Project 1: Impaired iNKT generation on deletion of chemokine receptors homing to the thymic medulla

Project 2: The requirement for co-stimulation in generation and homeostasis of conventional and memory-phenotype regulatory T-cells

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Abstract

Invariant natural killer T-cells (iNKT) are a subset of unconventional T-cells arising from the same pool of $CD4^+8^+$ double-positive (DP) precursors as conventional T-cells, but are distinct in their T-cell receptor (TCR) specificity - recognizing CD1d-bound glycolipids - as well as constitutive expression of memory cell markers and cytokine mRNA. Development of iNKT within the thymus is drastically different in terms of both the molecular requirements, and the transcriptional processes involved. However, like conventional thymocytes, they are known to undergo positive and negative selective processes to screen TCR functionality and auto-reactivity, albeit on contrasting cell types – DP thymocytes and dendritic cells respectively. Progressive maturational stages are defined by expression of activation and NK-cell markers. Conventional thymocytes up-regulate the chemokine receptors CCR4 and CCR7 post-positive selection, which control re-localization to the medulla, where they are negatively selected on self-antigens promiscuously expressed by medullary epithelial cells. On deletion of these chemokine receptors, development is unaffected, but auto-reactive T-cells escape deletion, and cause auto-immunity in the periphery. Our work has demonstrated that deletion of either receptor severely impedes thymic generation of iNKT, although only CCR4 is expressed on developing iNKT, suggesting localization of other $CCR7^+$ cell types with iNKT is vital to their development.

Section 1.0: Acknowledgements

I would like to thank the following for their help and support throughout this project – my project supervisors, Professor Graham Anderson and Dr. Nick Jones, as well as the entirety of the Anderson lab; William Jenkinson, Andrea White, Kyoko Nakamura, Sonia Parnell, Jennifer Cowan, Song Baik and Amanda Holland.

Section 2.0: Introduction

2.1: Efficient positive and negative selection are reliant on chemokine receptor signaling

The thymus is a primary lymphoid organ acting as the site of T-cell development and maturation, and is divided into two functional sub-compartments, the cortex and medulla, each of which is occupied by a unique population of thymic epithelial cells (TECs)[1,2]. Bone marrow derived precursors enter at the cortico-medullary junction, and traffic into the subcapsular region of the cortex in response to signaling through chemokine receptors CCR9 and CXCR4[3]. During this time, they undergo T cell receptor (TCR) gene rearrangements to form a clonally unique TCR[4], capable of binding a specific peptide antigen bound in a surface expressed major histocompatibility complex (MHC) molecule. Thymocytes also up-regulate the co-receptors CD4 and CD8 to become double positive (DP) cells[5]. At this stage, cells are selected to progress dependent on their successful formation of a TCR capable of binding MHC on the surface of cortical thymic epithelial cells (cTEC)[6]. Ligation provides thymocytes with essential survival signals in a process known as positive selection, and cells down-regulate either CD4 or CD8 (depending on the class of MHC bound)[7] to become single positive (SP cells), and concomitantly express high levels of CCR4 and CCR7[8].

CCR4 and CCR7 control trafficking of SP thymocytes into the thymic medulla, wherein they undergo another selective process – negative selection – in which they are screened for TCR auto-reactivity by medullary TEC (mTEC) and dendritic cells (DCs), and induced to apoptose on strong TCR-ligation by auto-antigen[5]. mTEC are capable of promiscuously expressing tissue-specific antigens (TSAs) for this purpose, partially under the control of the autoimmune regulator gene (Aire) in mature CD80^{hi} mTECs[9]. These antigens can also be acquired for presentation by DCs[10]. The ligands for CCR4 (CCL5, CCL17, CCL22) and CCR7 (CCL19, CCL21) are also expressed by mTEC in a partially Aire-dependent manner[11], although reduction in the Aire$^{-/-}$ thymus may in part be indirect, as mTEC expressing CCR7 ligands have recently been identified as a separate population to those expressing Aire[11,12]. Auto-immunity, as characterized by tissue-specific lymphocytic infiltrations, has been observed not only in mice where Aire has been deleted[11], but also CCR7$^{-/-}$ mice[13,14]; thymocyte development proceeds normally in both, but is largely restricted to the cortex[11,14] (more notably in the CCR7$^{-/-}$), allowing thymocytes with TSA-reactive TCRs to escape negative selection. Aire$^{-/-}$ and CCR7$^{-/-}$ thymi also display defective medullary development – mature CD80^{hi} mTEC largely fail to develop in the Aire$^{-/-}$[11], and medullary architecture is altered in the CCR7$^{-/-}$, producing small, non-continuous pockets of mTEC[14]. This is likely due to the reliance of mTEC maturation on provision of CD40-CD40L interactions by SP thymocytes[2].

2.2: Selection and development of iNKT in the thymus and periphery

Thymic selective processes are also imposed on invariant natural killer T-cells (iNKT). iNKT are an unconventional T-cell subset notable for their constitutive expression of memory and NK-cell markers[15], as well as cytokine mRNA, allowing rapid production and export of

cytokines upon activation[16]. This capacity allows iNKT to play dominant roles in both tolerance and immunity - they have been implicated in tumour immunity[17,18], transplant rejection[19] and asthma development[20], and their absence or malfunction has been implicated in several autoimmune diseases[21].

iNKT are derived from the pool of DP cells within the thymus[22], and are phenotypically indistinguishable from conventional DP thymocytes, except by TCR specificity. The iNKT TCR is produced at a low frequency (~$1:10^6$) from the random gene recombination events defining production of novel TCRs in early thymocyte development[23]. It is distinguishable from those of conventional T-cells as it binds the non-classical MHC molecule CD1d[24], which displays glycolipid, rather than peptide, antigens. The assembly of the iNKT TCR requires recombination of Vα14-Jα18, which is paired with Vβ8.2, Vβ7.2 or Vβ7.1, and hence is often referred to as semi-invariant[23]. Due to it's positioning, Jα18 recombination occurs late in DP development, which contributes to both the rarity of iNKT among developing thymocytes, as well as the susceptibility of iNKT development to deletion of factors which extend the life-span of DP cells[25]. iNKT hence require the E-protein HEB[25], and transcription factors RORgt[26,27,28] and c-Myb[29], which together contribute to expression of the anti-apoptotic molecule Bcl-X_L[23].

Upon TCR assembly, iNKT undergo positive selection within the cortex[30], as demonstrated by the complete absence of iNKT in CD1d$^{-/-}$ mice[21], and low affinity bias of the iNKT TCR upon transgenic overexpression of CD1d[31,32], wherein iNKT are reduced. Through studies involving deletion and transgenic expression of CD1d, this process was found to occur upon interaction with DP thymocytes, as iNKT selection proceeded normally when CD1d expression was driven by the promoter for the Lck gene[33], expression of which is

restricted to DPs. Negative selection of iNKT also occurs, mediated by DCs, and mice expressing GFP as a reporter for the TCR-signaling downstream element Nur77 show that iNKT TCRs possess greater self-reactivity than those of conventional T-cells[34], although the identity of selective antigens has yet to be fully elucidated. Similarly, deletion of DGKα or DGKζ – proteins inhibitory of TCR signaling – also triggers enhanced deletion of iNKT after normal positive selection[35], suggesting tight regulation of TCR signaling strength is of particular importance. Recent evidence suggests that the glycolipid antigen iGb3 contributes to positive selection[36], as well as β-GlcCer[37], as siRNA silencing of enzymes involved synthesis of the latter limits iNKT production. However, as some enzymes may be required in synthesis of multiple glycolipids, iNKT develop normally without iGb3, it is likely that other important selective antigens have yet to be identified[36]. Currently the exogenous antigen α-galactosylceramide (α-GalCer), which derives from a marine sponge, is used for both identification and activation of iNKT[38].

Positive selection of another unconventional T-cell subset, namely regulatory T-cells, is known to be heavily dependent receipt of co-stimulatory signals on TCR ligation[39]. This is similarly true of iNKT, which require the homotypic interactions of SLAM family members (namely SLAM and Ly108), for selection[40]. SLAM signaling is transduced into the cell via the SLAM adapter protein (SAP), which in turn activates Fyn kinase, although other mediators are likely involved, as the phenotypic loss of iNKT upon deletion is much greater in in the $SAP^{-/-}$ than $Fyn^{-/-}$ [41]. Signals provided during positive selection initiate a series of defined maturational stages specific to iNKT development, and defined by the expression of CD24, CD44 and NK1.1. iNKT uniformly down-regulate CD8 to become CD4 SP immediately after negative selection, and cells are identifiable immediately post-selection (stage 0) by

expression of CD24[42] - equivalent to $CD69^+$ conventional thymocytes[43]. HSA is then itself down-regulated (stage 1), and iNKT progressively up-regulate CD44 ($CD44^+NK1.1^-$, stage 2), before acquiring expression of the NK-cell marker NK1.1 ($CD44^+NK1.1^+$, stage 3)[31]. The majority of $CD44^+NK1.1^+$ iNKT in the thymus represent a mature resident population, retained in response to expression of CXCR3[44].

Development of iNKT can either be entirely confined to the thymus, or cells can exit to the periphery at stage 2, and progress to express NK1.1, although many peripheral iNKT remain $NK1.1^-$, and are functionally mature[45]. In either case, development is controlled by several transcription factors known to be integral to the development and function of other conventional and unconventional T-cell subsets, as well as external signaling by cytokines and co-stimulatory molecules[31]. Expression of the transcription factor promyelocytic leukemia zinc-finger protein (PLZF) in cells of haematopoietic lineage is largely restricted to iNKT, mucosal-associated innate lymphoid cells and some Yδ T-cells[31,46]. High levels of expression are induced by a combination of TCR and Ly108 signaling in all iNKT post-positive selection[15,40]. iNKT are greatly depleted upon deletion of PLZF – $PLZF^{-/-}$ iNKT show defective trafficking and cytokine production, stall at stage 0, and fail to down-regulate CD4 (normally lost by ~50% of iNKT in the wildtype)[15]. Conventional T-cells acquire memory markers and can produce IL-17 upon transduction of constitutively expressed PLZF[47]. PLZF is highly expressed in all thymic iNKT at stages 0-2, but undergoes T-bet mediated down-regulation when cells reach full maturity – all peripheral iNKT are $PLZF^{low}$ [15].

iNKT rely on two Tec kinases (Itk and Rlk)[25], as well as signaling through the Vitamin-D receptor[48], to initiate expression of the transcription factor T-bet[49], which is commonly associated with type 1 helper T-cells, but essential in proper iNKT development. Expression

of T-bet is initiated by co-stimulation through ICOS and CD28[50], the latter being required on both iNKT and conventional T-cells for intra-thymic proliferation of iNKT[42]. It is well established that iNKT undergo a period of intense proliferation in response to IL-15 early in their development during CD44 up-regulation[51]. Multiple transcription factors, including the cell-cycle regulator c-Myc[52], and NFκB family member RelA[53], are indispensible in this early proliferative burst, and iNKT are greatly reduced on their deletion[52,53]. Nuclear relocation of RelA through the classical NFκB pathway has been demonstrated to be of importance in inducing expression of the IL-receptor common γ-chain (γ_C), which sensitizes $CD44^{low}$ iNKT to IL-15- and, less significantly, IL-7-mediated proliferative responses in vitro[53]. IL-15 is trans-presented by high-affinity receptors on the surface of mTEC - where it drives extensive proliferation of early iNKT[54]. IL-15 expression by mTEC, as well as peripheral DCs and stromal cells, is also required for homeostasis of iNKT within the thymus (mature $NK1.1^+$ iNKT) and liver (all $CD44^{hi}$ iNKT) respectively, but not the spleen, where it's absence is likely compensated by other factors[36]. Another cytokine – TGFβ - is not required for thymic development of conventional T-cells, but is known to play a role in several developmental processes in iNKT; it drives early expansion and survival, and maintains expression of T-bet / CD122 in mature cells, enabling passage to the $NK1.1^+$ stage[31].

2.3: Extra-thymic maturation, and unique sub-populations

Although we have long been aware of the presence of phenotypically distinct populations of iNKT in terms of surface maker (NK1.1 and CD4) and cytokine expression, these have been usefully characterized to a greater extent in a recent paper by Watarai et al[16]. Thymic iNKT were divided on the basis of expression of IL-17RB (the IL-25 receptor) and

CD4, and unique homeostatic requirements and cytokine profiles were observed in IL-17RB$^-$ CD4$^{+/-}$, IL-17RB$^+$CD4$^+$ and IL-17RB$^+$CD4$^-$ iNKT[16]. IL-17RB$^-$ cells were entirely NK1.1$^+$ (whereas IL-17RB$^+$ are largely NK1.1$^-$) and the only population to express CD122, signifying their requirement for IL-15 in homeostasis[16]. Conversely IL-17RB$^+$ iNKT were normal in IL-15$^{-/-}$ mice[16]. Upon activation, IL-17RB$^-$ cells produced IFNγ in response to IL-12[16]. IL-17RB$^+$CD4$^+$ iNKT seemed to represent a significant portion of CD44^{low} (stage 0-1) cells - although many were also CD44$^+$NK1.1$^-$ (stage 2) – and required IL-25 to produce an array of Th2 (IL-4, IL-13), Th17 (IL-17, IL-22) and Th9 (IL-9, IL-10) cytokines[16,56]. The immature phenotype of these cells is unsurprising given that all iNKT are known to express CD4 immediately post-selection[27]. Finally, IL-17RB$^+$CD4$^-$ iNKT were the previously identified subset expressing RORgt, which produce IL-17, requiring IL-23 signaling[16,27,28].

