research Application System. All  
511 samples were collected with informed consent. Mononuclear cells from peripheral blood and  
512 tonsils were isolated as previously described (Zhao *et al.*, 2018) and cryopreserved before  
513 use.

514

515 **Murine tissue preparation**

516 Spleen, thymus and liver were harvested and smashed through a 45- $\mu$ m strainer to obtain  
517 single-cell suspensions before staining for flow-cytometry. LNs and lung were harvested and  
518 briefly digested (15 min at 37°C) with collagenase (1.5 mg/ml), DNase (100  $\mu$ g/ml) and  
519 NADase (6mg/ml) before filtering through a 45- $\mu$ m strainer. SI-LP was processed as  
520 described (Saez de Guinoa *et al.*, 2017). Briefly, small intestine (excluding Peyer's patches)  
521 was flushed with cold PBS, opened longitudinally and incubated for 20 min at 37°C in HBSS  
522 1 mM EDTA, 5% FCS. The supernatant containing epithelial cells and intraepithelial  
523 lymphocytes was discarded and the remaining tissue was incubated for 45 min at 37°C with  
524 collagenase and DNase as above and filtered through 45- $\mu$ m strainer. Cells from all the

525 tissues were resuspended in FACS buffer (PBS 1% BSA, 1% FCS) for flow-cytometry  
526 staining.

527

### 528 **Flow-cytometry**

529 Flow cytometry staining of mouse and human samples were performed in FACS buffer using  
530 the following antibodies from Biolegend or eBioscience unless specified otherwise: Anti-  
531 mouse antibodies: CD45R/B220 (RA 3-6B2), CD8 $\alpha$  (56-6.7), CD11b (M1/70), CD11c  
532 (N418), PLZF (9E12), ROR $\gamma$ t (Q31-378 BD Biosciences), T-bet (4B10), TCR $\beta$  (H57-587),  
533 V $\beta$ 7 (TR310), V $\beta$ 8.1/8.2 (KJ16), PD-1 (29F.1A12), CCR7 (4B12), Qa2 (1-1-2, BD  
534 Biosciences), CD25 (PC-61), Ki-67 (16A8), CD45.1 (A20), CD45.2 (104), ICOS (7E.17G9),  
535 CD4 (GK1.5), NK1.1 (PK136), CD27 (LG.3A10), CCR6 (29-2L17), CCR8 (SA214G2), IL-4  
536 (11B11), IFN- $\gamma$  (XMG1.2). Anti-human antibodies: CD3 (HIT3a), CD4 (A161A1), CD8 $\alpha$   
537 (HIT8a), CD25 (BC96), CD69 (FN50), V $\alpha$ 24 (C15/TCR V $\alpha$ 24), V $\beta$ 11 (REA559, Miltenyi  
538 Biotech), CD19 (HIB19), CD14 (HCD14). PBS57-loaded mouse and human CD1d tetramers  
539 were provided by the NIH Tetramer Core Facility. Incubations were performed on ice except  
540 for CCR7 staining in which cells were incubated with antibodies for 45 min at 37 °C. For  
541 transcription factor staining, cells were fixed and permeabilised with Foxp3/Transcription  
542 Factor Staining Buffer Set (eBioscience). For intracellular cytokine staining, cells were fixed  
543 and permeabilised with Fixation/Permeabilization Solution Kit (BD Biosciences). Dead cells  
544 were detected with Zombie fixable viability kit (Biolegend). Flow-cytometry data were  
545 collected on a Fortessa or Fortessa X20 flow cytometers (both from BD Biosciences) and  
546 were analysed with FlowJo software (TreeStar).

547

### 548 **Administration of antibiotics and EdU incorporation**

549 Mice were orally administrated a cocktail of antibiotics: 1 mg/ml Ampicillin, 1 mg/ml  
550 Gentamicin, 1 mg/ml Neomycin, 1 mg/ml Metronidazole and 0.5 mg/ml Vancomycin (all from  
551 Sigma-Aldrich) in filtered drinking water for 6 weeks (Jimeno *et al.*, 2018).

552 For EdU incorporation experiments mice were injected intraperitoneally with 500 µg of EdU  
553 for 2 consecutive days and EdU incorporation was detected with Click-iT Plus EdU Flow-  
554 cytometry assay kit (Life Technologies).

555

#### 556 **Lipids and tetramer loading**

557 CD1d tetramers were provided by the NIH Tetramer Core Facility. αGalCer and OCH were  
558 obtained from Enzo Life sciences. βGalactosylCeramide (C12; βGalCer) was from Avanti  
559 Polar Lipids. αGlucosylCeramide (C26; αGlcCer) and βGlucosylCeramide (C14; βGlcCer)  
560 were produced in house (University of Birmingham). Lipids were dissolved in 0.5% v/v  
561 Tyloxapol (Sigma) and loaded into CD1d at a three to six-fold molar excess.

562

#### 563 **Stimulation with lipid antigens and adoptive transfer**

564 For *in vivo* experiments mice were injected intravenously with 5 µg of OCH or 1 µg of  
565 αGalCer and sacrificed at the indicated time-points.

566 For *in vitro* stimulation experiments, single cell suspensions from the spleen were prepared  
567 as described above. Cells were cultured in complete media (IMDM, 10% FCS) and  
568 stimulated for 2.5 h in the presence of 5µg/mL αGalCer at 37°C. Brefeldin A (Biolegend) was  
569 added for the last 2 h of the incubation period. Cells were stained for detection of  
570 intracellular cytokines as described above.

571 For adoptive transfer experiments, donor mice were injected with anti-ARTC2 nanobody  
572 (Treg-protector, Biolegend) 15 min before tissue harvesting. Single-cell suspension from  
573 spleen and thymus were prepared and red blood cells were lysed by incubation with lysis  
574 buffer. Next, cells were incubated with biotinylated anti-B220 antibody followed by  
575 Dynabeads biotin binder magnetic beads (Invitrogen) according to the manufacturer's  
576 instructions. Cells were resuspended in PBS and injected intravenously into congenic  
577 recipient mice. Tissues were harvested for analyses 12 days after transfer.

578

579 **RNA sequencing**

580 RNA was extracted from sort-purified iNKT cells (TCR $\beta^+$ CD1d-tet-PBS57 $^+$ B220 $^-$ CD11b $^-$   
581 CD11c $^-$ CD8 $^+$ ) from the indicated tissues of WT mice using the RNAeasy micro kit (Qiagen)  
582 following manufacturer instructions. Library generation was performed according to  
583 manufacturer instructions using the Nugen Ovation ultralow kit. Libraries were barcoded and  
584 run on an Illumina HiSeq 2500 system with paired-end read lengths of 101 bp. Fastq files  
585 were trimmed using Cutadapt with a quality threshold of 10 before being aligned to and  
586 quantified against release GRCm38.p6 of the mouse genome with RSEM/Bowtie2. The raw  
587 counts were then imported into R/Bioconductor. DESeq2 was used to account for the  
588 different size factors between samples, and a model with main effects of tissue and sample-  
589 batch was used to find genes that were differentially expressed (false discovery threshold of  
590 0.01) either between pairs of tissues (Wald test) or not constant across all tissues (likelihood  
591 ratio test). The RNAseq data are available in the Gene Expression Omnibus (GEO)  
592 database with accession number GSE131420

593

594 **Analyses of TCR sequences**

595 MiXCR software was used to identify TCR sequences within the RNAseq data (Bolotin *et al.*,  
596 2013). The software performs CDR3 extraction, identifies V, D and J segments, assembles  
597 clonotypes, filters out or rescues low-quality reads. The obtained repertoires were further  
598 filtered to eliminate out-of-frame and stop codon-containing CDR3 variants. CDR3 length,  
599 CDR3 physicochemical properties (hydrophobicity (based on the Kyte–Doolittle scale (Kyte  
600 & Doolittle, 1982)), isoelectric point (pI, according to EMBOSS (Rice *et al.*, 2000)), frequency  
601 of polar (D, E, H, K, N, Q, R, S, T), aliphatic (A, I, L, V) or aromatic (F, H, W, Y) residues),  
602 CDR3 clonal size and V-J pairings were calculated using Brepertoire (Margreitter *et al.*,  
603 2018). CDR3 $\alpha$  and CDR3 $\beta$  sequence logos were generated on the Weblogo server (Crooks  
604 *et al.*, 2004) (<https://weblogo.berkeley.edu>) and provide a visual representation of amino  
605 acids enriched at different positions in the CDR3 sequences.

606

## 607 **Statistical analyses**

608 Statistical analyses were performed using Prism software (GraphPad). Unless specified  
609 otherwise, *n* represents the number of individual mice analysed in each experiment.

610 Statistical significance was determined using paired or unpaired two-tailed student's t test or  
611 ANOVA with multiple comparison Tukey test as specified in the figure legends. Correlation  
612 analyses were performed using Pearson correlation.

613

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620

621

622 The authors declare that they have no competing financial interests.

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## 628 **FIGURE LEGENDS**

629 **Figure 1. Different TCRV $\beta$  usage and clonal expansion in iNKT cells from several**  
630 **lymphoid tissues**

631 **(A)** Flow cytometry plots showing gating strategy (left) and quantification (right) for iNKT

632 cells expressing V $\beta$ 7, V $\beta$ 8.1/8.2 or V $\beta$ other (non-V $\beta$ 7 or V $\beta$ 8.1/8.2) in the depicted tissues of

633 WT C57BL/6. Bars represent mean +/- SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$   
634 paired  $t$ -test.  $n = 15$  mice from 5 independent experiments.

635 **(B)** Frequency of TRBV gene usage in iNKT cell TCR sequences. Frequencies are  
636 calculated from RNAseq data from 4 samples per tissue. Bars represent mean +/- SEM. \* $p <$   
637  $0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , ANOVA with Tukey's multiple comparison test.

638 **(C)** Gene usage plot (2D) showing TRAV-TRAJ (top) and TRBV-TRBJ (bottom) pairing for  
639 total TCR sequences obtained from iNKT cells isolated from the depicted tissues of WT  
640 mice. The circle size represents the percentage of sequences with each specific V-J pairing  
641 from the total TCR sequences for each tissue.

642 **(D)** Visual representation for aa enrichment at each position for CDR3 $\alpha$  (top) and CDR3 $\beta$   
643 (bottom) sequences for iNKT cells (pooled from all tissues). Analyses were performed with  
644 sequences of 15 or 14 aa for CDR3 $\alpha$  and CDR3 $\beta$  respectively. Graphics were generated  
645 with Weblogo.

646 **(E)** Median value for counts for CDR3 $\beta$  sequences identified in the depicted tissues. Data  
647 obtained from RNAseq and pooled from 4 samples per tissue. Data have been calculated  
648 using the counts for each CDR3 $\beta$  sequence and expressed as box-and-whisker diagrams  
649 depicting the median +/- lower quartile, upper quartile, sample minimum and maximum.  
650 \*\*\*\* $p < 0.0001$  Mann-Whitney test.

651 **(F)** Frequency of CDR3 $\beta$  clonotype usage in relation to the repertoire size for iNKT cells  
652 isolated from the depicted tissues (data obtained from RNAseq and pooled from 4 samples  
653 per tissue). Frequency of CDR3 $\beta$  sequences present once, twice, 3, 4 or 5 or more times  
654 are shown.

655 **(G)** Cumulative frequencies occupied by the 25 most prevalent CDR3 $\beta$  clonotypes for iNKT  
656 cells isolated from the depicted tissues. Data has been calculated for sequences from 4  
657 samples per tissue and represented as mean +/- SEM. \* $p < 0.05$ , paired  $t$ -test.

658

659 **Figure 2. Distinct TCRV $\beta$  usage for iNKT RTE**



660 **(A-B)** iNKT RTE were identified as GFP<sup>+</sup> cells in the tissues of RAG2<sup>GFP</sup> mice (6-9  
661 weeks/old). **(A)** Flow-cytometry (left) and quantification (right) showing the percentage of  
662 RAG2<sup>GFP+</sup> iNKT cells and the MFI for the GFP expression in RTE in the depicted tissues.  
663 **(B)** Frequency of Vβ7 or Vβ8.1/8.2-expressing GFP<sup>+</sup> and GFP<sup>-</sup> iNKT cells from the tissues of  
664 RAG2<sup>GFP</sup> mice.  
665 Bars represent mean +/- SEM. \**p*< 0.05, \*\**p*< 0.01, \*\*\*\**p*<0.0001 two-tailed unpaired (A) or  
666 paired *t*-test (B). n= 6 mice from 2 independent experiments.

667

### 668 **Figure 3. Increased activation and proliferation of LN iNKT cells**

669 **(A)** Plots show differentially expressed genes for pairwise comparisons from iNKT cells from  
670 the depicted tissues. A fold change cut-off of 1.5 and adjusted *p*-value cut off of 0.01 were  
671 applied to colour code differentially expressed genes on the plot (red). The numbers of  
672 differentially expressed genes are indicated in the graphs.

673 **(B)** Heat map showing RNAseq analyses of selected transcripts significantly changed in  
674 iNKT cells from thymus, mLN or iLN versus spleen. n=4 samples.

675 **(C)** Top, Representative flow-cytometry histograms showing GFP expression in iNKT cells  
676 (grey) and T cells (blue) from the depicted tissues of Nur77<sup>GFP</sup> mice. iNKT cells from the  
677 tissues of Nur77<sup>GFP-</sup> mice are shown as control (empty profile). Bottom, representative flow-  
678 cytometry plots showing CD25, ICOS and CCR8 expression in iNKT cells from the tissues of  
679 WT mice (grey) and control (blue).

680 **(D-E)** Ki-67 expression in iNKT cells and T cells from the depicted tissues of WT mice. Ki-67  
681 MFI for iNKT cells (E) is related to T cells from each tissue. Bars represent mean +/- SEM.  
682 \**p*< 0.05, \*\**p*< 0.01, two-tailed unpaired *t*-test. n=5 mice from 2 independent experiments.

683 **(F)** Quantification of EdU incorporation for iNKT cells from the depicted tissues after 48h of  
684 EdU administration *in vivo*. Bars represent mean +/- SEM. \*\**p*< 0.01, \*\*\*\**p*<0.0001 two-tailed  
685 unpaired *t*-test. n=6 mice from 3 independent experiments.

686 **(G)** Representative flow-cytometry plot showing EdU incorporation in iNKT cells (left) and  
687 frequency of V $\beta$ 7 or V $\beta$ 8.1/8.2-expressing EdU<sup>+</sup> and EdU<sup>-</sup> iNKT cells (right). n=4 mice  
688 **(H)** Heat map representation of the frequency of TRBV amongst low-abundance (1 copy) or  
689 more abundant (>2 copies) CDR3 $\beta$  sequences obtained from iLN and mLN as indicated.  
690 Data obtained from RNAseq and pooled from 4 samples per tissue.