This paper also went on to establish that, although immature (stage 1-2) iNKT were largely IL-17RB$^+$, some IL-17RB$^-$ cells were present[16]. These appeared to represent a distinct subset of precursors, as stage 1-2 IL-17RB$^+$ cells sorted into fetal thymic organ cultures ablated of haematopoietic cells failed to form mature IL-17RB$^-$ iNKT, and vice versa[16]. It is currently unclear at which early stage, and under what conditions these phenotypically distinct iNKT subsets initially diverge. CD4-reporter mice have shown that all CD4$^-$ iNKT originate from CD4$^+$ precursors[27], but not whether these precursors are common to CD4$^{+/-}$ cells, or if cells are differentially imprinted from the DP stage. It has recently been determined that TGFβ signaling, although a requirement in the development of all iNKT sub-populations, acts via different pathways to promote development. RORgt$^+$ iNKT from the IL-17RB$^+$CD4$^-$ subset are promoted to survive through a SMAD4-dependent pathway, and require TGFβ for peripheral homeostasis, whereas for RORgt$^-$ iNKT development occurs

independent of SMAD4, and survival requires IL-15 signaling[27]. It however remains unclear whether these findings on SMAD4 independence hold true if RORgt^{-} populations are divided based on IL-17RB expression.

Another recent paper used in situ thymocyte labeling to distinguish recent thymic emigrant (RTE) iNKT (which were Neuropilin-1^{+}) in the spleen and peripheral lymph nodes (LNs), and determined not only that these largely consist of IL-4- and IL-17-producing cells, but that the majority of peripheral IL-17^{+} cells were in fact RTE[45]. Although these cells were not phenotyped on the basis of surface marker expression, this suggests that mature NK1.1^{+} iNKT are minimal among those cells exported from the thymus, and require additional maturation steps to acquire cytokine expression, whereas NK1.1^{-} cells are largely functionally mature[45]. That IL-17^{+} cells were all Neuropilin-1^{+} in the spleen does not necessarily confirm that these cells are short-lived – it is also possible that RORgt^{+} cells either change cytokine expression over time, or instead relocate. In fact, tissue distribution of iNKT subtypes is quite varied[16], likely reflecting the availability of specific factors for homeostasis, as well as imprinted homing markers.

IL-17RB^{+}CD4$^{+/-}$ iNKT are in fact greatly enriched in the lung[20] in addition to the tissue draining LNs[16], largely as a result of expression of CCR4[20,56] (although CCR7 is also co-expressed). CCR4 is detected on these populations, but not IL-17RB^{-} iNKT, from generation in the thymus[16]. Airway hyper-responsivity (AHR), an autoimmune model of asthma, can be induced in mice through intra-nasal admission of α-GalCer as a result of IL-23-dependent cytokine production by iNKT[56,57], but is abrogated when mice are pre-treated with anti-CCL17/22 antibodies[20]. This effect is replicated when wildtype mice are reconstituted with CCR4$^{-/-}$ bone marrow[20], suggesting that chemokine receptor homing of iNKT to the lungs is

an absolute requirement of AHR development. However, to our knowledge, no study has as yet analyzed the importance of iNKT chemokine receptor expression in the context of intra-thymic development. This issue is particularly interesting knowing that iNKT are lost in mice with disrupted medullary development – this is true of both RelB$^{-/-}$ and NIK$^{-/-}$ mice[58], which present with defective medullary architecture, and the absence of CD80^{hi} mature mTEC[59]. Some data also suggests that iNKT development is impeded in Aire$^{-/-}$ mice[60], although reports are conflicting[61].

2.4: Project aims

The aims of this project were as follows:-

1. Identify the maturational stages associated with key events in iNKT development, including PLZF expression and proliferation
2. Phenotype iNKT populations in the thymus and periphery of wildtype mice
3. Determine whether medullary homing chemokine receptors (CCR4 and CCR7) are expressed in iNKT
4. Examine the effects of deletion of CCR4 and CCR7 on development and homeostasis of iNKT

Section 3.0: Methods

3.1: Animals

Wildtype C57/BL6 (B6) and B6 background Foxp3-GFP reporter mice were used as controls for B6 background CCR4$^{-/-}$, CCR7$^{-/-}$ and CCR4$^{-/-}$CCR7$^{-/-}$ mice. All mice were housed in

the BMSU under germ-free conditions, aged from 6-10 weeks, and sacrificed using a schedule 1 method of killing.

3.2: Media & reagents

RF10 medium was used for disaggregating tissues, as well as re-suspending cells in a final volume for the purpose of counting, and consisted of RPMI medium (Sigma) with 10% heat-inactivated fetal calf serum (FCS), 2% penicillin and streptomycin, and 1% glutamine. "FACS buffer" was used for suspension of antibody cocktails in staining for flow cytometry, and consisted of PBS with Ca^{2+} and Mg^{2+} (Sigma), with 3% FCS.

3.3: Tissue preparation for flow cytometry

Thymus, spleen and 2x inguinal lymph nodes were isolated and cleaned of excess fat and connective tissue under a light microscope using forceps. The cleaned organs were disaggregated between two frosted microscope slides, and pipetted through a fine gauze filter into a 25ml falcon tube, before centrifugation (1400rpm for 4 minutes as standard). Splenocytes were subjected to red blood cell (RBC) lysis through suspension in 2ml RBC lysis buffer (Sigma) at room temperature for 5 minutes, before neutralization with 2ml RF10 and centrifugation. The supernatant was removed, and cells re-suspended in a known volume of RF10 (5ml thymus & spleen, 1ml lymph nodes), before cells were counted using counting beads. This was done in a solution containing 10µl beads (10^4 beads), and 100µl cell solution, where cell numbers were calculated based the ratio of cells:beads in a plot of FSC vs. SSC. Cell suspensions were stored on ice at all times except where stated.

3.4: Staining for flow cytometry

Cells were aliquoted into 1.5ml eppendorfs ($5x10^6$ cells for samples, $2x10^6$ for single colours), centrifuged, and supernatant removed. Single colour samples - used for compensation of samples through the BD FACS-Diva software - were stained in 50µl, and samples 100µl of staining solution, which consisted of antibody cocktails suspended in FACS buffer. Samples were re-suspended in 200µl FACS buffer in FACS tubes, run on the BD LSR-Fortessa, and analyzed using FlowJo software v8.7.

All samples were first suspended in Fc block (CD16/32, BD biosciences, 1:100) for 20 minutes on ice. Cells were spun down and the supernatant removed prior to re-suspension in the first antibody stain. All stains were applied for 30 minutes on ice, with the exception of CCR7 PE, which was refrigerated for 2hrs, and all were washed in 1ml FACS buffer x1 between steps, and x2 between intracellular stains and prior to final re-suspension. Cells were surface stained for the following; CD4, CD8, CD24, CD44, TCRβ, NK1.1, CCR4 and CCR7 (see **table 1**). iNKT were picked up using a CD1d tetramer loaded with synthetic αGalCer - PBS57 loaded tetramer BV (NIH, 1:200), or empty tetramer BV as a control.

Permeabilization and fixation of cells for intracellular staining was conducted using the ”Foxp3 staining buffer kit” (eBioscience), wherein cells were suspended in 400µl “Fix / Perm” buffer (1:3 concentrate: diluent) for 40mins (refrigerated), and washed x2 in 1ml 1:9 “Perm / Wash” buffer. Intracellular staining was conducted using the following antibodies suspended in Perm/Wash buffer; Ki67 (or isotype control), mouse IgG1 anti-PLZF, rat anti-mouse IgG1 (see **table 1**). Rat anti-mouse secondary was absorbed in rat serum for 10 minutes on ice at 1:50, then diluted with Perm/Wash buffer for staining.

3.5: Cell sorting

Cells from 2x B6 thymi were isolated and stained in 1ml staining solution containing CD8 FITC (1:200) for 30 mins on ice. Thymocytes were enriched through sorting on the $CD8^-$ population, and then stained in 400µl for; NK1.1 PE (1:200), CD44 PeCy7 (1:2000), TCRβ APC Cy7 (1:100), and separately αGalCer loaded CD1d tetramer APC (1:200). Stained cells were then sorted by Moflo cell-sorter into three maturational subsets of $tetramer^+TCRb^{Int}$ iNKT; stage 0-1 ($CD44^-NK1.1^-$), stage 2 ($CD44^+NK1.1^-$) and stage 3 ($CD44^+NK1.1^+$). Purity was confirmed through analysis on BD FACS Diva software on the LSR-Fortessa, and cells were frozen down for storage.

3.6: Quantitative Polymerase chain reaction (qPCR)

mRNA was isolated and converted to cDNA according to protocol using the MACS One-step cDNA kit (Miltenyi Biotech). qPCR was performed using 2-300nM gene specific primers for β-actin, CCR4, CCR7, RANKL and CD40L in a reaction buffer containing Absolute qPCR Sybr mix without ROX reference dye (Abgene, AB-1158), dH_2O, and isolated cDNA in a volume of 15ul. Primer sequences for CCR4, CCR7, CD40L and RANKL are listed in ***table 2***. Target genes were quantified relative to the housekeeping gene β-actin, with respect to reaction efficiencies, as described by Pfaffl in 2001[62]. cDNA was obtained from sorted iNKT subpopulations (above), as well as stock solutions of DP and SP thymocytes, sorted according to expression of CD4 and CD8, with CD4 SP thymocytes further subdivided on the basis of CD69, CCR9 and CCR7 expression (early – $CD69^+CCR9^+CCR7^-$, intermediate - $CD69^+CCR9^-CCR7^+$, late - $CD69^-CCR9^-CCR7^+$).

3.7: Statistical analysis

Graphical representation of data and statistical analyses were accomplished using Prism 6 software (Graphpad), and all p values shown are the results of unpaired t-testing, where "ns" = non-significant, "*" = $p<0.05$, "**" = $p<0.01$, "***" = $p<0.001$, and "****" = $p<0.0001$.

4.0: Results

4.1: iNKT development is severely disrupted upon deletion of medullary homing chemokine receptors

iNKT can be detected by flow cytometry by staining using tetrameric CD1d complexes loaded with the synthetic glycolipid α-Gal-cer. When comparing iNKT populations in the thymus of wildtype B6 with $CCR4^{-/-}$, $CCR7^{-/-}$ and $CCR4^{-/-}CCR7^{-/-}$ (DKO) mice, we observed a remarkable reduction in the percentage of thymocytes displaying the iNKT TCR in all KO mice (***fig. 1.1***). In spite of the established role of CCR4 and CCR7 in medullary homing of positively selected cells[8,14], no overt defects in thymocyte maturation in KO models have been previously reported, and hence any proportional changes are likely to reflect actual numerical differences (unpublished data). However, altered thymic architecture, and limitations in the repertoire of negatively selected thymocytes have been observed in $CCR7^{-/-}$ mice[13]. We did observe slight increases in the proportion of $CD4^{+}8^{-}$ $TCR\beta^{Hi}$ thymocytes in the $CCR4^{-/-}$ and DKO thymus (***fig. 1d***), but these were unlikely to be large enough distortions to account for the reduction in iNKT observed.

After positive selection, iNKT uniformly down-regulate CD8 to become CD4 SP (although CD4 expression is then itself lost by ~50% of iNKT, ***fig. 1.2***), and follow a unique

Figure 1.1 – iNKT distribution in the thymus of CCR KO mice

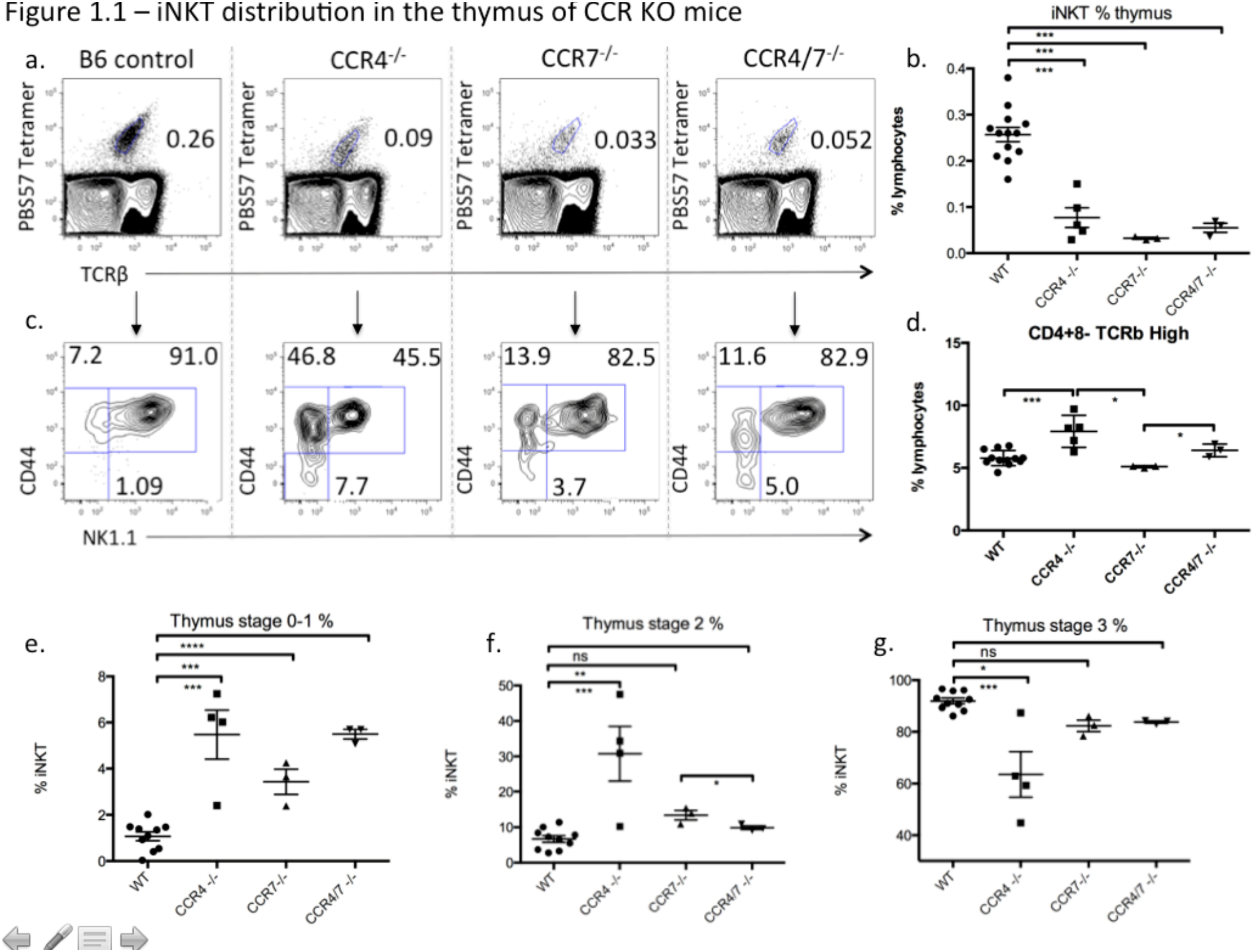
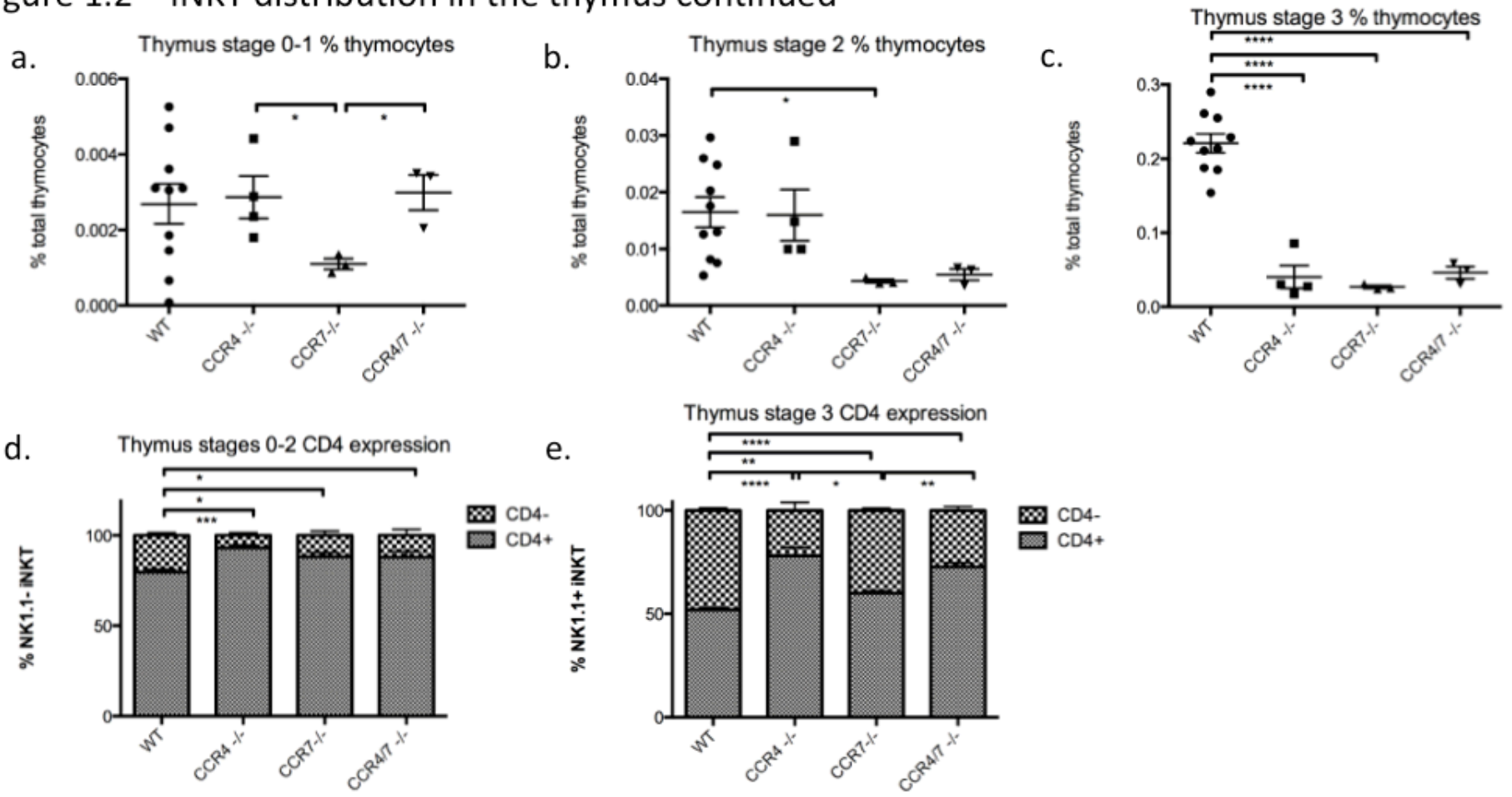


Figure 1.2 – iNKT distribution in the thymus continued



iNKT at maturational stages 1-3 are represented as a percentage of total thymocytes, rather than the % of iNKT in fig. 1.1 (**a-c**). CD4 expression on iNKT in the thymus is also displayed – CD4+ and CD4- iNKT are shown as a proportion of the NK1.1^{-} (stages 0-2, **d**) and NK1.1^{+} (stage 3, **e**) subsets.

sequential program of expression throughout development. The earliest stages are defined by expression of CD24 (Stage 0), which is down-regulated (Stage 1), prior to sequential up-regulation of CD44 (Stage 2) and NK1.1 (Stage 3). As expression of CD24 at the early stages is relatively heterogeneous, we divided iNKT maturational subsets into 3 groups; stage 0-1 ($CD44^{-}NK1.1^{-}$), stage 2 ($CD44^{+}NK1.1^{-}$) and stage 3 ($CD44^{+}NK1.1^{+}$). In the wildtype thymus, the vast majority (91.8 ± 1.0%) of cells are stage 3 (***fig. 1.1***). However, there were significant increases in the proportion of iNKT in the immature stages 0-1 in all of the KO models studied, as well as an increase in the percentage of iNKT at stage 2 in the $CCR4^{-/-}$ and $CCR7^{-/-}$, which was by far most evident in the $CCR4^{-/-}$, wherein only half of cells were $NK1.1^{+}$ (***fig. 1.1***). By examining iNKT at each stage of development as a proportion of total thymocytes, and assuming total cellularity is unchanged, it is possible to directly compare mature and immature populations between the KO models (***fig. 1.2***). This approach reveals iNKT to be lost from stage 0-1 onwards in the $CCR7^{-/-}$ thymus only, stage 2 onwards in DKO mice, and with only $NK1.1^{+}$ cells absent in the $CCR4^{-/-}$ (***fig. 1.2***).

4.2: Early stage expansion and expression of the transcription factor PLZF define iNKT development

Given the raised percentage of less mature subsets of iNKT in the thymus of CCR KO mice, we wished to analyze the events underpinning iNKT development that may be disrupted in the KOs. Using the marker Ki67 – a nuclear protein associated with proliferation – we examined the proliferation status of iNKT between developmental stages 0 and 3. Most (80%) of iNKT at stage 0-1 were actively proliferating, with fewer proliferating cells present

Figure 2 – Ki67 and PLZF expression in iNKT developmental stages, and NK1.1$^{+/-}$ populations