691

692 **Figure 4. The tissue of origin dictates the basal activation and TCR $\beta$  repertoire of all**  
693 **iNKT subsets**

694 **(A)** Analysis of iNKT cell populations in the tissues of WT C57BL/6 mice, showing flow-  
695 cytometry plots (A, left) and frequency (A, right) of NKT1 (ROR $\gamma$ t<sup>+</sup>PLZF<sup>lo</sup>T-bet<sup>+</sup>), NKT2  
696 (ROR $\gamma$ t<sup>-</sup>PLZF<sup>hi</sup>T-bet<sup>-</sup>) and NKT17 (PLZF<sup>int</sup>ROR $\gamma$ t<sup>+</sup>) cells. n=10 mice from 4 independent  
697 experiments

698 **(B)** Top, Representative flow-cytometry plots showing Ki-67, CD25 and ICOS expression in  
699 NKT1, NKT2 and NKT17 cells from the depicted tissues. Subpopulations were identified as  
700 in (A). Bottom, GFP expression in iNKT cell subsets from the depicted tissues from Nur77<sup>GFP</sup>  
701 mice. iNKT cell subsets were identified as described (Engel *et al.*, 2016): NKT1  
702 (CD27<sup>+</sup>NK1.1<sup>+</sup>), NKT2 (CD27<sup>+</sup>, NK1.1<sup>-</sup>, CD1d-Tet<sup>hi</sup>, CD4<sup>+</sup>), NKT17 (CD27<sup>-</sup>, CD4<sup>-</sup>, CCR6<sup>+</sup>).

703 **(C)** Frequency of V $\beta$ 7- or V $\beta$ 8.1/8.2-expressing iNKT cells within the NKT1 (top), NKT2  
704 (middle) or NKT17 (bottom) populations in the depicted tissues of WT mice. n=10 mice from  
705 4 independent experiments. Bars represent mean +/- SEM. \* $p$ < 0.05, \*\* $p$ < 0.01, \*\*\* $p$ < 0.001,  
706 \*\*\*\* $p$ <0.0001 paired  $t$ -test

707

708 **Figure 5. Differential lipid antigen recognition for iNKT cells from various lymphoid**  
709 **tissues**

710 **(A-D)** iNKT cells from the depicted tissues were co-stained with CD1d-tet-PBS57 and CD1d-  
711 tet- $\alpha$ GlcCer or CD1d-tet-OCH. **(A)** Flow-cytometry profiles. **(B)** Quantification of  $\alpha$ GlcCer<sup>-</sup>  
712 (left) or OCH<sup>-</sup> (right) iNKT cells. Bars represent mean +/- SEM. \* $p$ < 0.05, \*\* $p$ < 0.01, \*\*\* $p$ <

713 0.001, two-tailed paired *t*-test. **(C)** V $\beta$  usage for the depicted iNKT cell populations from the  
714 spleen. **(D)** Frequency of  $\alpha$ GlcCer<sup>+</sup> (left) or OCH<sup>+</sup> (right) iNKT cells was related to the  
715 frequency of V $\beta$ 8.1/8.2 usage for each sample. Pearson correlation analyses are shown for  
716 each graph. n=6-8 mice from 3-4 independent experiments.  
717 **(E-F)** TCR V $\beta$  repertoire and lipid antigen recognition of iNKT cells from the depicted tissues  
718 were analysed at indicated time points (weeks of age). **(E)** Frequency of iNKT cells  
719 expressing V $\beta$ 7, V $\beta$ 8.1/8.2 or V $\beta$ other (no V $\beta$ 7 or V $\beta$ 8.1/8.2) in the tissues of WT C57BL/6  
720 mice of 2, 6 or 11 weeks of age. n= 10-15 mice from 10 independent experiments. **(F)** Flow-  
721 cytometry profiles (left) and quantification of  $\alpha$ GlcCer<sup>-</sup> iNKT cells (right) for iNKT cells from  
722 the tissues of WT mice of 2 or 11 weeks of age as depicted. n=3 mice from 2 independent  
723 experiments. Bars represent mean +/- SEM. \**p*< 0.05, \*\**p*< 0.01, \*\*\**p*< 0.001, \*\*\*\**p*<0.0001  
724 two-tailed unpaired *t*-test.

725

726 **Figure 6. TCR repertoire of iNKT cells changes following immunisation and**  
727 **environmental challenges**

728 **(A-D)** Mice were injected with OCH (or PBS as control, c) and iNKT cells from spleen and  
729 lymph nodes were analysed 3 or 13 days later. **(A)** Flow-cytometry profiles showing Ki-67  
730 expression in all iNKT cells (left) and V $\beta$ 7, V $\beta$ 8.1/8.2 expression for iNKT cells expressing  
731 high (Ki-67<sup>hi</sup>, right) or low (Ki-67<sup>lo</sup>, middle) Ki-67 3 days after OCH administration. **(B-D)** Bar  
732 plots showing expression of Ki-67 **(B)**, frequency of iNKT cells **(C)** and percentage of iNKT  
733 cells expressing V $\beta$ 7 or V $\beta$ 8.1/8.2 **(D)** at the depicted time points in spleen (yellow), mLN  
734 (blue) or iLN (red). n=3-5 mice from 2-3 independent experiments.

735 **(E-F)** Frequency of iNKT cells expressing V $\beta$ 7 or V $\beta$ 8.1/8.2 in the tissues of mice (Thymus=  
736 grey; Spleen= yellow; mLN= blue; iLN= red) treated with antibiotics in the drinking water vs  
737 control mice **(E)** or SPF vs GF mice **(F)**. n=10 mice (E) and n=6 mice (F) from 2 independent  
738 experiments.

739 **(A-F)** Bars represent mean +/- SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\* $p < 0.0001$  two-  
740 tailed unpaired  $t$ -test.

741

742 **Figure 7. Distinct phenotype and TCR repertoire for human iNKT cells from different**  
743 **anatomical locations.**

744 **(A)** Mean percentage of iNKT cells (CD1d-tet-PBS57<sup>+</sup>CD3<sup>+</sup>) from total CD3<sup>+</sup>B220<sup>-</sup>CD14<sup>-</sup>  
745 cells in blood or tonsils.

746 **(B-C)** Flow-cytometry (left) and quantification (right) showing the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>  
747 and DN cells **(B)** or CD69<sup>+</sup> and CD25<sup>+</sup> cells **(C)** within the iNKT cell population in the  
748 depicted tissues.

749 **(D-E)** Flow-cytometry (left) and quantification (right) showing the percentage of V $\beta$ 11<sup>-</sup> iNKT  
750 cells within CD1d-tet-PBS57<sup>+</sup>V $\alpha$ 24<sup>+</sup> cells **(D)**; or the percentage of V $\alpha$ 24<sup>-</sup> cells within CD1d-  
751 tet-PBS57<sup>+</sup>V $\beta$ 11<sup>+</sup> cells **(E)**

752 **(A-E)** Data are expressed as box-and-whisker diagrams depicting the median +/- lower  
753 quartile, upper quartile, sample minimum and maximum. \* $p < 0.05$ , \*\*\*  $p < 0.001$ , two-tailed  
754 unpaired  $t$ -test. n=10 samples per tissue from 4 independent experiments.

755

756

## 757 SUPPLEMENTARY FIGURE LEGENDS

758 **Figure 1 - figure supplement 1. Tissue-dependent bias for TCRV $\beta$  usage in iNKT cells**

759 **(A)** Flow cytometry profiles showing iNKT cells (TCR $\beta$ <sup>+</sup>CD1d-tet-PBS57<sup>+</sup>) in the depicted  
760 tissues of WT and CD1d-KO mice. Numbers indicate percentage of iNKT cells within TCR $\beta$ <sup>+</sup>  
761 cells. **(B)** Quantification of iNKT cells expressing V $\beta$ 7, V $\beta$ 8.1/8.2 or V $\beta$ other (non-V $\beta$ 7 or  
762 V $\beta$ 8.1/8.2) in the depicted tissues of WT C57BL/6 mice. Bars represent mean +/- SEM. \* $p <$   
763 0.05, \*\* $p < 0.01$ , \*\*\*  $p < 0.001$  two-tailed unpaired  $t$ -test. n = 7 mice from 3 independent  
764 experiments. **(C)** iNKT cells enriched from spleen and thymus were adoptively transferred  
765 into congenic mice and TCRV $\beta$  usage in iNKT cells homing to various tissues was analysed

766 12 days later. Flow-cytometry plots show expression of V $\beta$ 7 and V $\beta$ 8.1/8.2 in iNKT cells from  
767 donor (CD45.2<sup>+</sup>) and recipient (CD45.1<sup>+</sup>CD45.2<sup>+</sup>) mice before or 12 days after transfer. Bar  
768 plots show frequency of donor and recipient iNKT cells expressing V $\beta$ 7 or V $\beta$ 8.1/8.2 in the  
769 depicted tissues. Bars represent mean  $\pm$  SEM. n=5 recipient mice from 2 independent  
770 experiments. \* $p$ < 0.05, \*\* $p$ < 0.01, \*\*\*  $p$ < 0.001 two-tailed unpaired  $t$ -test

771

772 **Figure 1 - figure supplement 2. Frequency of TRBV and TRAV gene usage in iNKT cell**

773 **TCR sequences. (A)** Total number of TRAV and TRBV sequences and percentages of  
774 unique sequences obtained from cells on the indicated tissues. **(B)** Heat map representation  
775 of the frequency of TRAV, TRAJ, TRBV and TRBJ sequences for iNKT cells isolated from  
776 the depicted tissues. Data obtained from RNAseq and pooled from 4 samples per tissue.

777

778 **Figure 1 - figure supplement 3. Physicochemical properties of CDR3 $\beta$  sequences.**

779 Median values for length, hydrophobicity, pI, and frequency of polar, aromatic or aliphatic aa  
780 in CDR3 $\beta$  sequences identified in the depicted tissues. Data are expressed as box-and-  
781 whisker diagrams depicting the median  $\pm$  lower quartile, upper quartile, sample minimum  
782 and maximum. Data obtained from RNAseq and pooled from 4 samples per tissue.

783

784 **Figure 2 - figure supplement 1. TCRV $\beta$  usage for iNKT cell precursors. (A)** iNKT cell

785 precursors were identified by flow-cytometry (left top) as CCR7<sup>+</sup>PD-1<sup>-</sup> cells. Q2a expression  
786 in iNKT cell precursors (empty profile) and non-precursors (grey, rest) is shown (left bottom).

787 Bar plot shows quantification of CCR7<sup>+</sup>PD-1<sup>-</sup> iNKT cells in the depicted tissues (right). \*\*\*\* $p$ <

788 0.0001, two-tailed unpaired paired  $t$ -test. n= 5 mice from 3 independent experiments. **(B)**

789 Flow-cytometry plots showing expression of CCR7, PD1, Qa2 and GFP in iNKT cells from

790 Rag2-GFP mice. **(C)** Frequency of V $\beta$ 7 or V $\beta$ 8.1/8.2-expressing iNKT cell precursors

791 (CCR7<sup>+</sup>PD-1<sup>-</sup>) and non-precursors (rest) from the tissues of WT mice. \* $p$ < 0.05, \*\* $p$ < 0.01,

792 two-tailed paired  $t$ -test. n= 5 mice from 3 independent experiments.

793

794 **Figure 3 - figure supplement 1. Gene expression analyses for iNKT cells from various**  
795 **tissues. (A)** Differentially expressed genes upregulated (red) or downregulated (blue) in  
796 iNKT cells from LNs vs. iNKT cells from spleen/thymus. A fold change cut-off of 1.5 and  
797 adjusted  $p$ -value cut off of 0.01 were applied to colour code differentially expressed genes  
798 on the plot. **(B)** Functional enrichment analysis of genes upregulated in LN iNKT cells (vs  
799 spleen/thymus iNKT; top) or in spleen/thymus iNKT cells (vs LN iNKT; bottom). The GO  
800 terms are shown for both sets of genes ranked by  $p$  values and fold-enrichments are  
801 depicted on the graph. Enrichment and  $p$  values (from a Fisher's exact test with Bonferroni  
802 correction) were calculated with PANTHER tools. **(C)** Frequency of  $V\beta 7$  or  $V\beta 8.1/8.2$ -  
803 expressing  $EdU^+$  or  $EdU^-$  iNKT cells from mLN (blue) or iLN (red).  $n=4$  mice from 2  
804 experiments.  $*p < 0.05$ , two-tailed paired  $t$ -test.

805

806 **Figure 4 - figure supplement 1. TCRV $\beta$  usage for iNKT cell subpopulations.** (Top)  
807 Analysis of iNKT cell populations in the tissues of WT C57BL/6 mice, showing flow-  
808 cytometry plots (left) and frequency (right) of  $CD4^+NK1.1^-$ ,  $NK1.1^+CD4^-$ ,  $CD4^+NK1.1^+$  or  $CD4^-$   
809  $NK1.1^-$  iNKT cells. (Bottom) Frequency of  $V\beta 7^-$  or  $V\beta 8.1/8.2$ -expressing iNKT cells within the  
810  $CD4^+NK1.1^-$ ,  $NK1.1^+CD4^-$ ,  $CD4^+NK1.1^+$  or  $CD4^-NK1.1^-$  populations in the depicted tissues of  
811 WT mice.  $n=5$  mice from 2 independent experiments. Bars represent mean  $\pm$  SEM.  $*p <$   
812  $0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ , ANOVA with Tukey's multiple comparisons test.

813

814 **Figure 5 - figure supplement 1. Differential lipid antigen recognition for iNKT cells**  
815 **from non-lymphoid tissues (A-D)** iNKT cells from liver, lung or SI-LP were co-stained with  
816  $CD1d$ -tet-PBS57 and  $CD1d$ -tet- $\alpha$ GlcCer or  $CD1d$ -tet-OCH. **(A)** Flow-cytometry profiles. **(B)**  
817 Quantification of  $\alpha$ GlcCer $^-$  (left) or OCH $^-$  (right) iNKT cells. Bars represent mean  $\pm$  SEM.  
818  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , two-tailed paired  $t$ -test.  $n=4$  mice from 2 independent  
819 experiments. **(C)**  $V\beta$  usage for the depicted iNKT cell populations from the liver. **(D)**

820 Frequency of  $\alpha$ GlcCer<sup>+</sup> (left) or OCH<sup>+</sup> (right) iNKT cells was related to the frequency of  
821 V $\beta$ 8.1/8.2 usage for each sample. Pearson correlation analyses are shown for each graph.

822

823 **Figure 6 - figure supplement 1. Changes in the iNKT cell TCR repertoire following**  
824 **immunisation with  $\alpha$ GalCer.** Mice were injected with  $\alpha$ GalCer (or PBS as control) and  
825 iNKT cells from spleen and lymph nodes were analysed 3 days later. **(A)** Flow-cytometry  
826 profiles showing Ki-67 expression in all iNKT cells (left) and V $\beta$ 7, V $\beta$ 8.1/8.2 expression for  
827 iNKT cells expressing high (Ki-67<sup>hi</sup>, right) or low (Ki-67<sup>lo</sup>, middle) Ki-67 3 days after  $\alpha$ GalCer  
828 administration. **(B-D)** Bar plots showing expression of Ki-67 **(B)**, frequency of iNKT cells **(C)**  
829 and percentage of iNKT cells expressing V $\beta$ 7, V $\beta$ 8.1/8.2 or V $\beta$ other **(D)** at the depicted time  
830 points in spleen (yellow), mLN (blue) or iLN (red). n=3 mice from 2 independent  
831 experiments. Bars represent mean +/- SEM. \* $p$ < 0.05, \*\* $p$ < 0.01, \*\*\*  $p$ < 0.001, \*\*\*\* $p$ <0.0001  
832 two-tailed unpaired  $t$ -test.