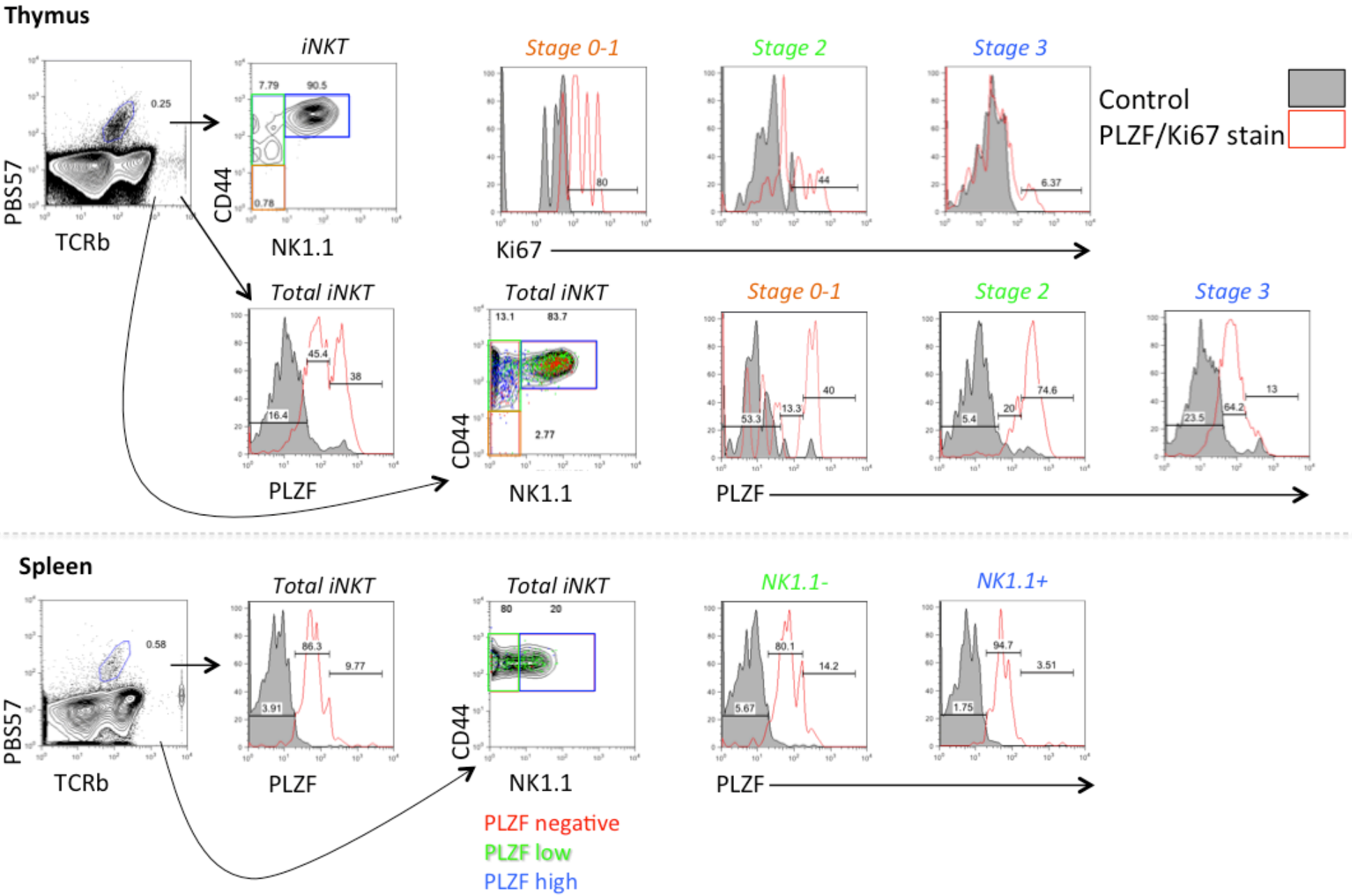


Figure 3 – CCR4 and CCR7 expression in iNKT

Thymus

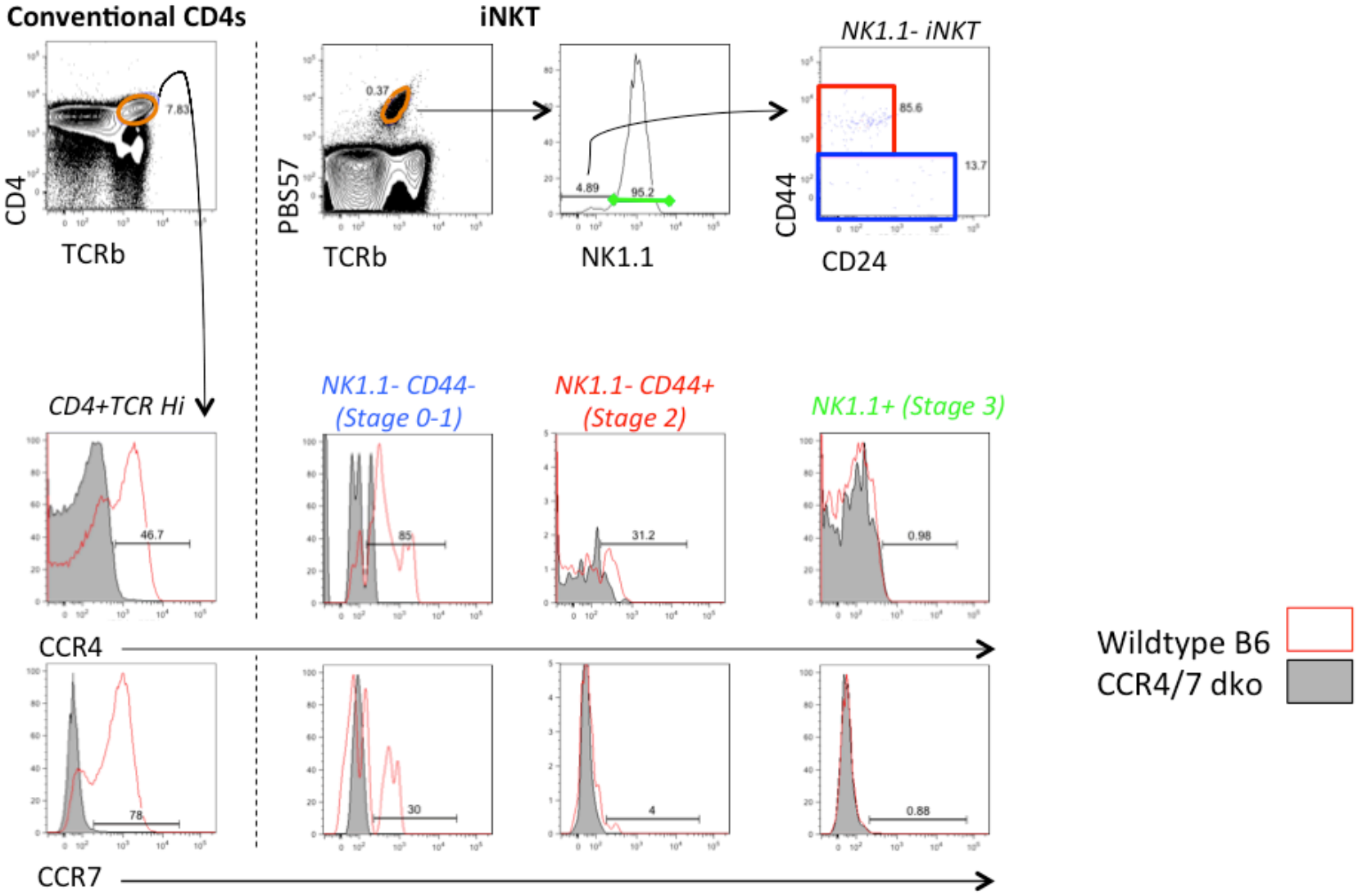
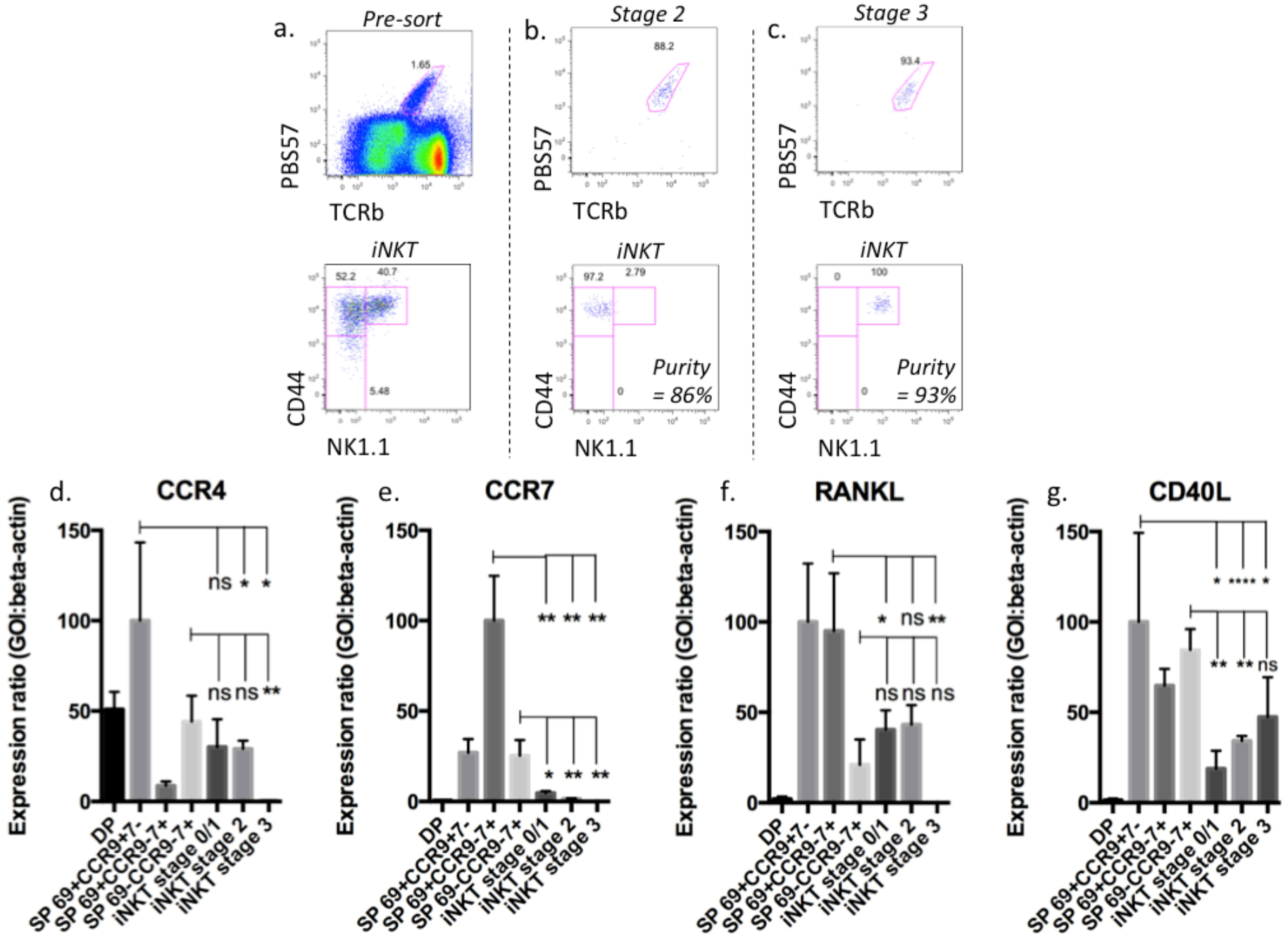


Figure 4 – sorting thymic iNKT into maturational stages, and PCR analysis of CCR, CD40L and RANKL expression



as cells matured (44% at stage 2, 6% at stage 3) (***fig. 2***). This suggests that an early proliferative burst occurs immediately after positive selection on cortical DP thymoctes.

The transcription factor promyelocytic leukaemia zinc finger protein (PLZF) is known to be the master transcription factor for the iNKT lineage[15], and is both necessary and sufficient for adoption of the distinct iNKT phenotype[47]. The vast majority of thymic iNKT were shown to be $PLZF^{+}$ (84%), with those cells separated into two distinct populations – $PLZF^{Hi}$ and $PLZF^{Lo}$ (***fig. 2***). PLZF expression is induced at the earliest stages of development, as 40% of stage 0-1 cells are $PLZF^{+}$, expressing high levels of the protein (***fig. 2***). High expression of PLZF persists into stage 2, wherein it is down-regulated in progression to stage 3. Expression of low levels of PLZF appears phenotypic of mature iNKT, as all peripheral (splenic) iNKT are $PLZF^{Lo}$ (***fig 2***). Early proliferation and PLZF expression, and subsequent down-regulation are key events in iNKT development that may be disrupted in the CCR KO thymus.

4.3: CCR4, but not CCR7, is directly expressed by cortically selected iNKT

CCR4 and CCR7 are involved in relocation to the thymic medulla prior to negative selection, and hence are co-expressed on SP4 thymocytes (***fig. 3***). FACs analysis of iNKT developmental stages showed significant expression of CCR4 on iNKT at stages 0-1, with expression reduced with increasing maturity, as only residual levels were detected at stage 2, and protein expression was absent by stage 3 (***fig. 3***). CCR7 protein was only detected on a minority (30%) of stage 0-1 cells, and at neither of the later stages (***fig. 3***). These findings were confirmed through PCR of sorted iNKT populations (***fig. 4***), wherein CCR4 mRNA comparable to some subsets of conventional SP4s was present in stages 0-1 and 2, but