833

834 **Figure 6 - figure supplement 2. Cytokine secretion by iNKT cells relates to**  
835 **TCRV $\beta$  usage.** Splenocytes were stimulated *in vitro* with  $\alpha$ GalCer (left, *in vitro*) or WT mice  
836 were injected *in vivo* with  $\alpha$ GalCer (right, *in vivo*) and cytokine production was measured by  
837 intracellular staining 2h later. Flow cytometry graphs and quantification (bar plots) showing  
838 the frequency of IL-4 or IFN- $\gamma$ -producing iNKT cells within the V $\beta$ 7<sup>+</sup>, V $\beta$ 8<sup>+</sup> or V $\beta$ other<sup>+</sup>  
839 populations. n=7 mice (*in vitro*) and n=4 mice (*in vivo*). Bars represent mean +/- SEM. \* $p$ <  
840 0.05, \*\* $p$ < 0.01, \*\*\* $p$ < 0.001, ANOVA with Tukey's multiple comparisons test.

841

842

843 **Figure 6 - figure supplement 3. Frequency of iNKT cells after antibiotic treatment.**

844 Frequency of iNKT cells in the tissues of mice (Thymus= grey; Spleen= yellow; mLN= blue;  
845 iLN= red) treated with antibiotics in the drinking water vs control mice. n=10 mice from 2

846 independent experiments. Bars represent mean +/- SEM. \*\* $p < 0.01$  two-tailed unpaired  $t$ -  
847 test.

848

## 849 SOURCE DATA FILES

850 **Figure 1- source data 1.** Different TCRV $\beta$  usage and clonal expansion in iNKT cells from  
851 several lymphoid tissues

852 **Figure 2- source data 1.** Distinct TCRV $\beta$  usage for iNKT RTE

853 **Figure 3- source data 1.** Increased activation and proliferation of LN iNKT cells

854 **Figure 4- source data 1.** The tissue of origin dictates the basal activation and TCR $\beta$   
855 repertoire of all iNKT subsets

856 **Figure 5- source data 1.** Differential lipid antigen recognition for iNKT cells from various  
857 lymphoid tissues

858 **Figure 6- source data 1.** TCR repertoire of iNKT cells changes following immunisation and  
859 environmental challenges

860 **Figure 7- source data 1.** Distinct phenotype and TCR repertoire for human iNKT cells from  
861 different anatomical locations.

862

863 **Figure 1 - figure supplement 1- source data 1.** Tissue-dependent bias for TCRV $\beta$  usage  
864 in iNKT cells

865 **Figure 1 - figure supplement 2- source data 1.** Frequency of TRBV and TRAV gene  
866 usage in iNKT cell TCR sequences.

867 **Figure 1 - figure supplement 3- source data 1.** Physicochemical properties of CDR3 $\beta$   
868 sequences.

869 **Figure 2 - figure supplement 1- source data 1.** TCRV $\beta$  usage for iNKT cell precursors

870 **Figure 3 - figure supplement 1- source data 1.** Gene expression analyses for iNKT cells  
871 from various tissues.



872 **Figure 4 - figure supplement 1- source data 1.** TCRV $\beta$  usage for iNKT cell  
873 subpopulations.  
874 **Figure 5 - figure supplement 1- source data 1.** Differential lipid antigen recognition for  
875 iNKT cells from non-lymphoid tissues  
876 **Figure 6 - figure supplement 1- source data 1.** Changes in the iNKT cell TCR repertoire  
877 following immunisation with  $\alpha$ GalCer  
878 **Figure 6 - figure supplement 2- source data 1.** Cytokine secretion by iNKT cells relates to  
879 TCRV $\beta$  usage.  
880 **Figure 6 - figure supplement 3- source data 1.** Frequency of iNKT cells after antibiotic  
881 treatment.

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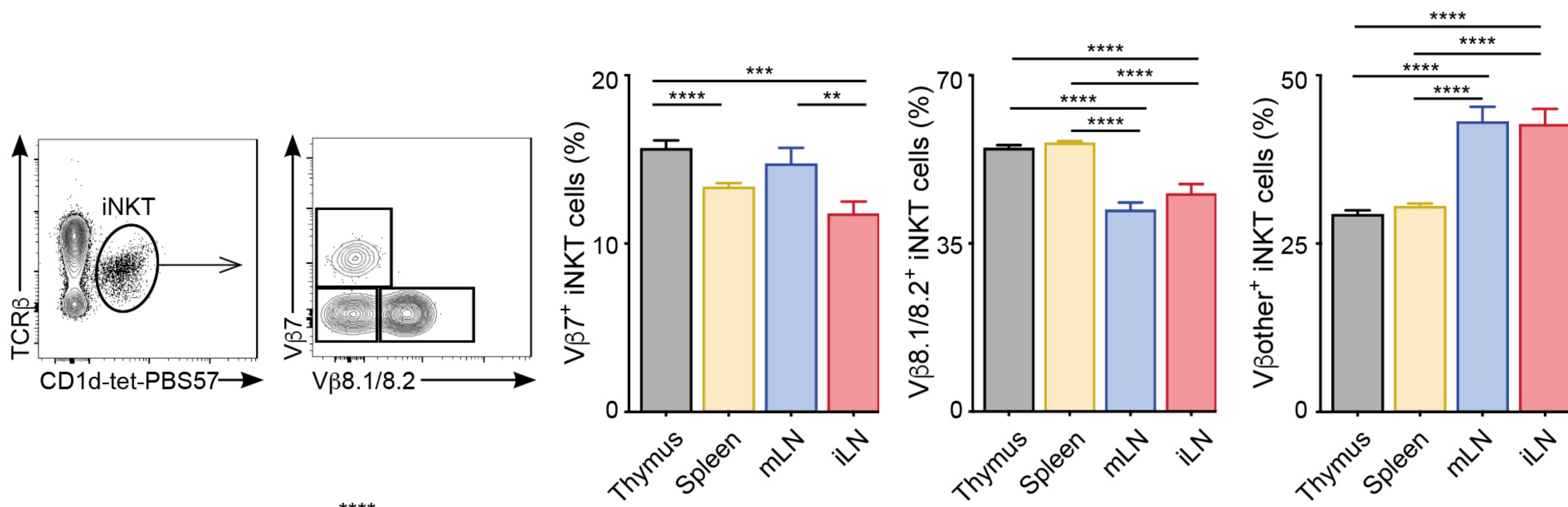
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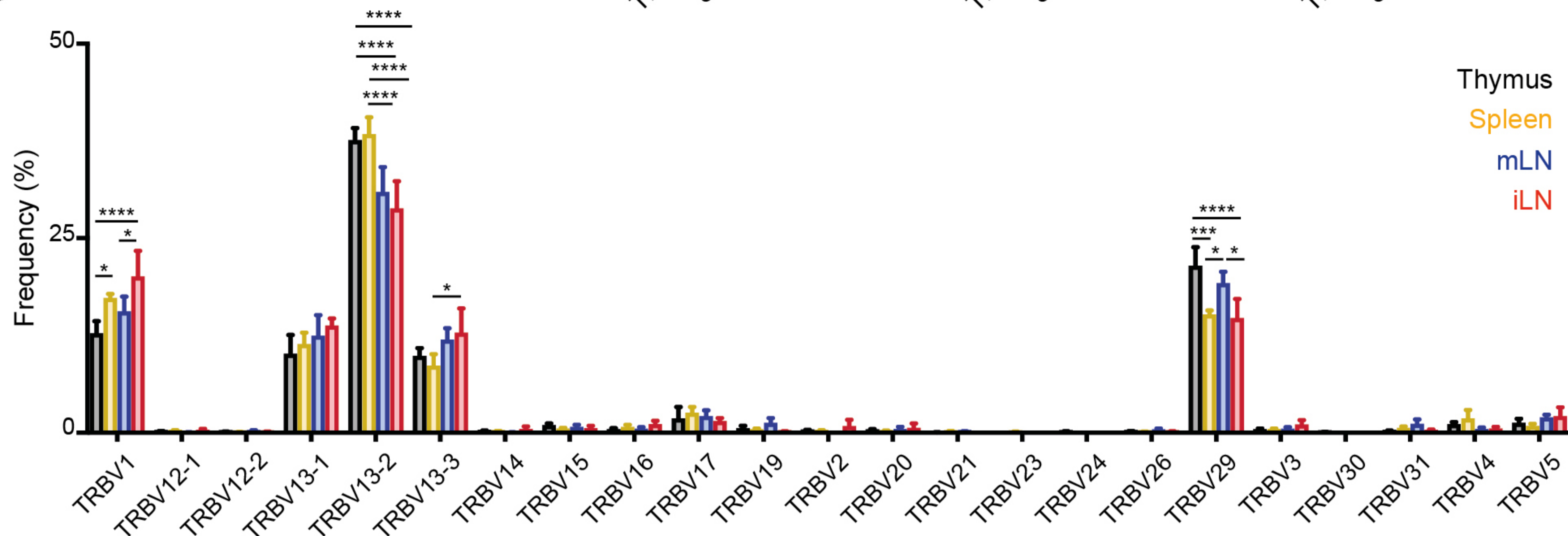


# FIGURE 1

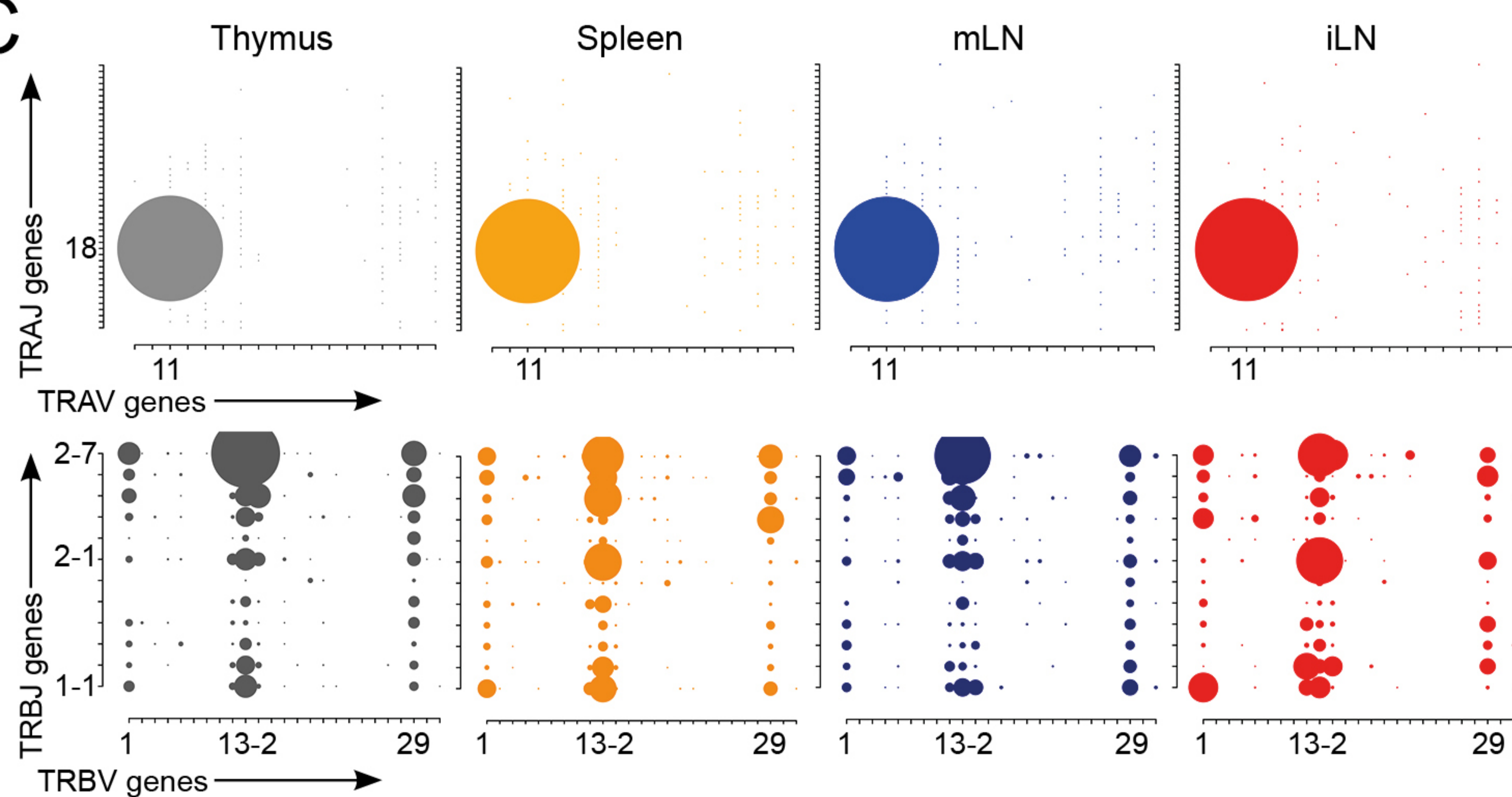
**A**



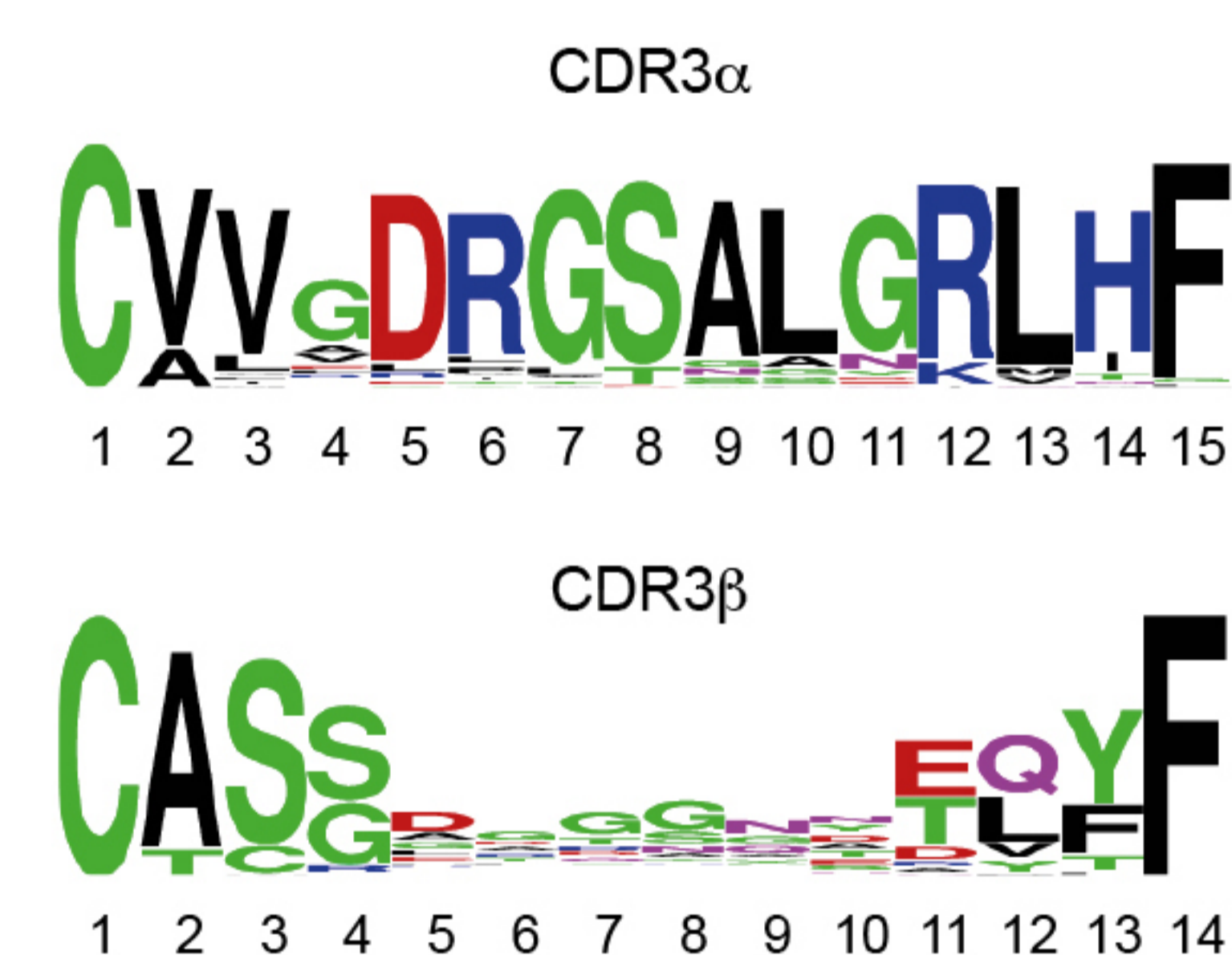
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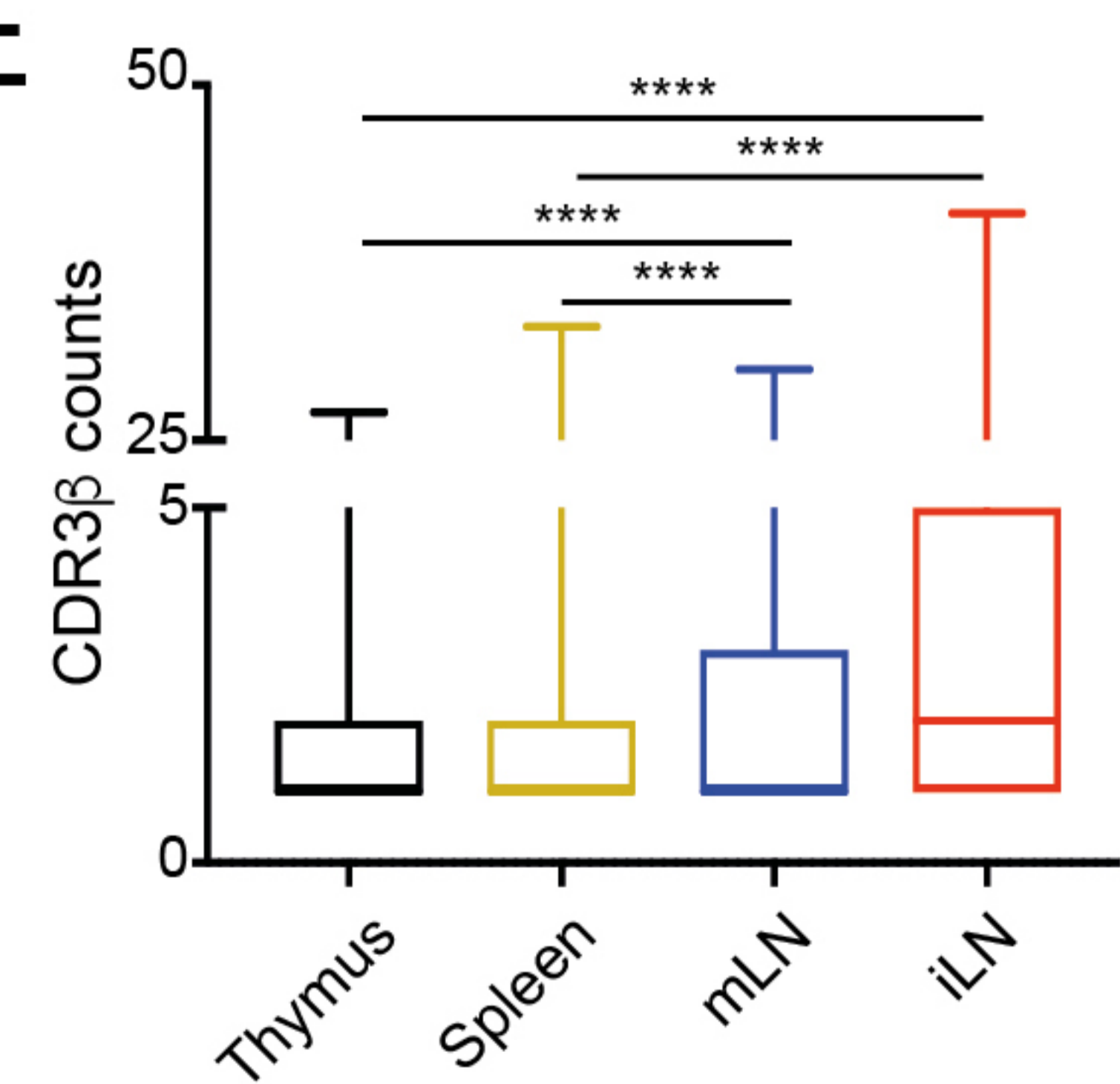
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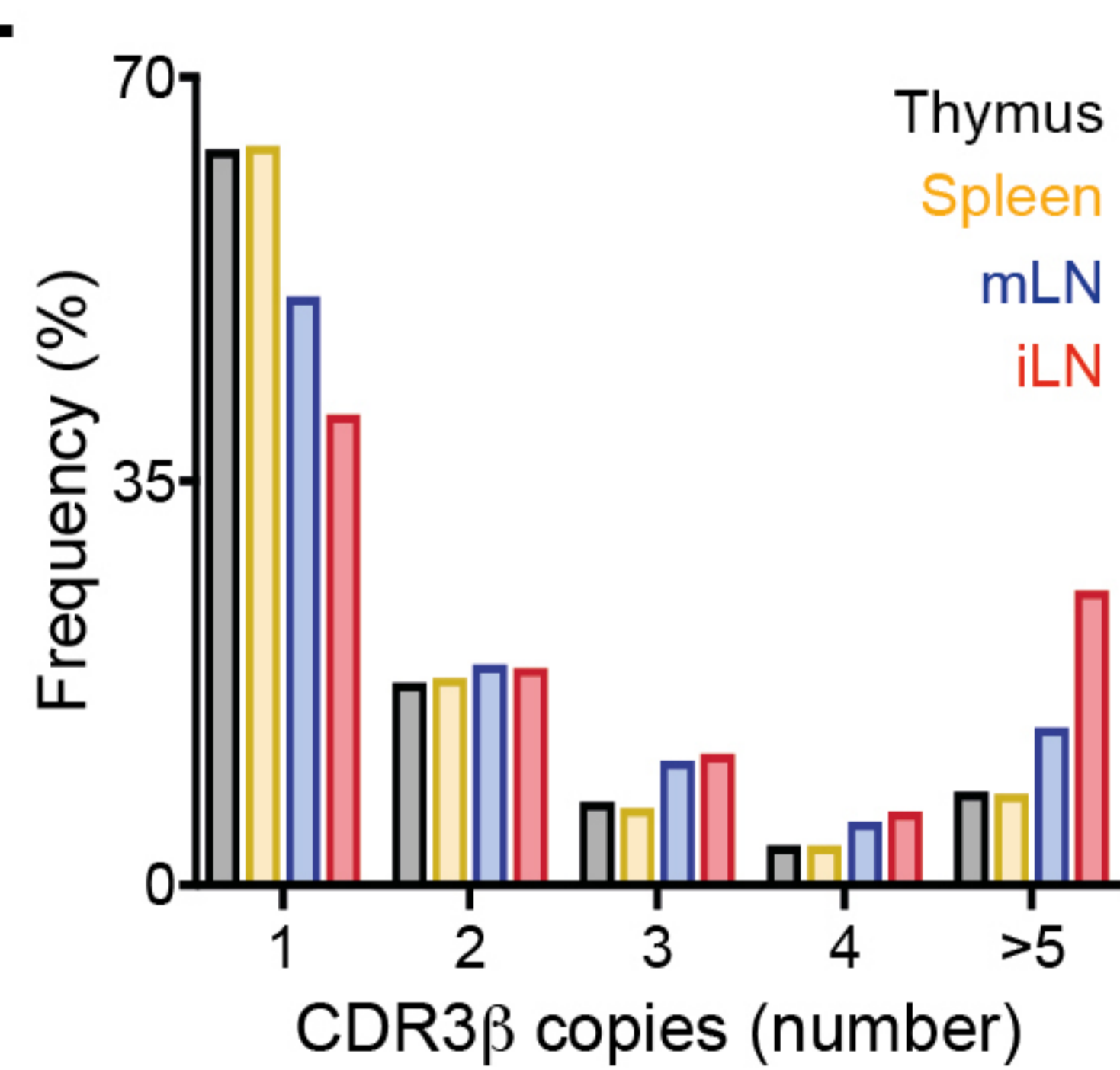
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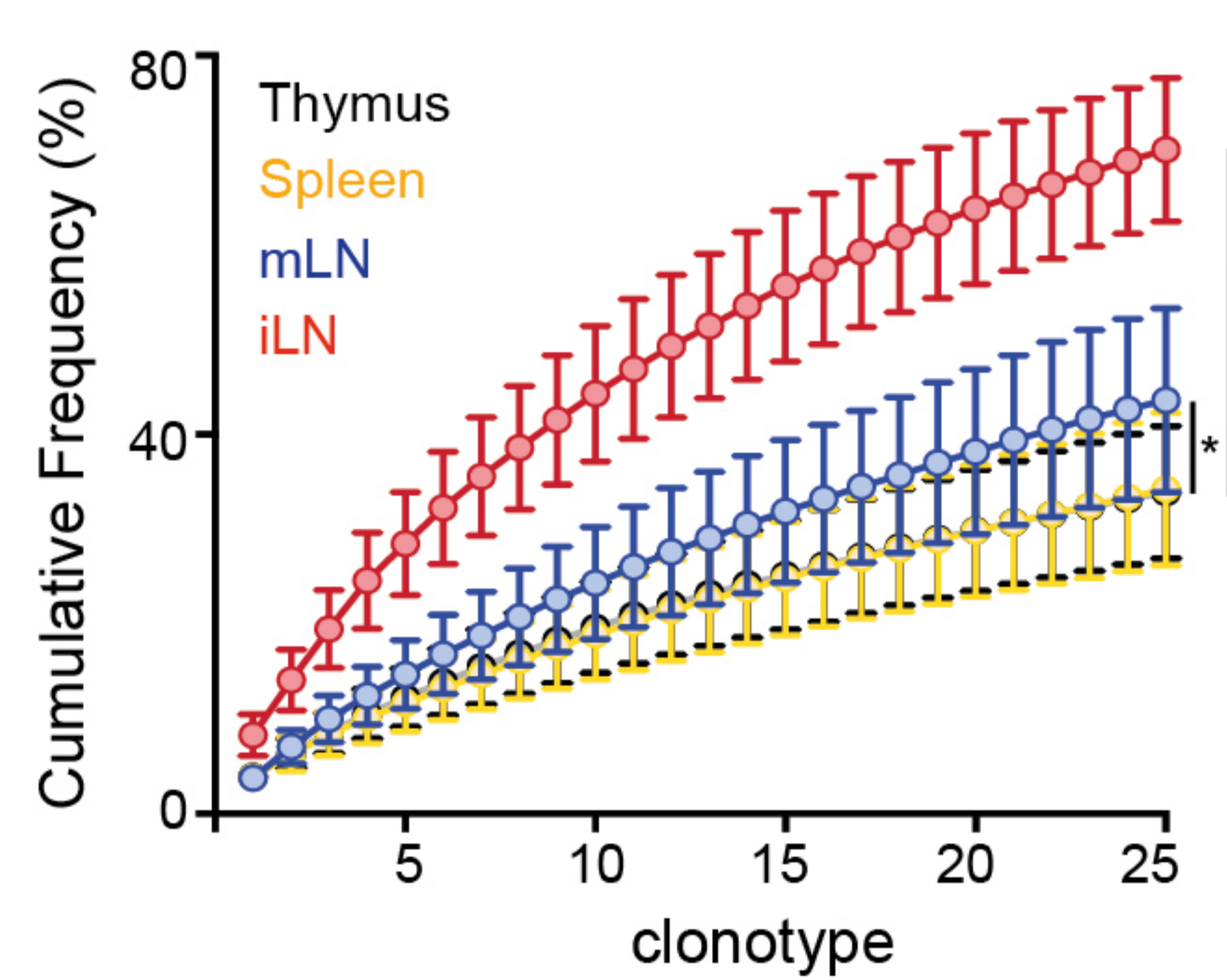
**E**