Figure 5 – iNKT distribution in spleen of CCR KO mice

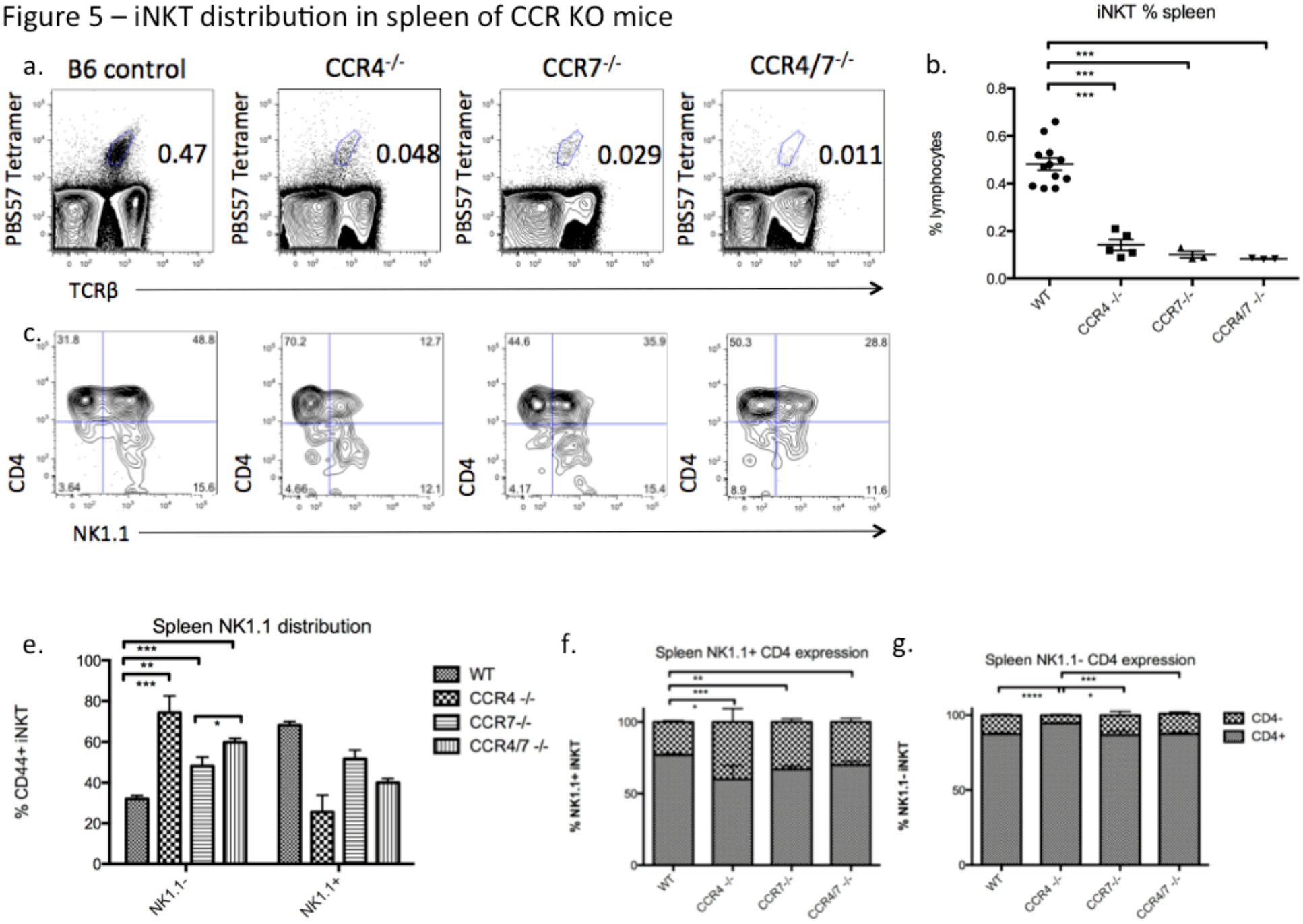
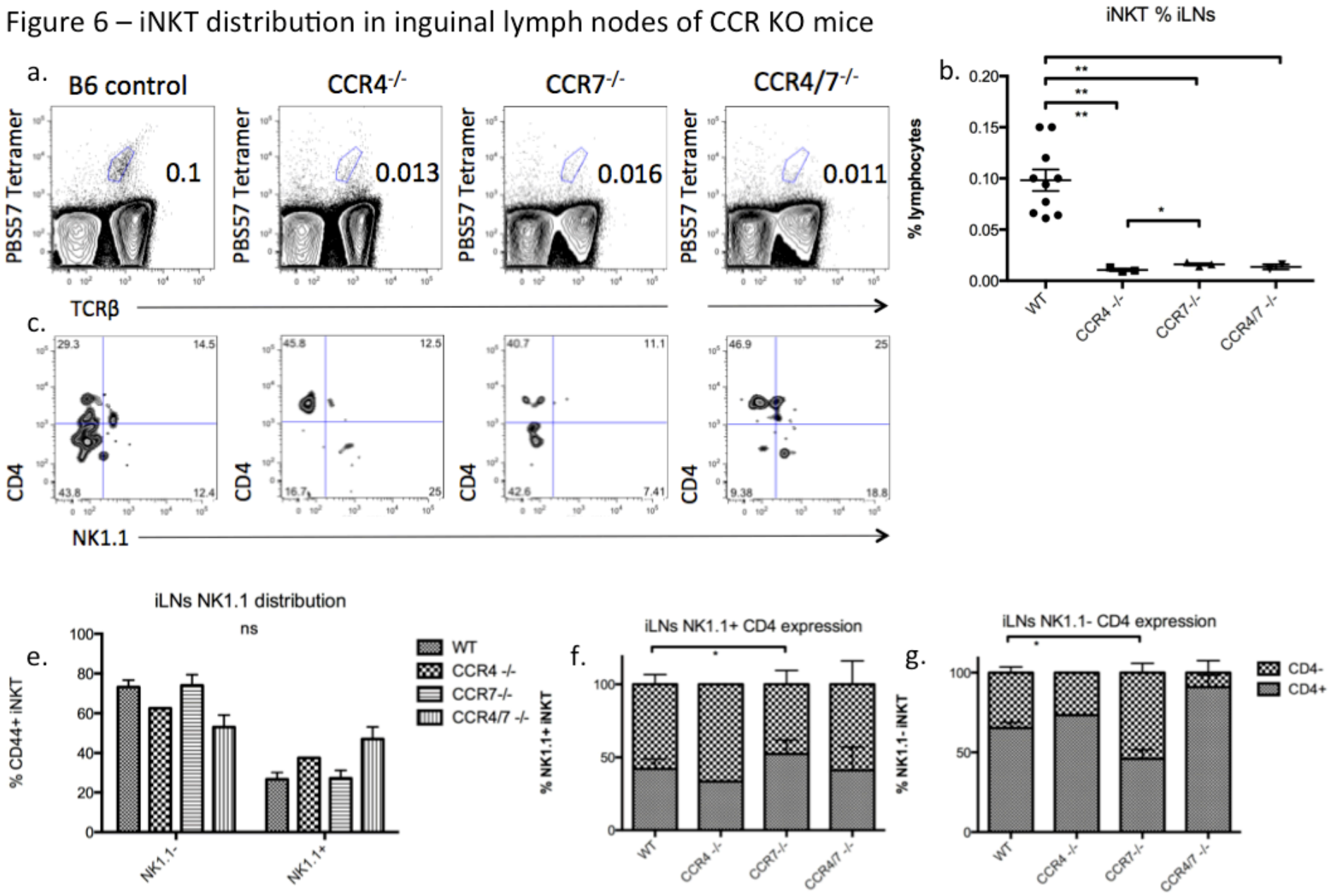


Figure 6 – iNKT distribution in inguinal lymph nodes of CCR KO mice

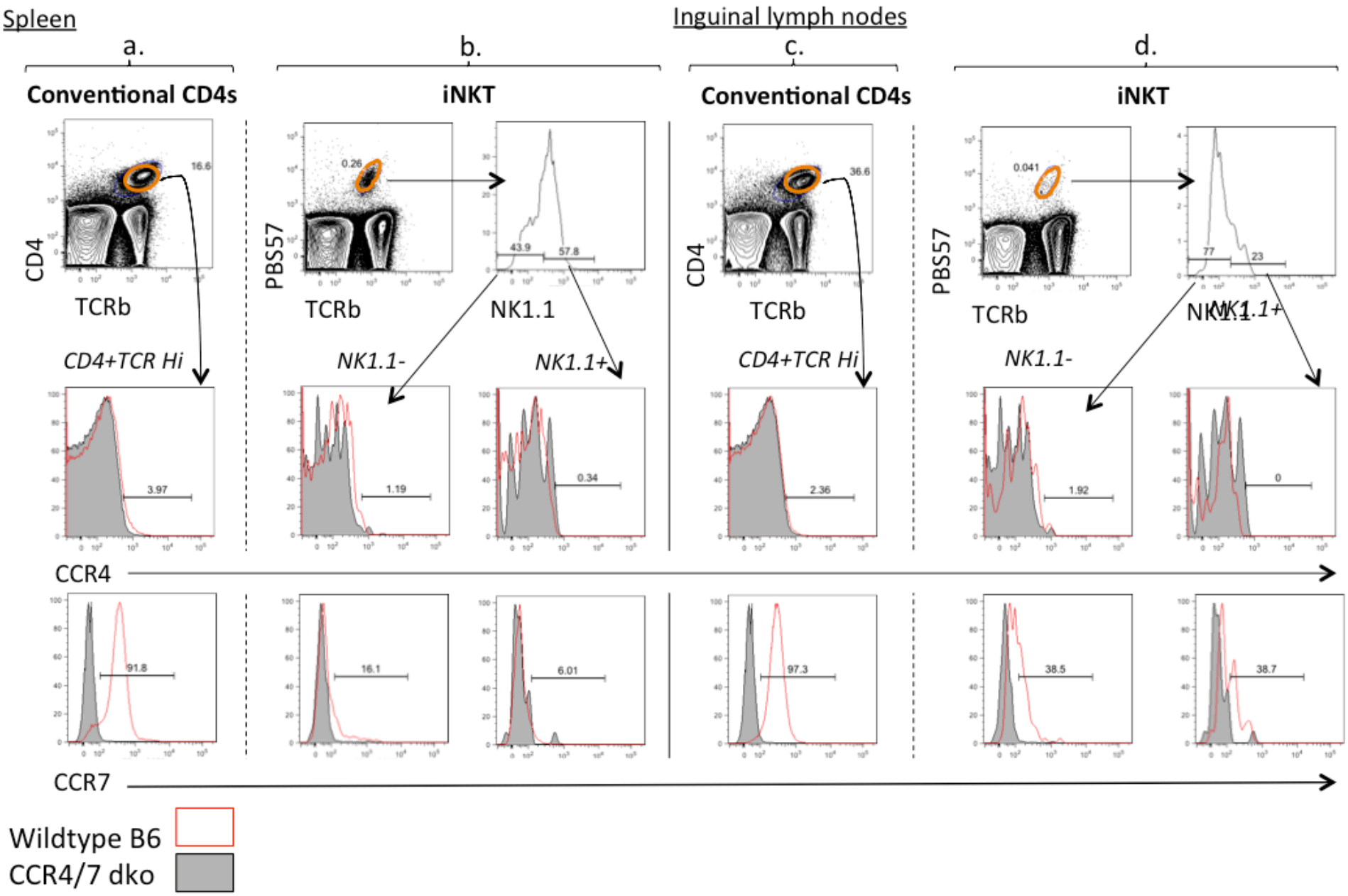


entirely absent in stage 3 iNKT. CCR7 mRNA was barely detectable, even at the earliest stages of maturation (***fig. 4***), suggesting that CCR7 expression is very transient at low levels, and unlikely to play a direct role in iNKT development. Sorted populations of iNKT were also tested for expression of mRNA for RANKL and CD40L – two signals known to influence mTEC development. Stage 0-2 iNKT (but not $NK1.1^+$ cells) express RANK-L at levels equivalent to those seen on early CD4 SPs, and iNKT were seen to express CD40L at all maturational stages, albeit at levels significantly lower than early CD4 SPs (***fig. 4***). CD40L did however appear to be up-regulated slightly on maturation, and $NK1.1^+$ iNKT expressed CD40L at levels equivalent to late $CD69^-$ SP CD4s (***fig. 4***).

4.4: The thymic defect in iNKT is not made up in the periphery

Peripheral iNKT are largely phenotypically mature (although some $CD44^+NK1.1^-$ present will still go on to express NK1.1), and can to some extent be divided into subsets with unique cytokine expression based on the markers CD4 and NK1.1. Populations of iNKT in both the spleen and inguinal lymph nodes (iLNs) of CCR4, CCR7 and DKO mice were examined by FACS. The peripheral iNKT were again dramatically reduced proportionally – the defect in thymic production was in no way made up through peripheral expansion in any of the KO models (***figs 5-6***). The drop in numbers of iNKT present in the periphery was also accompanied by a shift in NK1.1 expression – only 31.8±1.5% of iNKT are $NK1.1^-$ in the wildtype spleen, but this percentage was significantly higher in each of the CCR KOs, particularly the $CCR4^{-/-}$ (***fig. 5***). Among the $NK1.1^-$ population, there was also a relative reduction in the NK1.1/CD4 double negative population in the CCR4 KO spleen (***fig. 5***).

Figure 7 – CCR4 and CCR7 expression in peripheral iNKT



Analysis of iNKT in the iLNs yielded no such shift in NK1.1 expression (***fig. 6***) - although fluctuation in CD4 expressing cells was observed in the $CCR7^{-/-}$, the very low number of cells present largely devalues any phenotypic analysis. Similarly, it is difficult to ensure loss of iNKT numerically in the $CCR7^{-/-}$ and DKO spleen, as CCR7 is required for entry of lymphocytes into the lymph nodes, and hence the percentage drop seen may in part be attributable to retention of conventional lymphocytes in the spleen. This may be particularly relevant given the very low proportion of iNKT in the wildtype iLNs (***fig. 6***), as reflected in the relatively low proportion of $CCR7^{+}$ iNKT in the spleen (16.1% vs 91.8% of conventional CD4s) (***fig. 7***).

Phenotypic analysis of the iNKT present in the spleen may be similarly impeded. Firstly, iNKT in the iLNs are largely $NK1.1^{-}$ (73.2±3.4%, ***fig. 6***), conversely to splenic iNKT (31.8±1.5 $NK1.1^{-}$, ***fig. 5***), and hence retention would be likely to increase the proportion of $NK1.1^{-}$ cells. Secondly, CCR4 acts as a lung homing receptor for both conventional T cells and iNKT, and hence negligible $CCR4^{+}$ cells were observed among conventional splenic CD4s, nor iNKT (***fig. 7***). Again splenic retention could at least in part explain the reduced proportion of iNKT in the spleen and iLNs (the lungs are relatively sparsely populated by iNKT[16]), as well as the shift towards $NK1.1^{-}$ cells, as these are relatively enriched in the lungs (50.2%[16]).

Section 5.0: Discussion

5.1: Expression of medullary-homing chemokine receptors on iNKT

We have observed a dramatic reduction in the proportion of $tetramer^{+}TCR\beta^{+}$ iNKT in the thymus, spleen and iLNs of mice lacking CCR4 and/or CCR7. Although we were unable to determine the number of cells present in these experiments, subsequent pilot experiments

(not shown) have shown these proportional changes to correspond to actual numerical absence, rather than the expansion of other populations, and thymic cellularities were comparable to the wildtype. Although development of conventional T-cells continues as normal in CCR4$^{-/-}$ (unpublished data) and CCR7$^{-/-}$ mice[14], these chemokine receptors are known to play an important role by controlling relocation of positively selected populations to the medulla[8,14]. This allows negative selection of potentially auto-reactive thymocytes through exposure to mTEC-derived self-antigens, and the TCR repertoire of peripheral T-cells in CCR7$^{-/-}$ mice is known to contain auto-reactive cells capable of triggering tissue-specific auto-immunity[13].