**F**

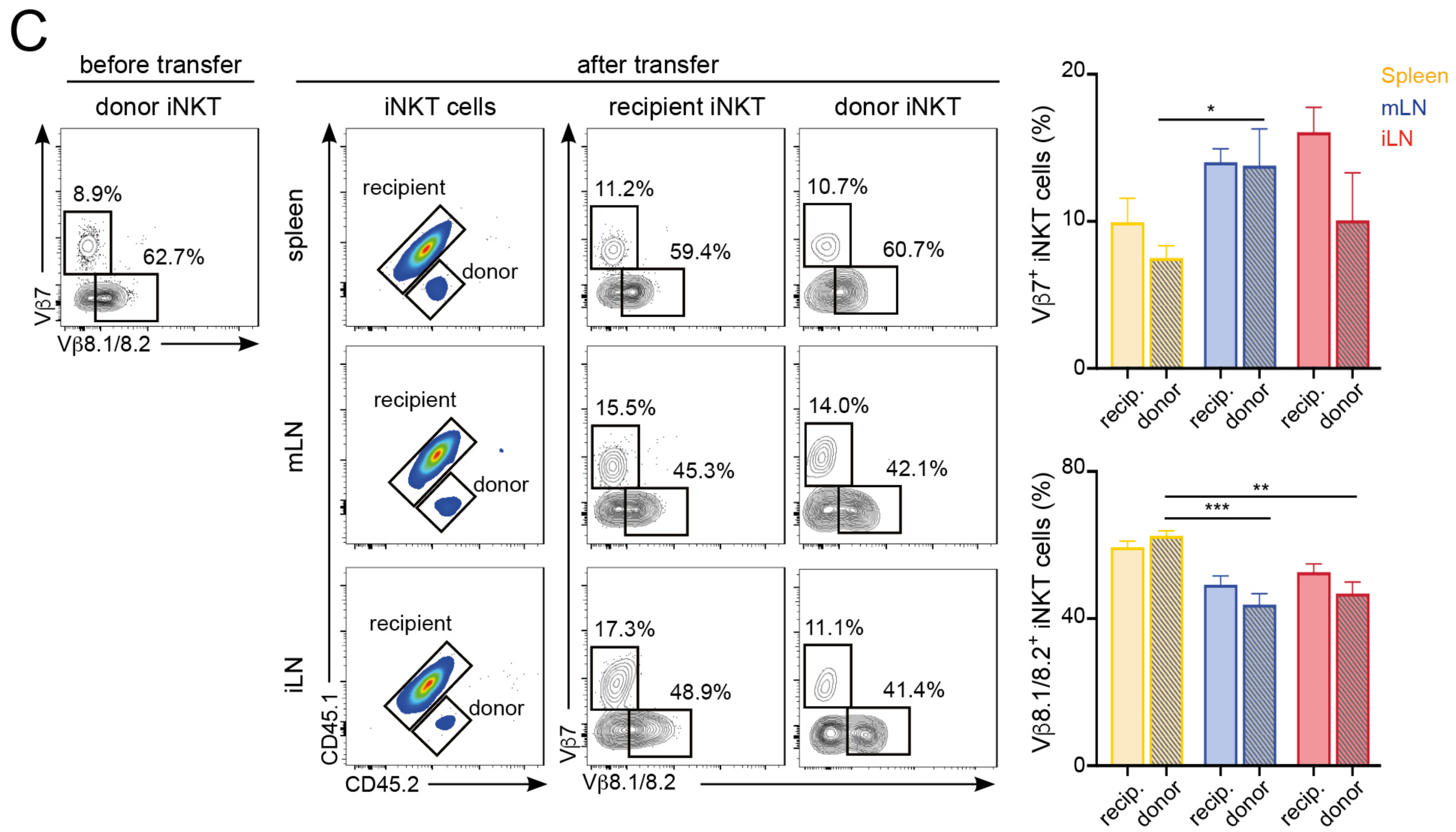
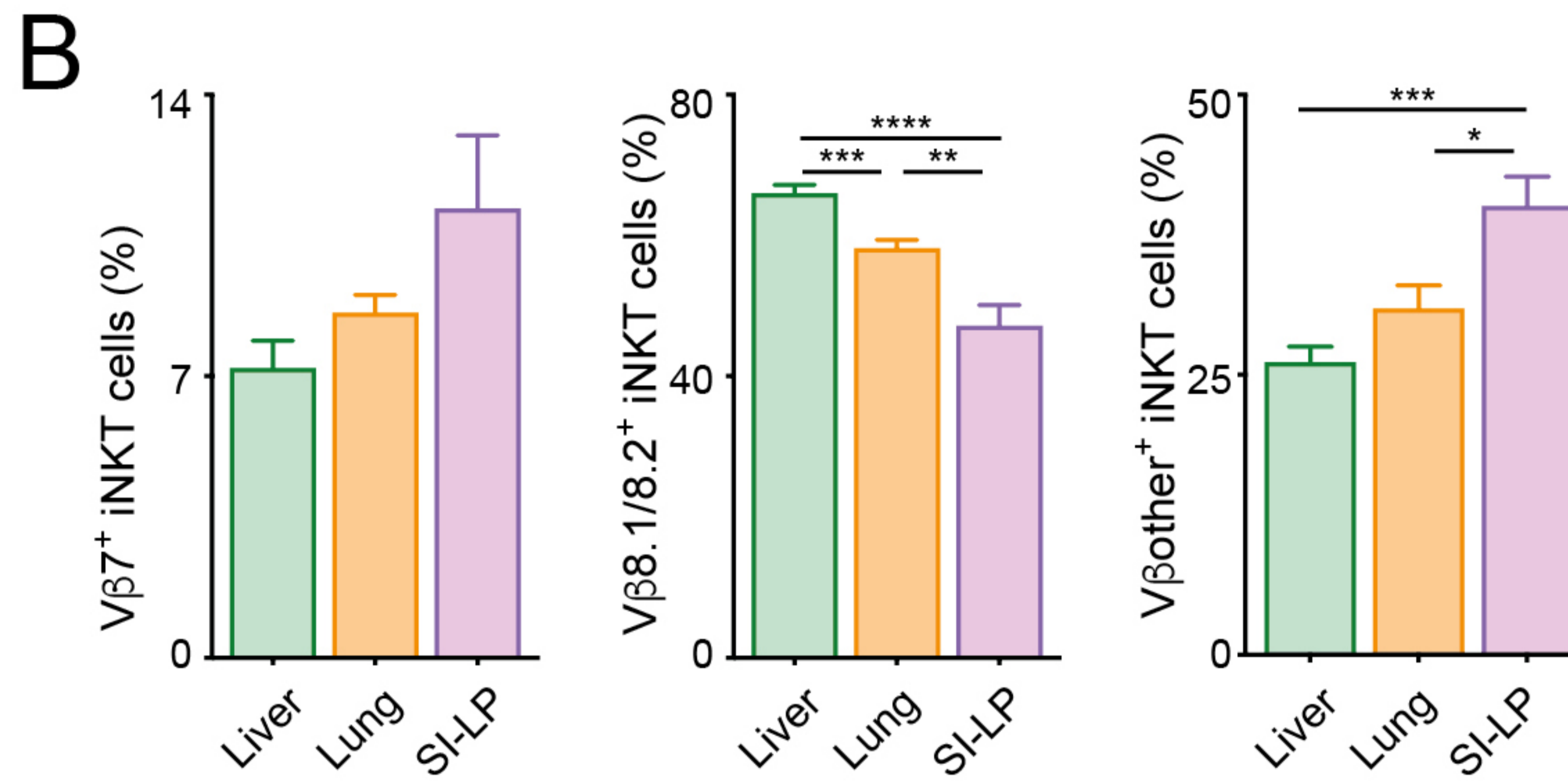
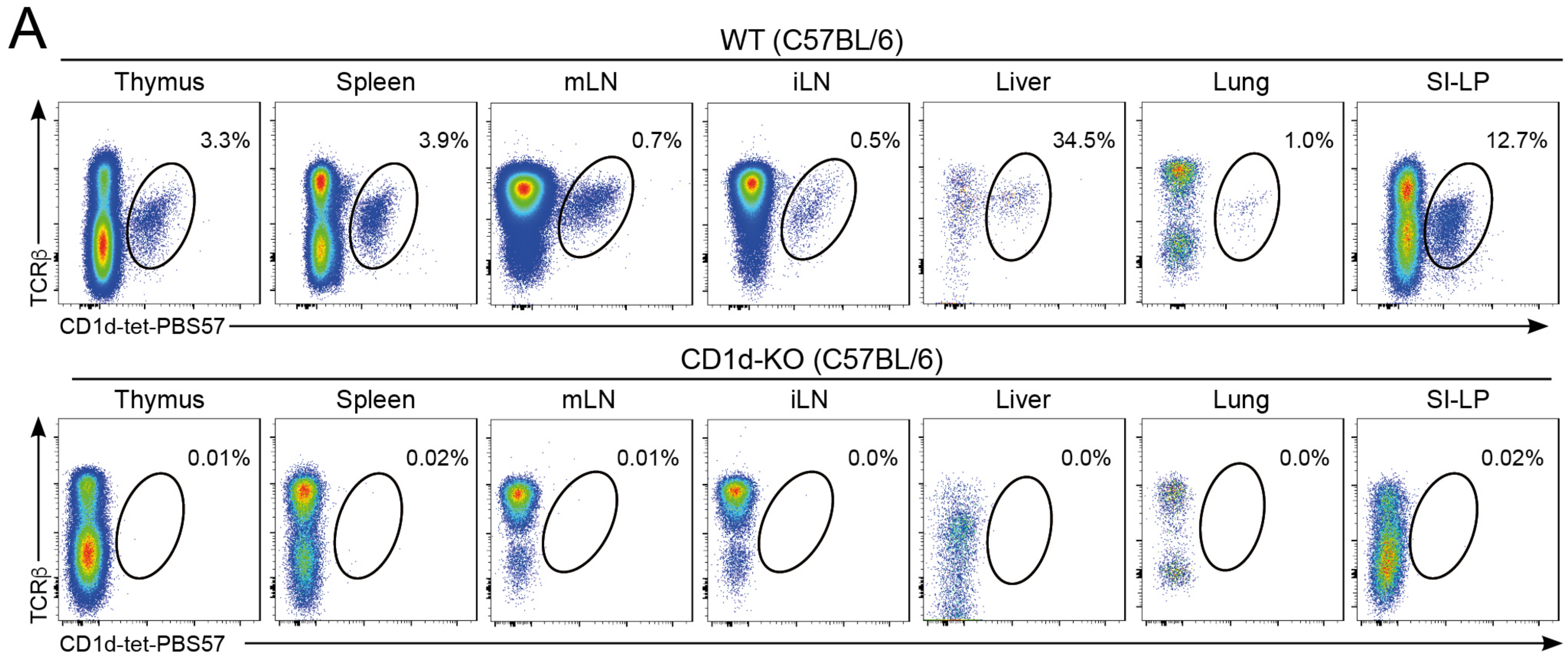


**G**





# FIGURE 1- FIGURE SUPPLEMENT 1



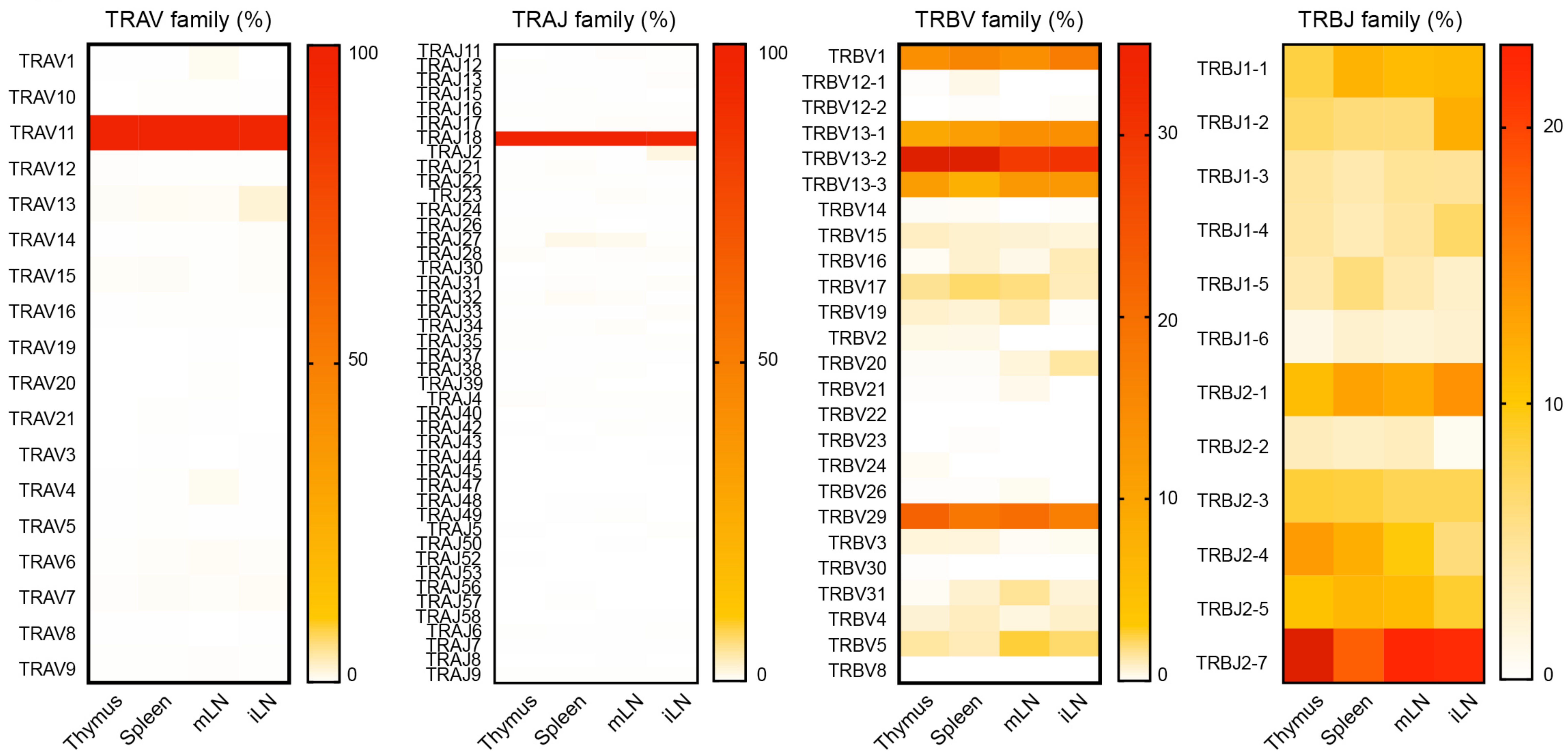


# FIGURE 1- FIGURE SUPPLEMENT 2

A

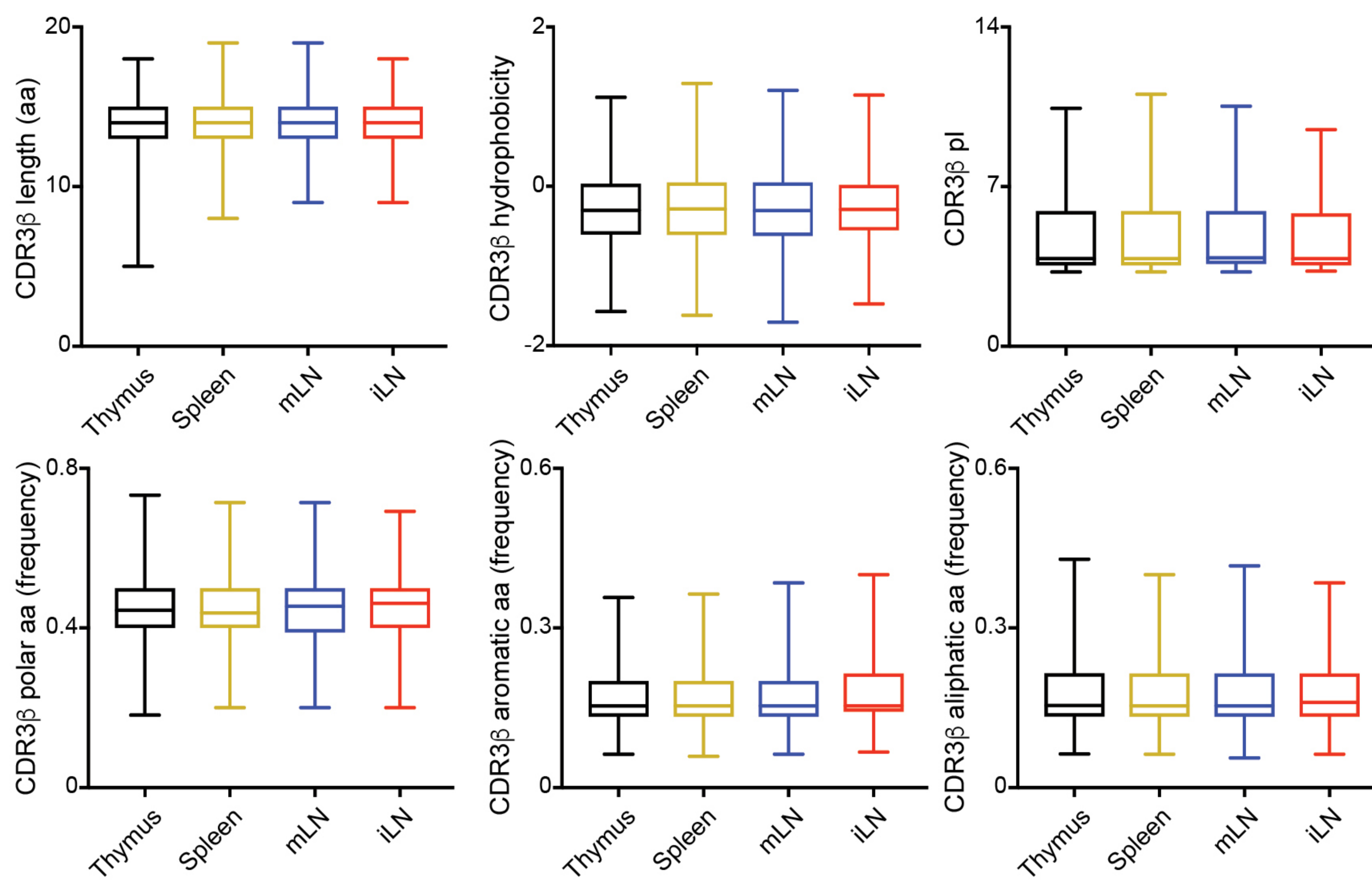
SAMPLE	TRAV	TRAV unique (%)	TRBV	TRBV unique (%)
Thymus	24463	225 (0.92%)	1697	849 (50.03%)
Spleen	13957	207 (1.48%)	1732	881 (50.87%)
mLN	17968	188 (1.05%)	2013	763 (37.90%)
iLN	13854	141 (1.02%)	1475	361 (24.47%)
<b>Total</b>	<b>70242</b>	<b>761</b>	<b>6917</b>	<b>2854</b>

B



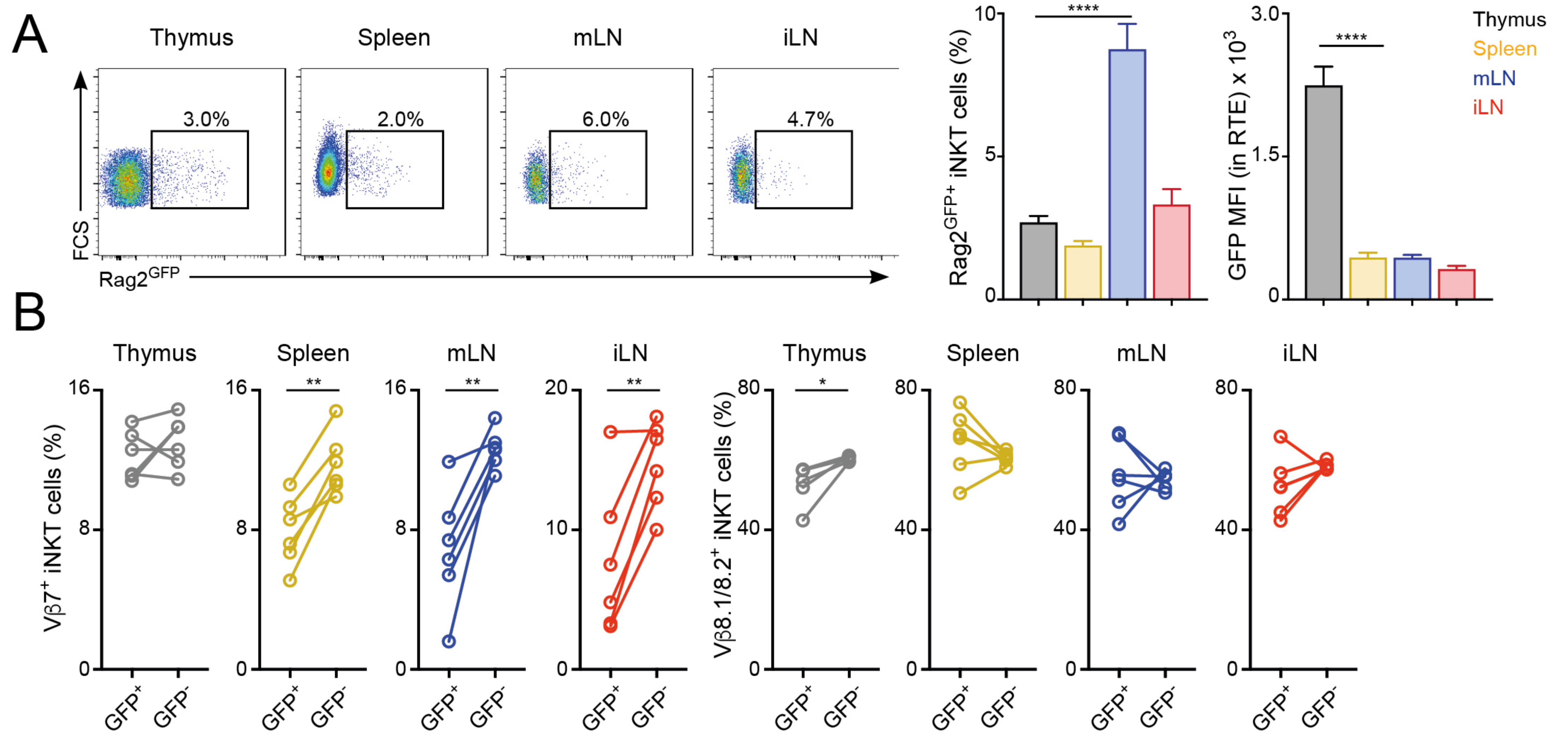


# FIGURE 1- FIGURE SUPPLEMENT 3



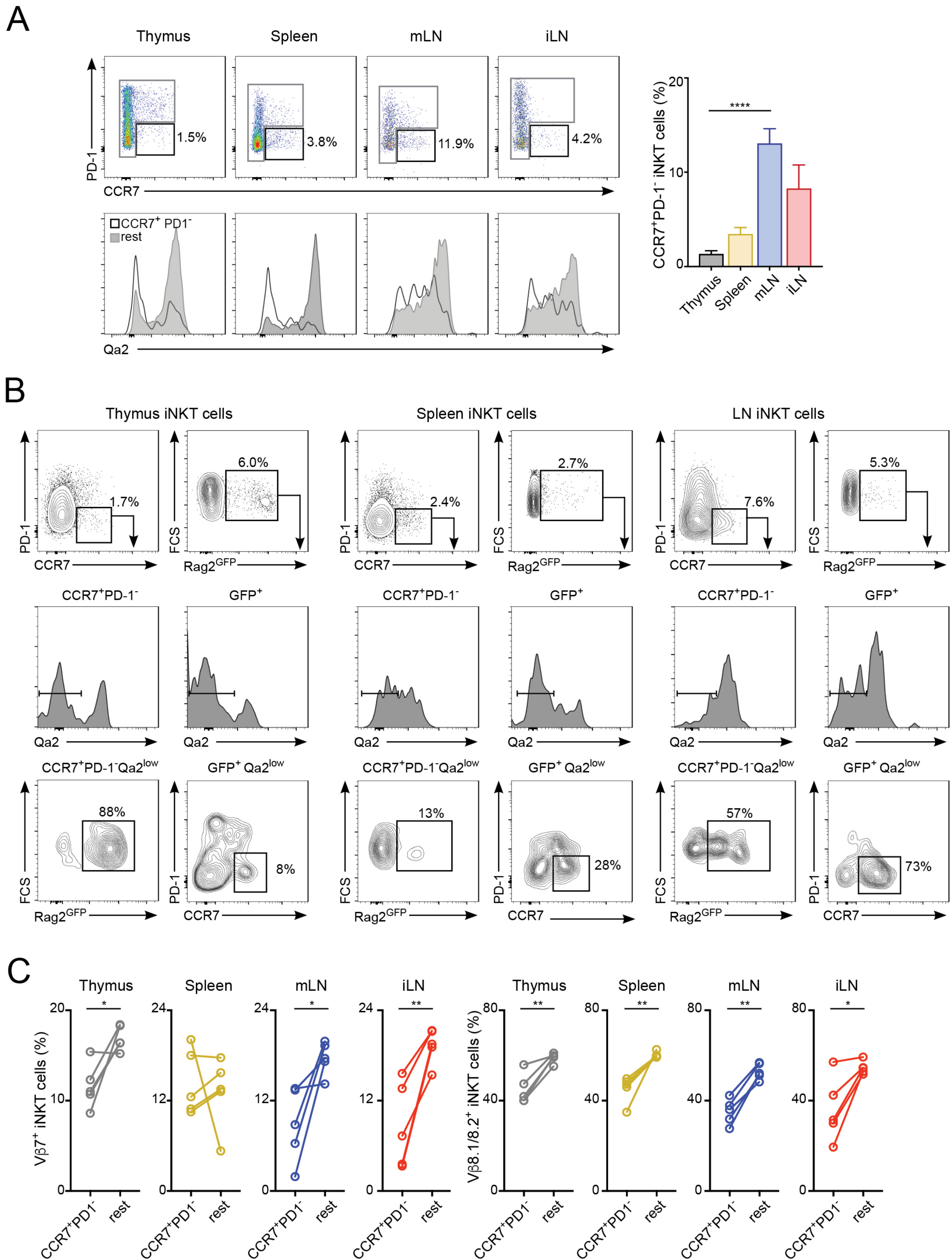


# FIGURE 2



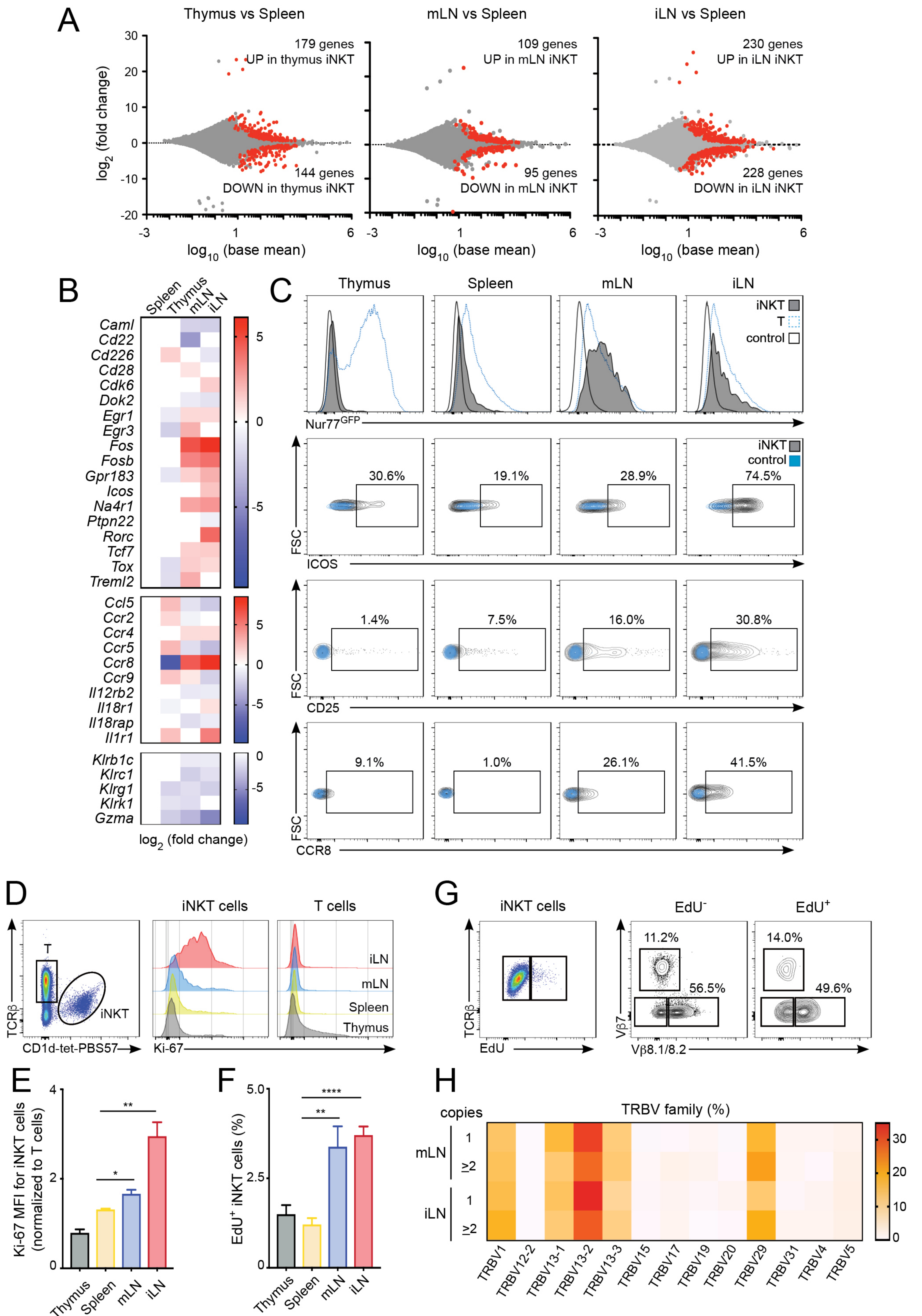


# FIGURE 2 - FIGURE SUPPLEMENT 1



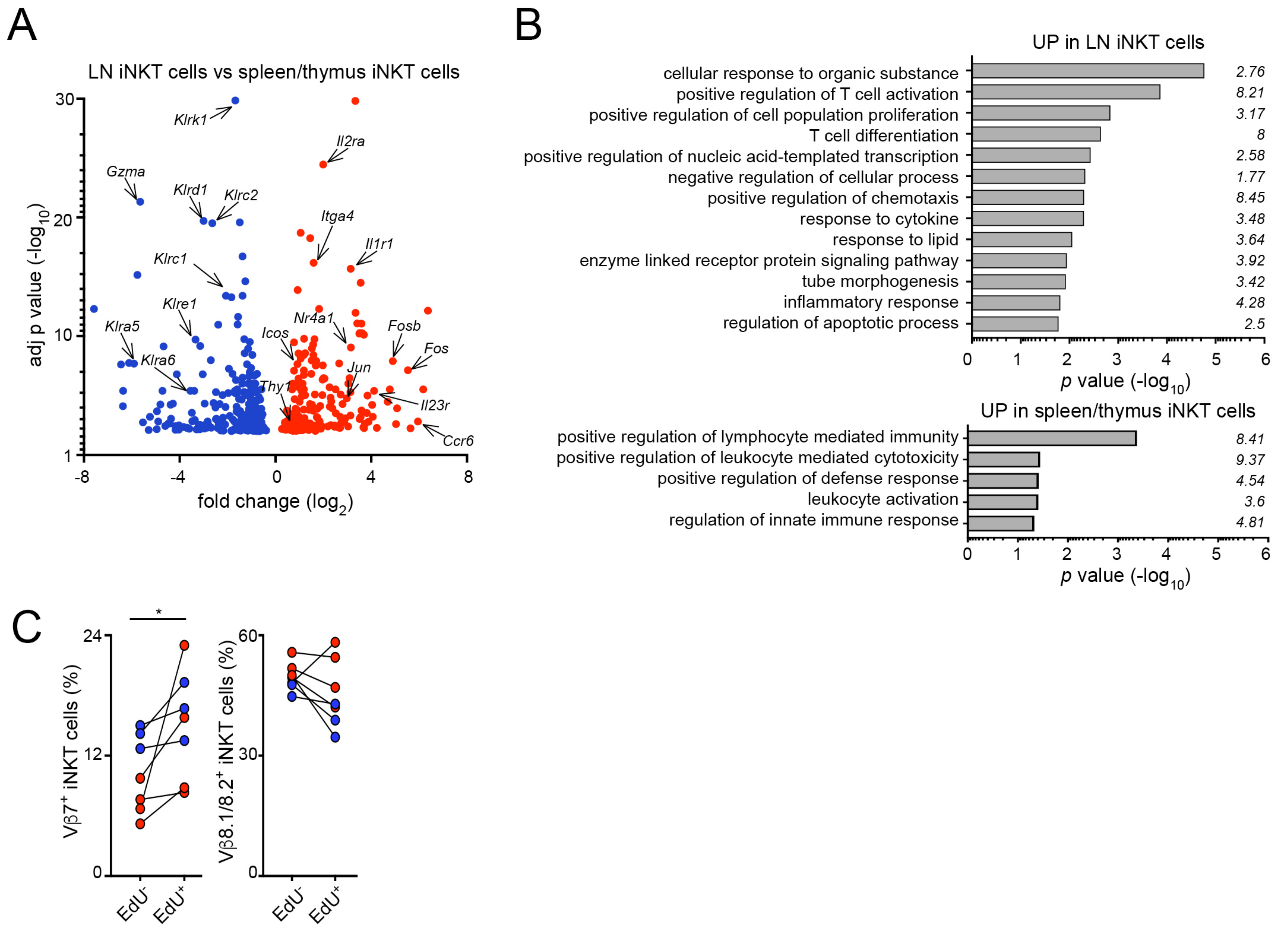


# FIGURE 3



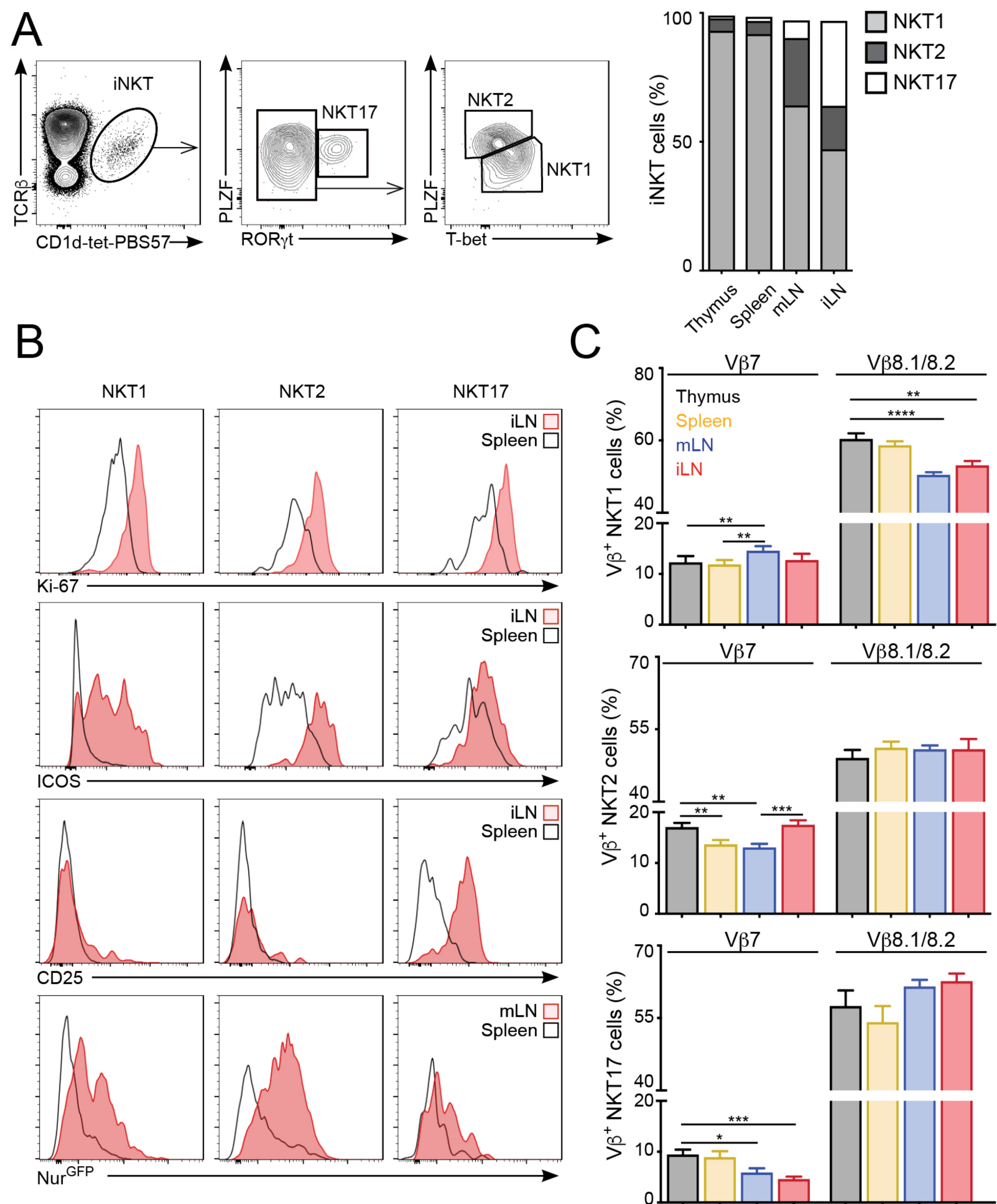