Through both flow cytometry, and PCR using isolated mRNA from sorted iNKT populations, we were able to detect expression of CCR4 in early, NK1.1^{-} populations of iNKT (stages 0-2) in the thymus, but not mature NK1.1^{+} cells. All positively selected iNKT are known to express the transcription factor PLZF[15], however we have detected PLZF^{-} cells among stage 0-1 cells, suggesting some conventional SPs are present in our gating. As expression of CCR7 was only detected on very few stage 0-1 cells in FACS, and not by PCR of sorted populations where gating was more frugal, this suggests that the CCR7^{+} cells may be contaminating SPs. Our findings largely agree with a recent study, which suggests that these receptors are co-expressed on a newly identified population of IL-17RB^{+} iNKT, which are CCR4^{hi}CCR7^{low}, and lie entirely within the NK1.1^{-} bracket in the thymus, but not IL-17RB^{-} cells, which are NK1.1^{+} [16].

That thymic development of iNKT is heavily reduced in CCR4, CCR7 and DKO mice is interesting in the context of previous work on the co-stimulatory requirements of these cells. Multiple studies have shown that signaling through homotypic interactions of SLAM

family members SLAM and Ly108 is essential for positive selection of iNKT[29,40]. Signals from SLAM are transduced by the adapter protein SAP, which activates Fyn kinase[41]. One of the downstream targets of Fyn kinase is DOCK2[63], a protein with known involvement in cytoskeletal regulation, which also lies downstream of chemokine receptors[14]. Deletion of any of these proteins impedes iNKT development[40,41,63], and it may emerge that the effects in DOCK2$^{-/-}$ cells are in part a consequence of the absolute dependence of iNKT on chemokine receptor signaling.

5.2: Potential explanations of disrupted iNKT development in CCR KO mice

As we failed to observe significant CCR7 expression on iNKT, the defect in their development we observed in CCR7$^{-/-}$ mice must be indirect. iNKT in these mice, but puzzlingly not the DKOs, were reduced as a proportion of total T-cells from the very earliest stages (0-1), suggesting a problem in their selection, and assumption of the iNKT phenotype. Selection of iNKT requires interaction with DP thymocytes[64], which co-express CD1d and SLAM family members[29,40], the former of which presents glycolipid antigens[24]. iNKT are positively selected on the condition they have successfully formed a TCR capable of recognizing CD1d-antigen complexes, which is achieved only by recombination of a limited selection of gene segments[25]. iNKT TCRs have a common α-chain (Vα14-Jα18), and a limited array of β-chains (Vβ8.2, Vβ7.1, Vβ7.2), and are hence termed semi-invariant[25]. As the α-chain rearrangement required must occur late on in DP recombination, iNKT are highly susceptible to factors that reduce the $t_{1/2}$ of DP cells[29]. The agonist ligands for positive selection themselves have so far remained somewhat elusive, although evidence for the involvement of iGb3 and βGlcCer is mounting[24,65].

It is well established that development of conventional T-cells proceeds as normal in $CCR7^{-/-}$ mice, but SP thymocytes, which we have confirmed strongly express both CCR4 and CCR7, fail to relocate to the medulla after negative selection[13,14]. These cells may contribute to the failure of iNKT selection by competing for survival factors, and hence reducing the lifespan of DP thymocytes, although normal DP numbers are present in $CCR7^{-/-}$s[14]. Gene expression data-browser Immgen suggests that expression of CD1d persists at similar levels on SP thymocytes[66], although little is known about the capacity of these cells to express agonists and co-stimulatory molecules, and indeed mediate positive and negative selection of iNKT. Dendritic cells are known to produce differential arrays of glycolipids dependent on their state of activation[67], and hence it seems conceivable that SP thymocytes could express ligands at a higher density, or with a higher affinity, or instead differentially express co-stimulatory molecules: factors which could raise the propensity for negative selection of iNKT upon retention of these cells in the cortex. Through transgenic over-expression of CD1d, Chun et al.[32] established that a bone marrow (BM)-derived population, rather than thymic epithelial cells, was responsible for negative selection of iNKT through creation of $CD1d^{-/-}$→WT and WT→$CD1d^{-/-}$ BM chimeras. The paper then went on to establish that DCs could negatively select iNKT when co-transferred with BM cells into an irradiated lymphopenic host, as greater deletion was seen on transgenic overexpression of CD1d in sorted DCs, but didn't address the possibility that similarly bone marrow-derived thymocyte populations could make a contribution[32]. Greater difficulty lies in proving negative selection by SPs, as they are derived from the same precursors as iNKT themselves, and would likely require overexpression of CD1d under the control of a promoter specific to SP thymocytes.

Another possibility for the absence of early iNKT in CCR7$^{-/-}$ mice may be transient expression of CCR7 prior to down-regulation of CD8 – this would account for the discrepancy in CCR7 expression we observed between FACS, wherein DP cells already to have up-regulated TCR expression were included in our analysis, and PCR, where sorted populations were enriched for CD8^{-} cells. It is well established that DP thymocytes are confined to the thymic cortex, but although rare DP thymocytes do express CCR7[68], a role for it's expression has yet to be established. Cortical expression of CCL21 - a CCR7 ligand – does occur, but is restricted to the areas surrounding the vasculature[69]. Using CD11c-YFP reporter mice, combined with two-photon laser scanning microscopy, Ladi et al. recently identified a population of DCs that co-localize with CCL21 in the cortex[69]. Interactions between cortical DCs and DP thymocytes were frequent, and observed to be largely dependent on CCR7 expression[69]. The purpose of these interactions is currently unknown, but as CCR7 expression, like the iNKT TCR, is rare in DP thymocytes, it would be interesting to establish whether iNKT are part of the make-up of CCR7^{+}DP thymocytes. As tetramer staining in tissue sections has proven difficult, determining the cortical localization of iNKT immediately post-positive selection in respect to CD11c^{+} DCs may be possible using antibodies against PLZF and CD24.

In contrast to the CCR7$^{-/-}$, we observed positive selection of iNKT to continue at normal levels in mice lacking both CCR4 and CCR7, but these cells were proportionally reduced in the thymus from stage 2. During stage 1-2, iNKT up-regulate CD44, and undergo rounds of proliferation[52], as we have confirmed using the nuclear marker Ki67. Ki67^{+} cells represented the majority of stage 0-1 iNKT, but were less abundant after up-regulation of CD44, and a minority of cells among the NK1.1^{+} population. Multiple intracellular

components have been identified as essential for this proliferative burst, including cell cycle regulator c-Myc[52,70], and NFκB member RelA[53], which controls γ_C expression, sensitizing CD44^{low} iNKT to IL-15 and IL-7 signaling[53], which along with TGFβ appear to drive expansion[27,31]. Co-stimulatory signals are important in initiating this process – in spite of normal numbers of CD24^{+} post-selection iNKT, these cells fail to expand in both CD80/86$^{-/-}$ and ICOSL$^{-/-}$ mice[50] (although this second finding has recently been contradicted[57]), and expression of CD28, the receptor for CD80/86, is required on both iNKT and conventional T-cells for optimal proliferative responses[42,50].

Although it is difficult to speculate why iNKT are successfully positively selected in these mice, but not the CCR7$^{-/-}$, it is tempting to suggest that these cells fail to receive the retinue of cytokine and co-stimulatory signaling required for proliferation – this could easily be established by staining the few cells present for Ki67 or by intra-thymic BrDu injection. Within the thymus, IL-15, CD80/86 and ICOSL are both largely expressed by mTEC and DCs in the medulla[55,71,72], whereas TGFβ is ubiquitously expressed in most tissues. As CCR4 expression is likely required by iNKT to undertake the cortex-medulla transition, cortical retention and limitation of these factors would seem a likely cause for the failure of these cells to expand. However, stage 0-2 iNKT are present in proportions equivalent to the WT in CCR4$^{-/-}$ mice – here retention of SP thymocytes in the medulla is likely to be less severe, as their chemotaxis towards CCR7 ligands is much stronger than CCR4[8]. It may therefore be the case that SP thymocytes - which up-regulate some IL-receptors on positive selection[73] – remain in the cortex alongside iNKT in DKO mice, and out-compete iNKT for factors driving their proliferation which are more abundantly expressed in the medulla.

Large numbers of iNKT were seen to express CCR4, although expression was lost with increasing maturity, and absent in NK1.1^{+} cells in the thymus. iNKT were seen in normal proportions within total thymocytes at stages 0-2, but NK1.1^{+} cells were conspicuously absent. A similar phenotype has previously been observed in Aire$^{-/-}$ mice[60], where medullary production of the ligands for CCR4 (and CCR7) is reduced[11]. It therefore seems likely, given the involvement of CCR4 in trafficking of conventional SP thymocytes to the medulla, that a factor expressed exclusively within the medulla is essential for the up-regulation of NK1.1 expression on mature populations of iNKT. However, it would be difficult to conclusively prove that this defect is intrinsic to iNKT without selective CCR4 deletion – this could in theory be achieved through generation of a transgenic mouse expressing CRE recombinase under the control of PLZF promoter, although other unconventional T-cell types would be similarly affected[31]. As mentioned above, PLZF could be a candidate for locating iNKT in sections of WT and CCR4$^{-/-}$ thymi.

Progression to stage 3 is known to be dependent on CD1d[33,48], as TCR signaling activates RelA, and subsequently CD122 expression, for formation of the IL-15 receptor[53]. Although homeostasis of NK1.1^{+} iNKT requires IL-15[55], continued antigen recognition is not required, as demonstrated by the persistence of NK1.1^{+} cells transferred into CD1d$^{-/-}$ mice[33]. Similar transfer experiments could be used to investigate whether it is survival or formation of NK1.1^{+} cells in the thymus that is compromised by CCR4 deletion. In either case, if IL-15 is the limiting factor, admission of exogenous IL-15R-stimulating antibody would be expected to restore the presence of NK1.1^{+} cells (as IL-15 requires trans-presentation[55], exogenous IL-15 alone is unlikely to be effective). We have confirmed previous reports[15] that iNKT down-regulate PLZF expression when reaching full maturity, and all mature thymic and peripheral

iNKT are PLZF^{low}. PLZF controls the expression of memory markers, including those involved in tissue localization[47], and is also believed to control the down-regulation of CD4 in iNKT development[47], as all PLZF$^{-/-}$ iNKT are CD4^{+}. Intriguingly, we observed a significant skew in CD4 expression in NK1.1^{-} thymocytes towards CD4^{+} cells. NK1.1^{+}CD4^{-} cells, which were proportionally reduced (most notably in the CCR4$^{-/-}$), largely consist of IL-17RB^{+}RORgt^{+} iNKT capable of producing IL-17[16], although few precursors to NK1.1^{+} cells are likely present.

As PLZF likely plays a role in both switching off CD4 expression, as well as generating NK1.1^{+} iNKT, it is tempting to assume that expression of PLZF is dysregulated in CCR4$^{-/-}$ iNKT, although this has been difficult to corroborate, as iNKT are so few in the CCR4$^{-/-}$ thymus, and both cell numbers and tetramer staining are substantially lost in the permeabilization process involved in PLZF staining. That CD4^{-} cells are proportionally lost in all CCR$^{-/-}$ mice may reflect differential requirements of the newly established IL-17RB^{+} subset of iNKT. These cells develop normally in IL-15$^{-/-}$ mice[16], utilize alternative pathways in TGFβ signaling during development[27], and their cytokine secretion is defined by CD4 expression – CD4^{+} cells produce Th2, Th9 and Th17 cytokines, whereas CD4^{-} express IL-17[16]. It has been suggested that these cells evolve from a distinct developmental pathway, as early RORgt^{+} iNKT cannot go on to form RORgt^{-} or NK1.1^{+} populations in culture[28]. That both the proportion of iNKT of the CD4^{-}NK1.1^{-} phenotype and mature NK1.1^{+} iNKT as a whole are reduced suggests that both pathways are severely inhibited upon CCR deletion.

As iNKT are seen to be so severely depleted in the thymus it seems highly likely that the reduced proportions observed in the spleen and iLNs were as a result of failed generation, rather than problems with homeostatis, especially as experiments by our lab have demonstrated limited rebound expansion among iNKT in lymphopenic mice

(unpublished data). The reduction of iNKT among T-cells was equivalent in all three KO mice, with the exception of the CCR4$^{-/-}$ iLNs, which were slightly more depleted of tetramer^{+} cells, potentially reflecting the severe skew in CD4 expression in the thymus of these mice, as NK1.1^{-}CD4^{-} cells largely localize to the lungs and LNs[16,45]. The reduction in iNKT was also most dramatic in the iLNs out of all 3 tissues, which is unsurprising, as the majority of LN iNKT are thought to be recent thymic emigrants[45], and would therefore directly reflect thymic output.