# FIGURE 3- FIGURE SUPPLEMENT 1



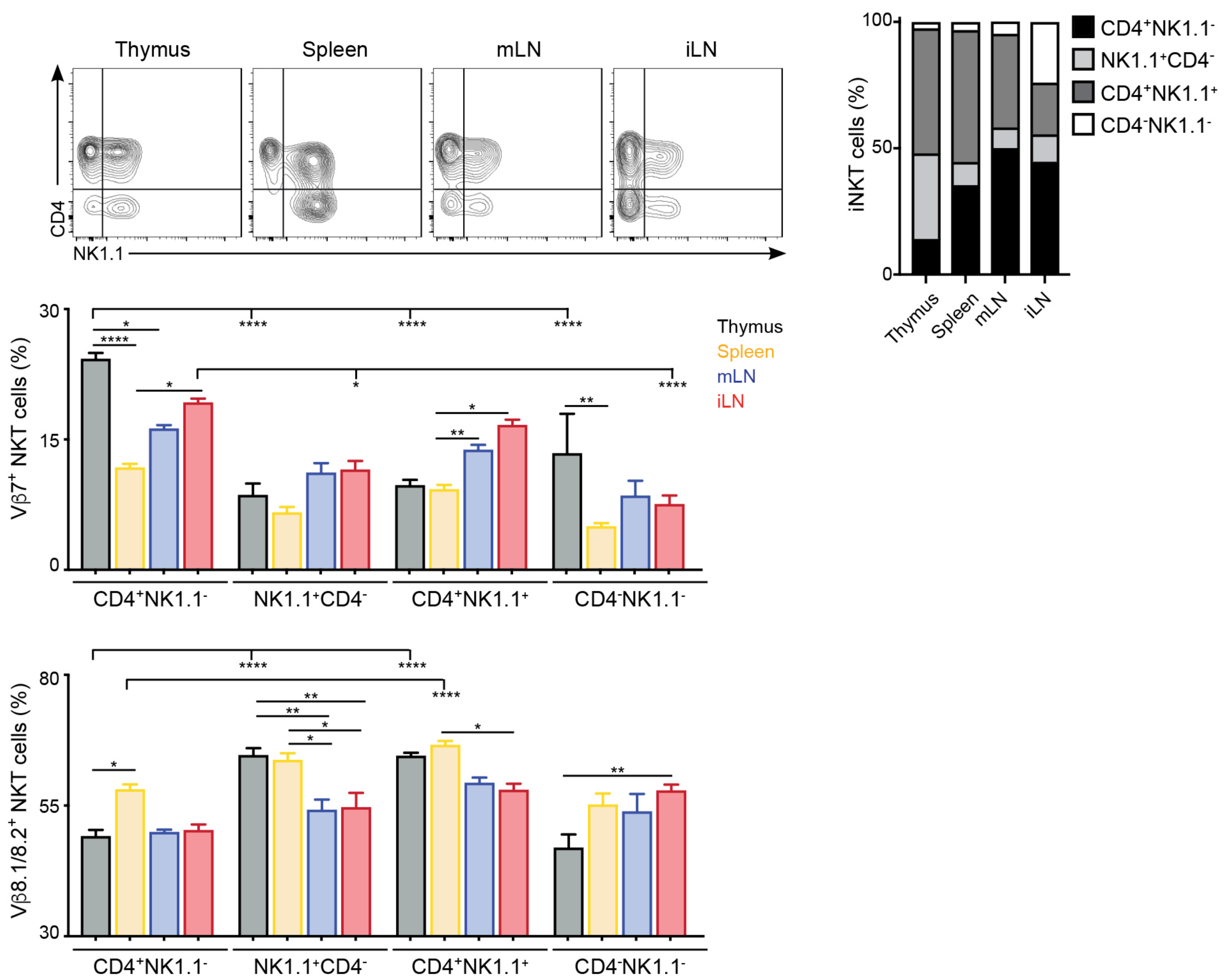


# FIGURE 4



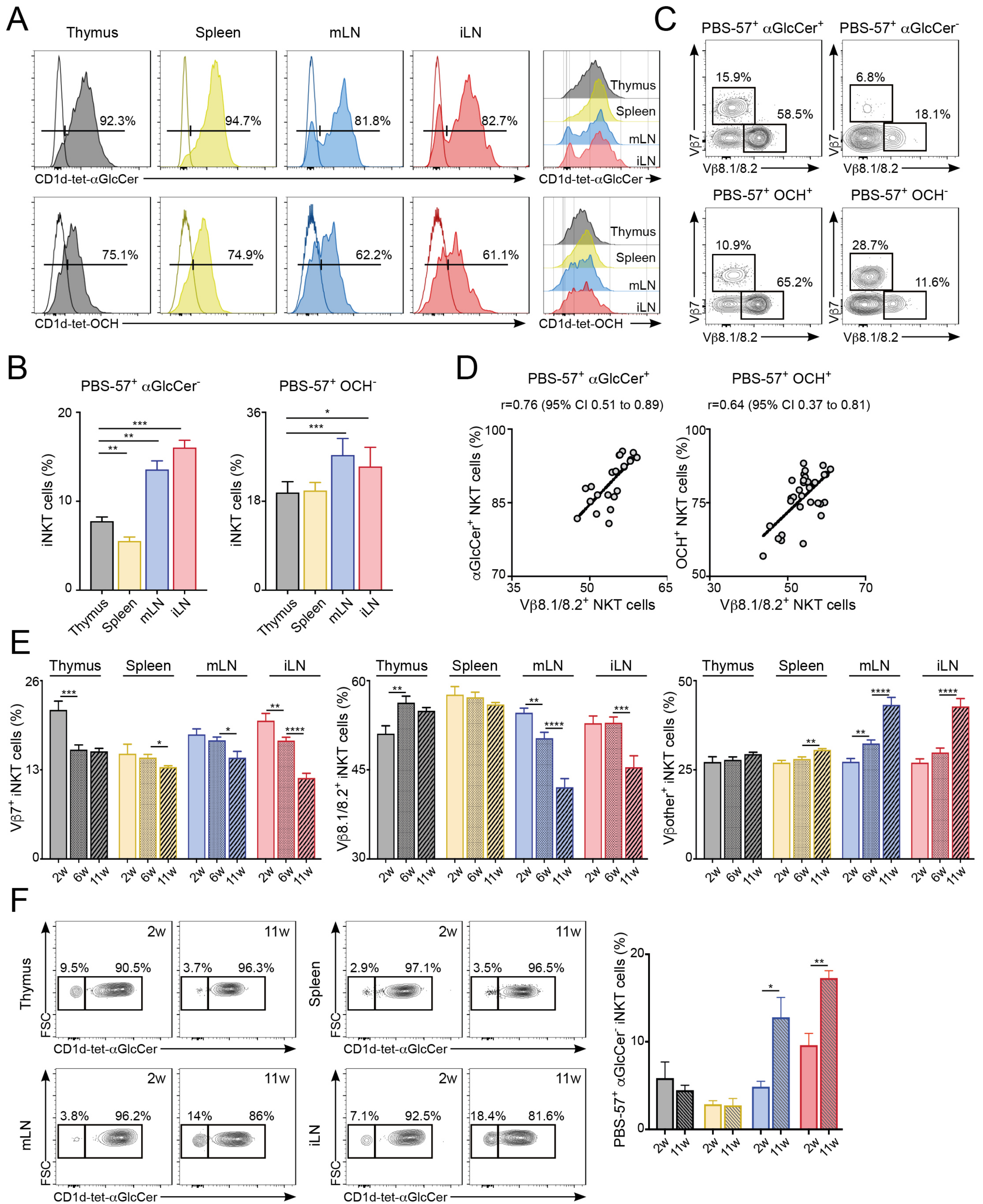


# FIGURE 4 - FIGURE SUPPLEMENT 1



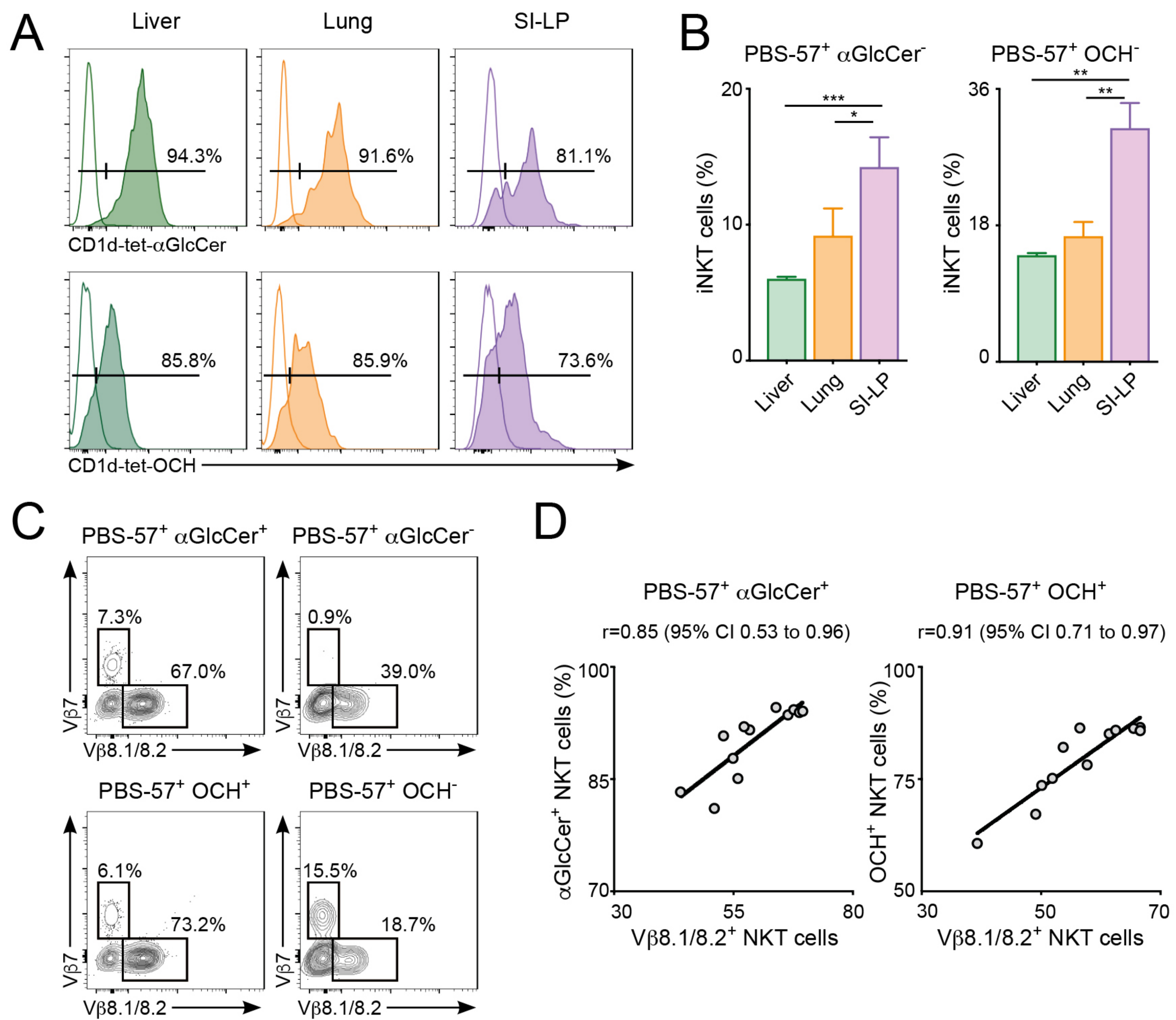


# FIGURE 5



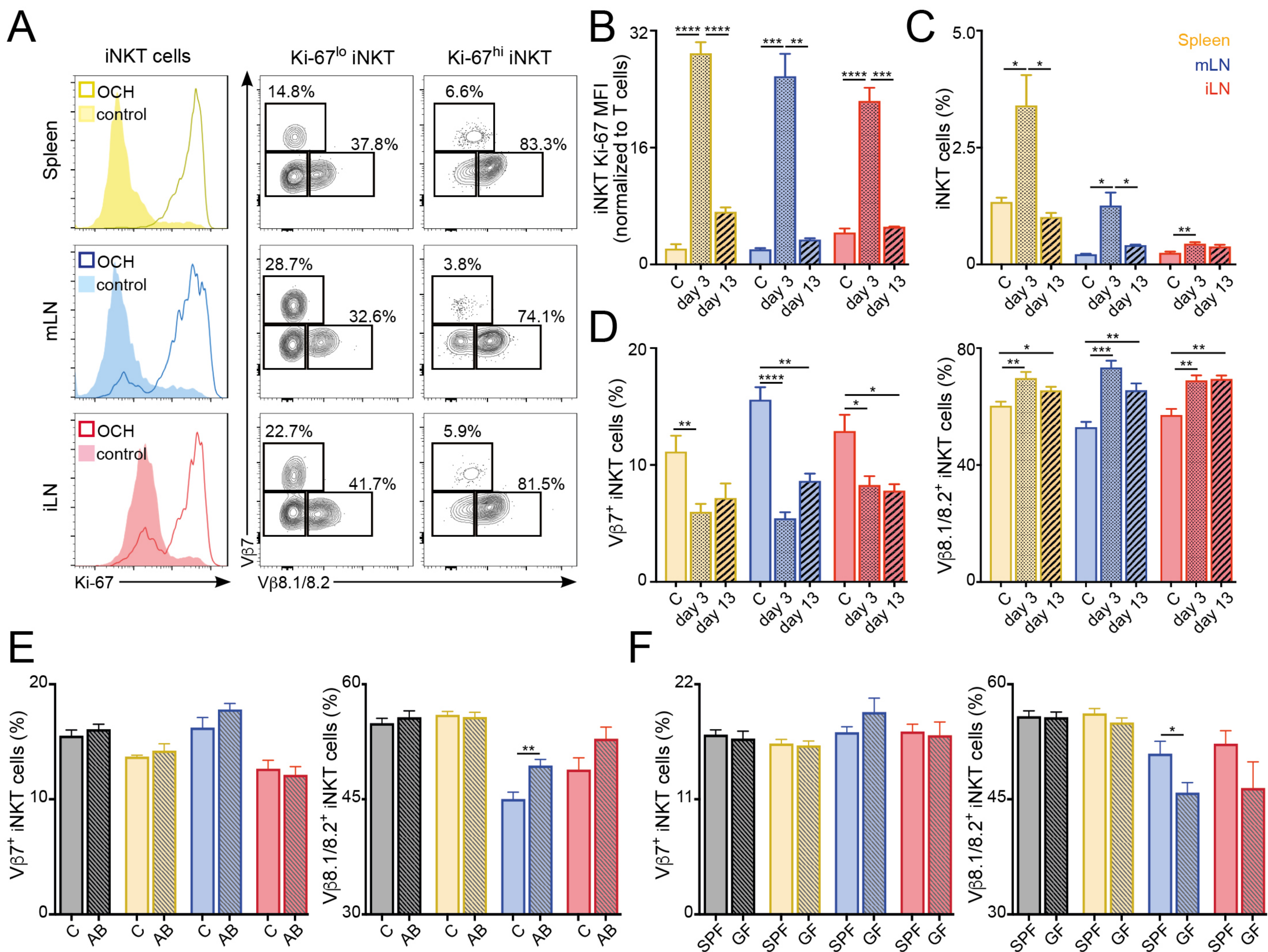