Without confirming numbers of iNKT rather than percentages, it remains difficult to determine whether these are genuinely smaller populations of cells, as CCR4 and CCR7 are homing molecules in the periphery, directing T-cells to the lungs and skin, and LNs respectively[16,20]. Failure of conventional cells to enter their target tissues could raise their cell numbers in the spleen, diluting iNKT. Similarly, CCR4 and CCR7 are homing receptors for iNKT, thought to be expressed exclusively by NK1.1^{-}IL-17RB^{+} cells[16]. Consistent with this, we found significant levels of expression of CCR7 on iNKT in the lymph nodes (largely NK1.1^{-}), but very low levels in the spleen, where the majority are NK1.1^{+}. We failed to detect CCR4 expression on peripheral iNKT, likely as a result of these cells homing to the lungs. The representation of these populations in the periphery could be fully elucidated using IL-17RB as a marker, and obtaining accurate total cell counts.

5.3: Conclusion

Our finding that CCR4 expression is required on iNKT specifically for stage 3 progression, known to be dependent on medullary-expressed IL-15, suggests that relocation of cells to the medulla plays an important role in the maturation of iNKT. iNKT have been

found to be reduced in mice with defects in medullary development – RelB$^{-/-}$ [58], NIK$^{-/-}$ [58], Aire$^{-/-}$ [60] and now CCR7$^{-/-}$ mice, which are known to have altered medullary architecture[14]. It is possible that this comes as a result of failure of SP thymocytes to enter the medulla, as they are known to provide CD40L, a signal required for mTEC maturation[2]. However, we have observed NK1.1^{+} iNKT, which are known to be a resident population within the thymus[44], to express both CD40L and RANKL, the latter of which is utilized by lymphoid tissue inducer cells (LTi) to initiate development of the mature medulla[2]. Thymic size is reduced by ~25% in PLZF$^{-/-}$ mice, and NK1.1^{-} iNKT have also been shown to be capable of producing IL-22[16], a trophic factor also produced by LTi, and thought to induce TEC turnover for post-stress thymic recovery[74]. It is therefore possible that, as well as being affected by dysregulation of thymic stromal cell development, iNKT contribute to maturation and proliferation of TEC populations, which are disrupted in their absence in CCR7$^{-/-}$ mice.

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Table 1:
Antibodies for FACS

Antibody	Surface (S) / Intracellular (IC)	Concentration	Supplier / Clone
CD4 V500	S	1 : 200	BD Horizon, RM4-5
CD8 APC	S	1 : 800	eBioscience, 53-6.7
CD24 PerCP Cy5.5	S	1 : 1000	eBioscience, M1/69
CD44 A700	S	1 : 100	eBioscience, IM7
TCRb 780	S	1 : 100	eBioscience, H57-597
NK1.1 PE	S	1 : 200	eBioscience, PK136
CCR4 APC	S	1:200	BioLegend, 2G12
CCR7 PE	S	1:50	eBioscience, 4B12
PLZF mouse monoclonal IgG1	IC	1:50	Santa Cruz, D-9
rat α-mouse IgG1 APC	IC	1:200	BioLegend, RMG1.1
Ki67 PeCy7	IC	1:400	BD Pharmingen, B56

Table 1:
Primers used in qPCR

Gene name	Genbank Accession Number	Forward primer	Reverse primer	Product length
β-actin	NM_007393	Predesigned (Qiagen)		149
CCR4	NM_009916.2	AACAGAGCAGTGCGCATGAT	CGTTGTACGGCGTCCAGAA	67
CCR7	NM_007719.2	TATCCGTCATGGTCTTGAGC	TATCCGTCATGGTCTTGAGC	351
CD40L	NM_011616.2	CCTTGCTGAACTGTGAGGAGA	CTTCGCTTACAACGTGTGCT	Not found
RANKL	NM_011613.3	CACACCTCACCATCAATGCTGC	GAAGGGTTGGACACCTGAATGC	394

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Abstract

Natural regulatory T-cell (nTreg) are a T-cell subset developed in the thymus which possess the unique capability to actively suppress antigen-specific CD4 responses in the periphery, which have been shown to be essential in the prevention of auto-immunity through deletion of their lineage-defining transcription factor Foxp3. $Foxp3^+$ nTreg possess T-cell receptors (TCRs) with high auto-reactivity relative to conventional cells, and are believed to require strong TCR-ligation and co-stimulation during negative selection for the production of $CD25^+$ precursors. More recently, it has been demonstrated that peripheral Treg are a heterogenous population, consisting of death-prone, as well as highly suppressive effector-memory subsets, which can be separated on the basis of expression of the activation markers ICOS and CD44. In this study, we have demonstrated $ICOS^+CD44^+$ memory Treg to have unique homeostatic requirements from conventional CD4 memory cells, which depend on provision of co-stimulation by TNF superfamily members CD30L and OX40L for survival. We did however discover a role for OX40 signaling in development of nTreg, as well as establishing redundancy in mTEC expression of the co-stimulatory molecules CD80/86 and ICOSL, the former of which has been identified as a key molecule for generation of both nTreg precursors and $ICOS^{hi}CD44^{hi}$ Treg.

Section 1.0: Acknowledgements

I would like to extend my gratitude to the following people for their help and support throughout this project – my project supervisors, Professors Graham Anderson and Peter Lane, as well as the entirety of the Anderson and Lane labs; William Jenkinson, Andrea White, Kyoko Nakamura, Sonia Parnell, Jennifer Cowan, Song Baik, Amanda Holland, David Withers, Emily Halford, Fiona Gaspal, Emma Mackley, Claire Marriott, and Maher Nawaf.

Section 2.0: Introduction

2.1: Thymic development of natural regulatory T cells

Regulatory T cells (Treg) are a phenotypically distinct population characterized by their expression of CD25, as well as the transcription factor Forkhead Box P3 (Foxp3)[1]. Treg are capable of mediating Foxp3 dependent antigen specific suppression of T cell responses through secretion of cytokines (including IL-10 and TGFβ)[2] and expression of cell surface receptors (CTLA-4)[3]. The suppressive function of Treg has been repeatedly demonstrated to be essential in preventing T cell mediated auto-immunity, as observed upon neonatal thymectomy[4] (as the appearance of peripheral Treg is delayed relative to conventional T cells), and particularly severely in $Foxp3^{-/-}$ and Foxp3 mutant "scurfy" mice[5]. Two main pathways for Treg production are known; Foxp3 expression can be triggered intrathymically from immature thymocyte populations to produce natural Treg (nTreg, generation of which

is the main focus of this study), or instead induced in naïve peripheral T cells (iTreg) through interaction with tolerizing dendritic cell (DC) populations.

Thymic T cell development is a multistage process, consisting of checkpoints involving recognition of antigen bound MHC complexes on the surface of thymic epithelial cell (TEC) populations via the newly formed T cell receptor (TCR). The thymus is divided into two sub-compartments; the cortex and the medulla, which play residence to, and are defined by, unique populations of TEC, cortical (cTEC) and medullary (mTEC)[6,7]. Bone marrow derived T cell precursors enter the thymus at the cortico-medullary junction, and pass through the cortex while undergoing several key developmental steps that result in formation of a TCR of unique specificity[8], as well as up-regulation of CD4 and CD8, which act as co-receptors for MHC class II and class I respectively[9]. These cells are described as CD4/CD8 double positive (DP), and require survival signals from ligation of a successfully formed TCR by cTEC displayed MHC in a process known as positive selection[10]. These cells then down-regulate either CD4 or CD8 to become single positive (SP) thymocytes, and traffic to the thymic medulla, where they undergo further selection by mTEC and DCs[11].

mTEC are capable of promiscuously expressing tissue restricted self-antigens, partially under the control of the autoimmune regulator (Aire)[12], allowing determination of the fate of SP thymocytes based on strength of their recognition of self-antigen, and hence the potential for auto-reactivity in the periphery. Medullary DCs are also capable of obtaining and cross-presenting these antigens[13], and contribute to selection[14]. If TCR signaling strength is over a certain threshold level, thymocytes are deleted, however some cells escape this process[11], hence the requirement for Treg. The TCR repertoire of Treg is distinct from that of conventional $CD4^+$ T cells, possessing higher self-reactivity[15]; formation

of $CD25^{+}Foxp3^{-}$ Treg precursors is induced by strong TCR ligation below the threshold for negative selection[15]. Treg are known to be reduced in the $Aire^{-/-}$ thymus[16], although recent data suggest that this is through loss of the Aire dependent DC chemo-attractant XCL1, and the absence of DC accumulations in the thymic medulla, rather than auto-antigen expression[17].

Selection of Treg is thought to be a 2-step process[18]; the first step consists of commitment to the Treg lineage, mediated by strong TCR ligation[15] and essential CD80/86-CD28 costimulation[18,19,20] (common to murine and human Treg development[21]), causing up-regulation of CD25 - the alpha chain of the IL-2 receptor – and c-Rel dependent formation of Treg precursors[22]. This in turn renders newly formed precursors receptive to γ_c cytokine signals, which trigger Foxp3 expression in precursors[18], with IL-2 dependency a lasting trait of mature nTreg[23], which are marked by expression of Neuropilin-1[24]. Several factors expressed in the thymic medulla have been shown to play an important role in production of precursors, and induction of Foxp3 expression. Deletion of CD80/86 greatly reduces thymic precursors[20], and has a secondary effect on the development and homeostasis of $Foxp3^{+}$ cells as it reduces conventional CD4 production of IL-2[18]. CD70 is another co-stimulatory ligand expressed by mTECs and DCs. It is expressed on all $Aire^{+}$, and some $Aire^{-}$ mTEC, although it is not yet known whether $CD70^{+}Aire^{-}$ cells are precursors yet to initiate Aire expression[25]. It does however appear certain that expression on both mTEC and $CD8\alpha^{+}$ DCs is essential for development of normal numbers of nTreg, with the defect in $CD27^{-/-}$ thymi causing loss of $Foxp3^{+}$ cells rather than precursors, although Foxp3 expression itself is CD27 independent[25].

CD40-CD40L interactions are also thought to play crucial but indirect roles in homeostasis of thymic Treg. $CD40^{-/-}$ mice show greatly reduced thymic Treg in the adult, but not newborn thymus, a puzzle solved neatly by analysis of $CD40^{-/-}$RAG-2p-GFP Treg, wherein GFP expression lingers in cells ($t_{1/2}$ 56hrs) after TCR rearrangements, allowing determination of newly formed and resident/recirculating populations in the thymus[23]. 60% of Treg were seen to be GFP^-, and it was this resident population only which was affected upon deletion of CD40, apparently as a result of depleted IL-2 expression[23]. The loss of CD40 had a minimal effect on generation of new Treg[23], but the observation of a dominant GFP^- subset of Treg in the thymus poses the question of whether these cells are resident to the thymus, undergo prolonged intrathymic maturation relative to conventional cells, or recirculate from the periphery.

The medulla is thought to play a central role in the selection of Treg; ontogeny correlates to medullary development in newborns[26], mice with disrupted medullary development have known defects in Treg[26] and expression of some important co-stimulatory molecules such as CD70 is restricted to the medulla[25]. However it is worth noting that $Foxp3^+$ cells are seen among DP thymocytes – be it in greatly reduced proportions – and may contribute to the pool of SP cells. A recent paper by our lab provided great evidence for medullary dependence through grafting of $Relb^{-/-}$ thymi (which fail to develop mTEC) under the kidney capsule of wildtype mice[27]. In these mice, generation of $Foxp3^-$ precursors and Treg were both greatly inhibited, but a low level of generation was still detected[27], possibly reflecting the contribution of cortical selection. Altered TCR repertoire selection and abrogation of diabetes in NOD mice upon the deletion of the thymus restricted serine protease PRSS16, expression of which is dominant in cTEC[28], suggests that cTEC are capable

of producing unique auto-antigens for CD4 selection. It has also been shown that CD80, which provides essential signaling for formation of Treg precursors, is highly expressed on $TSCOT^+$ cTEC[29]. Liston et al. demonstrated direct evidence of cortical selection in 2008[30] – production of DP $Foxp3^+$ thymocytes was observed in mice where thymic MHC-II expression was restricted to cTEC – these cells could mature to become CD4 SP upon purification and were detected in the cortex upon chemokine signaling abrogation by pertussis toxin[30]. Hence, although the thymic medulla is the main player in selection of Treg, many of the factors required for their generation are common to mTEC and cTEC, and it seems likely that both contribute to the pool of developing nTreg.

2.2: Effector/memory phenotype Treg

There is a growing consensus that, analogous to the development of effector and memory T cells upon dangerous antigenic challenge, Treg similarly mature phenotypically to produce effector/memory cells with enhanced proliferative and suppressive capabilities. Darrasse-jèze et al. reported specific expansion of $CD44^{hi}$ Treg in the draining lymph nodes (dLNs) of mammary tumours[31]. These Treg were antigen specific, as attested by models expressing transgenic TCRs recognizing haemagglutinin (HA) antigen – these cells were only expanded in dLNs of HA^+ mammary tumours, and tumour clearance by transferred conventional effector/memory T cells was only effective early after implantation[31]. This was recently elucidated in another model by Malchow et al., who used mice reconstituted with a low percentage of MJ23 TCR transgenic bone marrow in addition to wildtype[32]. This TCR recognizes an Aire-dependent prostate associated antigen, and nTreg of this specificity were

observed to be enriched in the dLNs anatomically associated with the prostate in male, but not female (which lack the prostate), mice[32].