# FIGURE 5- FIGURE SUPPLEMENT 1



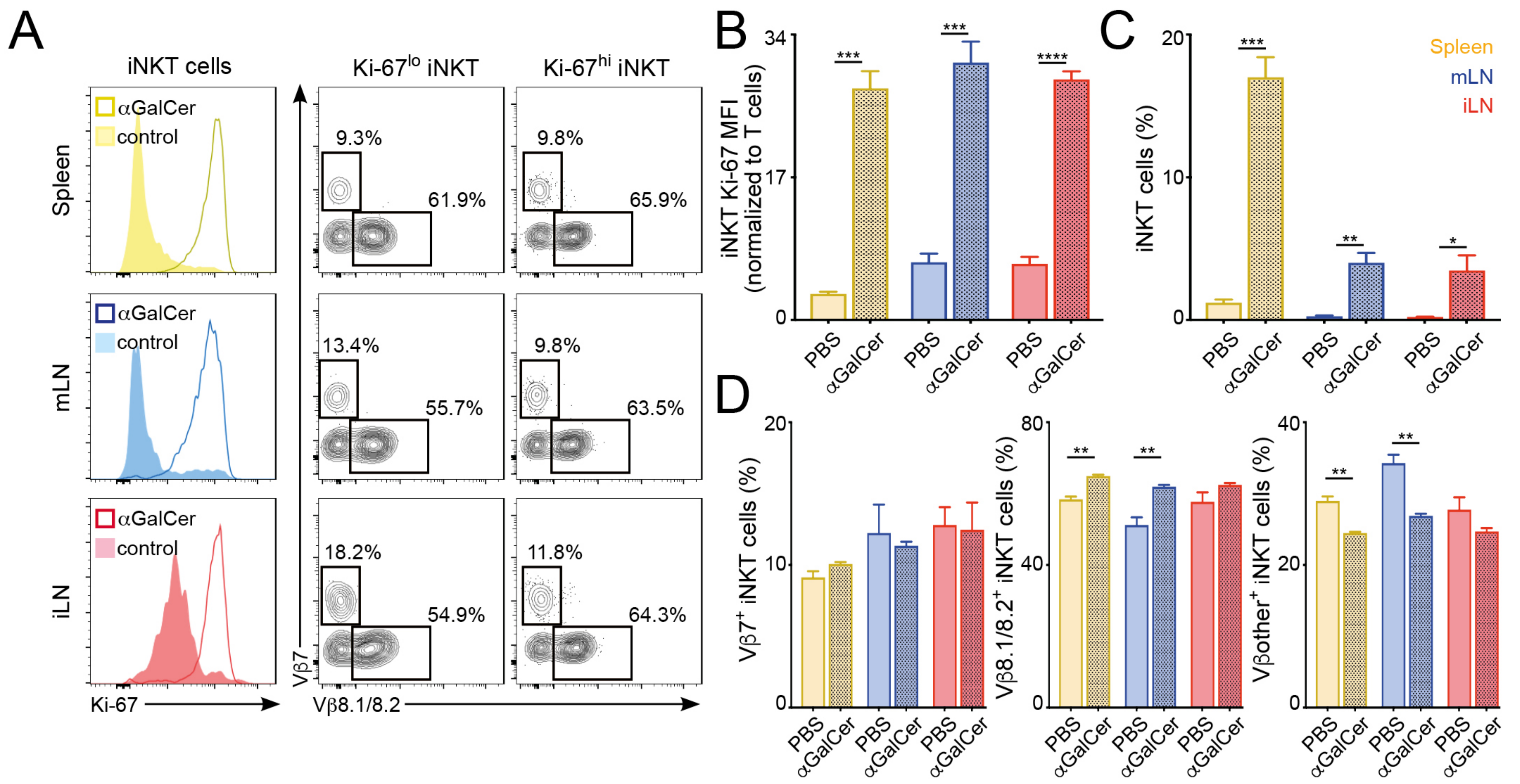


# FIGURE 6



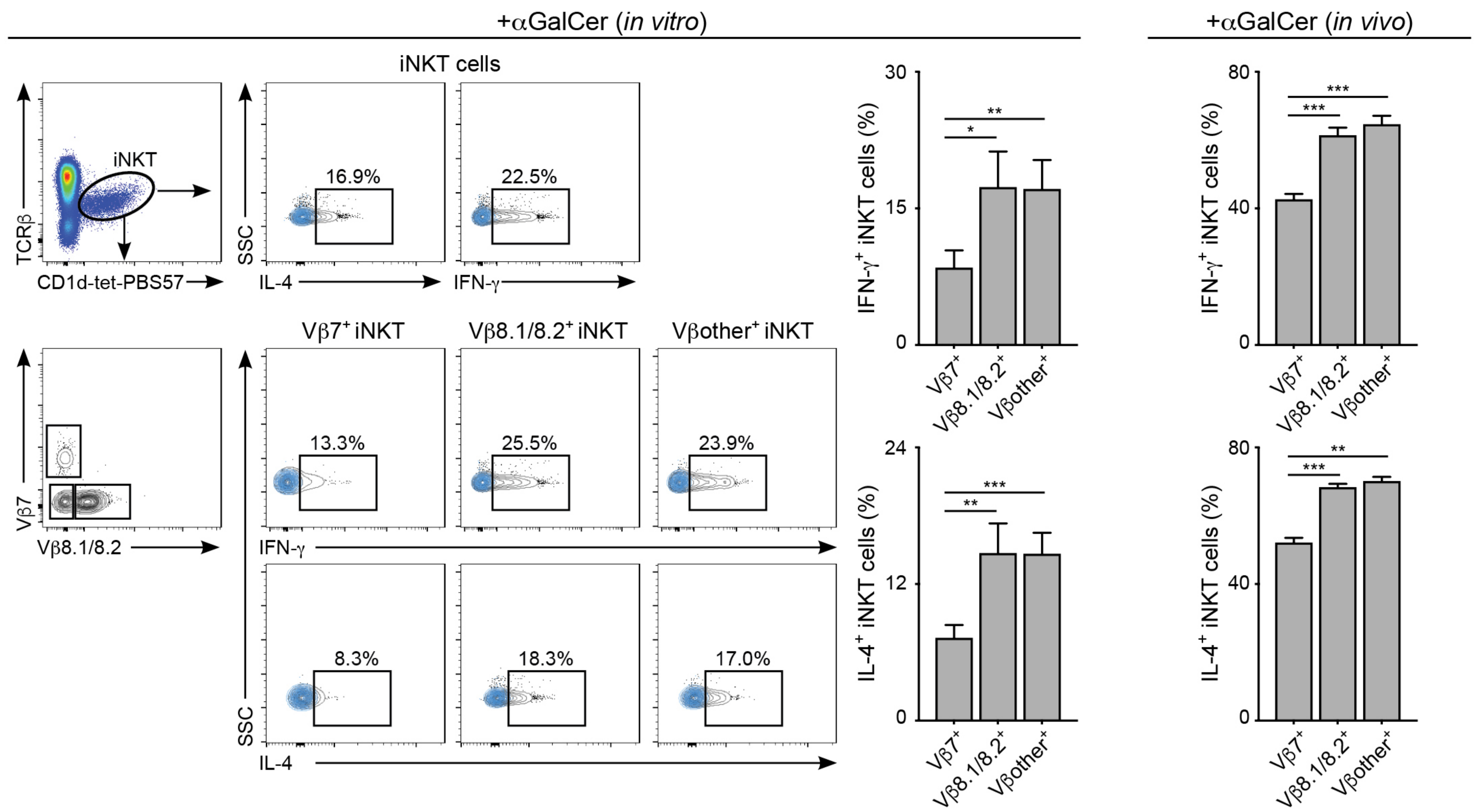


# FIGURE 6- FIGURE SUPPLEMENT 1

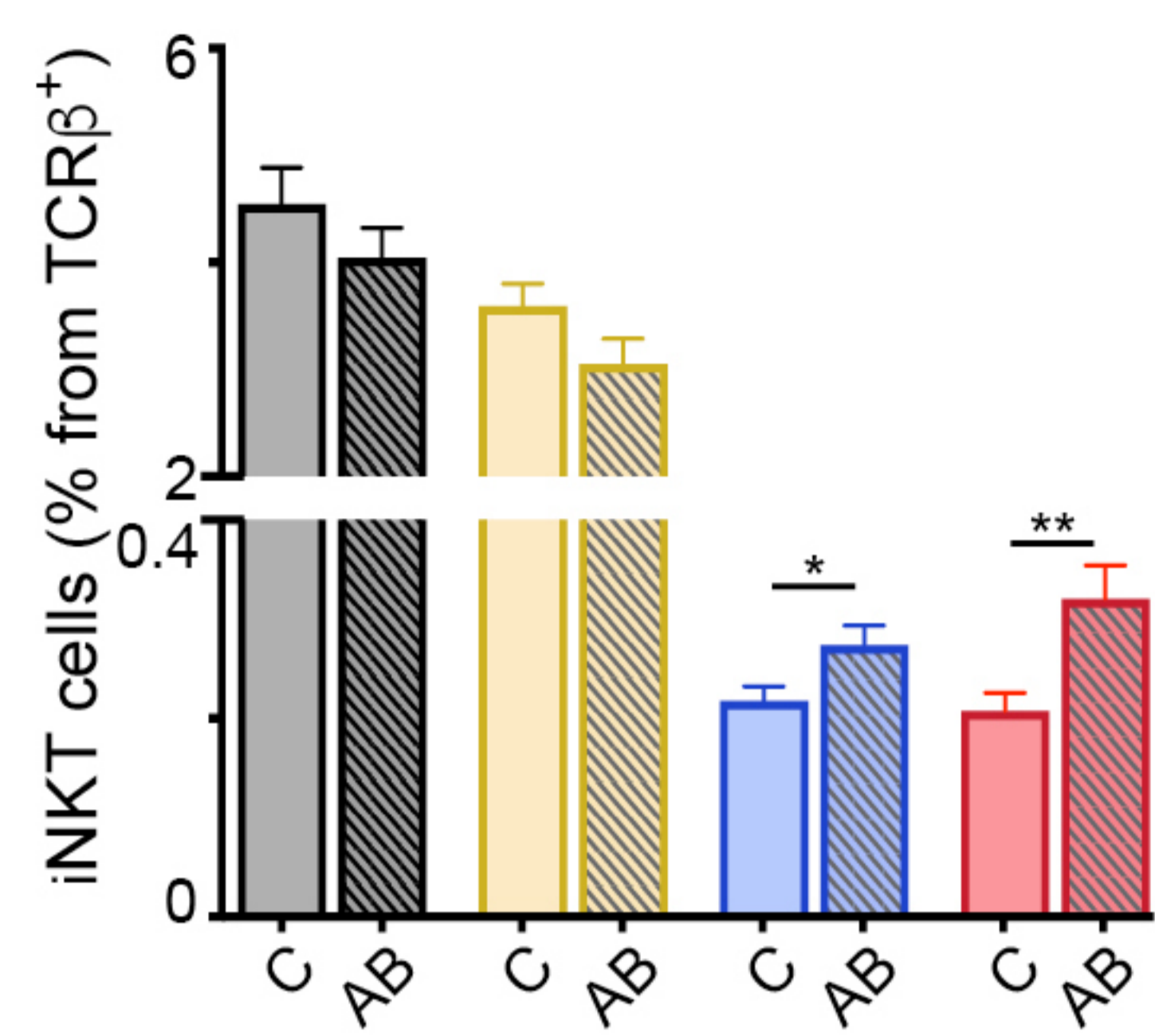




# FIGURE 6- FIGURE SUPPLEMENT 2



# FIGURE 6- FIGURE SUPPLEMENT 3





# FIGURE 7