Although these systems have shown the expansion and maintenance of antigen specific Treg upon antigenic challenge, the best evidence for memory function analogous to conventional memory cells has come through Rosunblum et al.'s[33] model of inducible OVA expression in the skin under the keratin-5 promoter. In this system, OVA is constitutively expressed in the thymus under control of Aire, allowing selection of OVA specific Treg from DO11 TCR transgenic cells[33]. However, expression in the skin is inducible through administration of oral doxycycline, triggering T-cell mediated inflammation of the skin, which gradually self-resolves through expansion of antigen-specific Treg[33]. Upon second administration of doxycycline, skin inflammation is either inapparent, or quickly resolves as a result of small, but rapidly expanding populations of antigen specific Treg persisting in the tissue itself[33].

Another recent paper has implicated Inducible T-cell Costimulator (ICOS) as being vital to the expansion of antigen specific Treg. Tolerizing mucosal immunization with OVA was followed by selective expansion of $ICOS^+$ Treg throughout the periphery, but particularly in the lungs of wildtype mice[34]. Tolerance to challenge with OVA+LPS was achieved in wildtype, but not $ICOS^{-/-}$ mice, or mice treated with ICOS blocking antibody prior to tolerization, wherein airway hyper-reactivity was observed as a result of failed accumulation of IL-10 producing antigen-specific Treg[34]. ICOS deficiency has also been linked with tissue specific auto-immunity in NOD mice, which presented with reduced Treg numbers, together with extensive neuromuscular auto-immunity upon it's deletion[35].

The central role of Treg possessing an effector memory phenotype - ie. those expressing ICOS and/or CD44 - may be explained by qualitative differences, as $ICOS^+$ Treg appear to have superior proliferative and suppressive capabilities, with the $ICOS^-$ subset being comparatively death-prone upon activation[36]. Differential cytokine expression and homeostatic requirements have also been ascribed to $ICOS^{+/-}$ subsets in humans; $ICOS^+$ Treg produce high IL-10, and require ICOSL on plasmocytoid DCs, whereas $ICOS^-$ Treg express mainly TGFβ and rely on CD80/86 expression by myeloid DCs for survival[37]. Antigen experienced Treg have hence been shown to possess unique functional capabilities, can contribute to immunological memory, and are phenotypically distinct.

2.3: Homeostatic requirements of conventional memory T cells

Conventional CD4 memory cells are formed upon antigenic stimulation with adjuvant, and are long-lived, allowing them to rapidly expand to produce effector cells on subsequent challenge with the same antigen. They can be subdivided into effector (T_{EM}) and central (T_{CM}) memory cells based expression of CD62L[38], with $CD4^+$ murine T_{EM} distinguishable as $CD62L^-IL7R\alpha^{Hi}$ (as well as $CD44^+CD27^+$), bearing receptors for entry into inflamed tissues (CCR6)[39]. The homeostatic requirements for memory cells differ considerably from conventional CD4s. T_{EM} express high levels of IL7Rα, and are dependent on IL-7 signaling for their survival[40]. IL-7 is also known to play a less fundamental role in homeostasis of naïve conventional T cells and Treg[41,42]. Similarly, ICOS is an important co-stimulatory molecule in homeostasis of memory cells as well as expansion of antigen specific T cells[43]. Importantly, it has also been recently discovered that CD4 memory cells are entirely dependent on signaling through another set of TNF superfamily members - OX40 and CD30 -

expression of which is triggered in subpopulations of effector cells after antigenic challenge. OX40 expression is also known to be induced by IL-7 signaling[44].

Double knockout (DKO) mouse models lacking CD30 and OX40 fail to maintain newly generated effector cells, and DKOs crossed with $Foxp3^{-/-}$ mice completely lack the normally severe autoimmune phenotype[45]. The ligands for CD30 (CD30L) and OX40 (OX40L) are up-regulated in inflammatory tissues, but importantly have been shown to be expressed by lymphoid tissue inducer cells (LTi), as regulated in part by TLR-2/4 (both)[46] and IL7Rα (OX40L)[40] signaling. LTi have been shown to play roles in development of lymphoid tissue architecture, and their absence – as shown in mice lacking RORgt, a signature transcription factor for LTi – results in the failure to develop lymph nodes, and incorrect thymic architecture[47]. It has long been known that LTi are capable of interacting with primed effector cells[44], but only recently has it become apparent that CD4 memory cell survival relies entirely on receipt of CD30L/OX40L signals from LTi. This was shown eloquently by Withers et al.[48] in a series of experiments, wherein mice lacking LTi alongside T and NK cells ($CD3\varepsilon^{tg26}RORgt^{-/-}$) were irradiated, and reconstituted with bone marrow from either genotype matched, or $CD3\varepsilon^{tg26}$ donors[48]. These mice were hence identical, with the exception of the LTi produced by $CD3\varepsilon^{tg26}$ bone marrow – pre-made $CD4^{+}$ memory cells specific to 2W1S peptide were unable to survive in those mice lacking LTi, but persisted normally in $CD3\varepsilon^{tg26}$ reconstituted hosts[48]. Memory cells hence have very specific homeostatic requirements, and require both soluble factors from stromal cells, as well as TNF superfamily member signaling for survival.

2.4: Project aims

The aims of this project were as follows;

1. Analyse expression of Treg- and activation-associated markers in memory phenotype Treg
2. Identify and compare the tissue locations of $ICOS^{+/-}$ Treg sub-populations
3. Determine the requirement for OX40 and CD30 in homeostasis of memory phenotype Treg
4. Produce bone marrow chimeras to investigate specific requirements for mTEC expressed factors (CD80/86 & ICOSL) in development of all Treg populations

Section 3.0: Methods

3.1: Animals

Wildtype BoyJ & C57/BL6 mice were used as controls for Treg phenotyping of C57/BL6 background $OX40^{-/-}$, $CD30^{-/-}$, $CD30^{-/-}/OX40^{-/-}$, $CD80/86^{-/-}$ and $ICOSL^{-/-}$ mice in flow cytometry experiments. $CD45.1^{+}$ BoyJ mice were used as bone marrow donors to distinguish donor from host cells. All mice were housed in the BMSU under germ-free conditions, aged from 6-10 weeks, and sacrificed using a schedule 1 method of killing.

3.2: Media & reagents

RF10 medium was used for disaggregating tissues, as well as re-suspending cells in a final volume for the purpose of counting, and consisted of RPMI medium (Sigma) with 10% heat-inactivated fetal calf serum (FCS), 2% penicillin and streptomycin, and 1% glutamine.

"FACS buffer" was used for suspension of antibody cocktails in staining for flow cytometry, and consisted of PBS with Ca^{2+} and Mg^{2+} (Sigma) with 3% FCS. Staining buffer for tissue section staining consisted of PBS (from tablets - Sigma) with 2% powdered Bovine Serum Albumin (BSA).

3.3: Tissue preparation for flow cytometry

Thymus, spleen and peripheral lymph nodes (PLNs; including inguinal, mesenteric, axial, brachial and cervical) were isolated and cleaned of excess fat and connective tissue under a light microscope using forceps. The cleaned organs were disaggregated between two frosted microscope slides (all PLNs were pooled unless specified), and pipetted through a fine gauze filter into a 25ml falcon tube, before centrifugation (1400rpm for 4 minutes as standard). Splenocytes were subjected to red blood cell (RBC) lysis through suspension in 2ml RBC lysis buffer (Sigma) at room temperature for 5 minutes, before neutralization with 2ml RF10 and centrifugation. The supernatant was removed, and cells re-suspended in a known volume of RF10 (5ml thymus & spleen, 1ml lymph nodes), before cells were counted using counting beads. This was done in a solution containing 10µl beads (10^4 beads), and 100µl cell solution, where cell numbers were calculated based on the ratio of cells:beads in a plot of FSC vs. SSC. Cell suspensions were stored on ice at all times except where stated.

3.4: Staining for flow cytometry

Cells were aliquoted into 1.5ml eppendorfs ($5x10^6$ cells for samples, $2x10^6$ for single colours), centrifuged, and supernatant removed. Single colour controls - for compensation of

samples through the BD FACS-Diva software - were stained in 50µl, and samples 100µl of staining solution, which consisted of antibody cocktails suspended in FACS buffer. All samples were re-suspended in 200µl FACS buffer in FACS tubes, run on the BD LSR-Fortessa, and analyzed using FlowJo software v8.7. Cells were spun down and the supernatant removed prior to re-suspension in the first antibody stain. All stains were applied for 30 minutes on ice, and washed in 1ml FACS buffer x1 between steps, and x2 between intracellular stains and prior to final re-suspension.

Treg phenotyping was performed using α-CD4, CD8, CD25, CD44, CD69, TCRb, ICOS, Neuropilin-1 (Nrp-1) and Foxp3 as stated in ***table 1***, where Nrp-1 Biotin was picked up with a separate staining step using Streptavidin PeCy7 (1:800). Bone marrow (BM) chimeras were stained using α-CD4, CD25, CD44, CD45.1, CD69, TCRb, ICOS and Nrp-1 as shown in ***table 1***. Permeabilization and fixation of cells for intracellular staining was conducted using the ”Foxp3 staining buffer kit” (eBioscience), wherein cells were suspended in 400µl “Fix / Perm” buffer (1:3 concentrate: diluent) for 40mins (refrigerated), and washed x2 in 1ml 1:9 “Perm / Wash” buffer diluted with dH_2O. Intracellular staining was conducted using Foxp3 PE (1:100) suspended in 100ul “Perm / Wash” buffer on ice for 30 mins.

3.5: Bone Marrow Chimeras

Cells for BM chimeras were prepared under a culture hood in sterile conditions. BM was flushed using a needle filled with RF10 from long-bones of legs dissected from $CD45.1^+$ BoyJ mice. Single cell solution was filtered and spun for 6min at 1400rpm. RBC lysis was performed by suspension in 5ml Gey’s solution (Sigma) for 5min on ice. Cells were washed in

10ml RPMI, and re-suspended in 15ml for counting, before centrifugation and suspension at a concentration of 10^8/ml.

T-cells were depleted using magnetic beads; 10^7 cells were suspended in anti-Thy1.2 biotin (eBioscience, Clone 53-2.1) on ice for 30 minutes and then washed before SA-conjugated magnetic beads were added at $4x10^7$/ml. Cells were then passed through a magnetic column to remove T-cells, collected in PBS and placed in orbital and rotating shakers at room temperature (15min each), before a second passage down the magnetic column. The supernatant (Thy1.2 depleted) was stained for CD3 and Thy1.2, and analysed by FACS to confirm depletion, and equal numbers of cells prepared for injection into each recipient (200µl/mouse).

Wildtype C57/BL6, CD80/86$^{-/-}$ and ICOSL$^{-/-}$ recipient mice (all CD45.2$^+$) were sub-lethally irradiated with 2x450 rads after 7 days treatment with the antibiotic baytril, and intravenously injected with Thy1.2-depleted BoyJ BM. After 6 weeks, mice were drained of serum by cardiac puncture under isofluorane anaesthetic without recovery, and thymus, spleen and PLNs were dissected and prepared for flow cytometry as above.

3.6: Confocal microscopy

Tissues were isolated and cleaned as for flow cytometry (above), before freezing in OCT compound on aluminium foil using dry ice. Tissues were stored at -80°c prior to use. Tissue was sectioned to a width of 7µm using a cryostat, and cut sections were transferred to microscope slides, fixed by immersion in acetone for 30 mins, air dried (30min), and kept at -20°c until stained.

Staining was conducted using antibody cocktails suspended in PBS with 2% BSA. Tissue was first blocked using 10% horse serum for 15min, before pipetting off. All stains were applied in 75µl total volume per spot for 30mins in a dark, humidified chamber at room temperature, and then bathed in PBS for 10 minutes to wash. Primary stains were applied as above; secondary and tertiary stains were incubated in 1:20 normal mouse serum at double concentration for 30min, then diluted to staining concentration by addition of PBS 2% BSA, to minimize non-specific binding (see ***table 2*** for staining regimes). After the final wash step, slides were bathed in DAPI solution for 20sec, and then washed in serial PBS tubes. Slides were then removed of excess fluid, and placed on a glass cover slip coated with Prolong Gold mounting medium, which was sealed using clear nail polish. Slides were air dried, before storage at -20°c. Imaging was performed with the Zeiss LSM 780 confocal microscope.

3.7: Statistical analysis

Graphical representation of data and statistical analyses were accomplished using Prism 6 software (Graphpad), and all p values shown are the results of unpaired t-testing, where “ns” = non-significant, “*” = $p<0.05$, “**” = $p<0.01$, “***” = $p<0.001$, and “****” = $p<0.0001$.

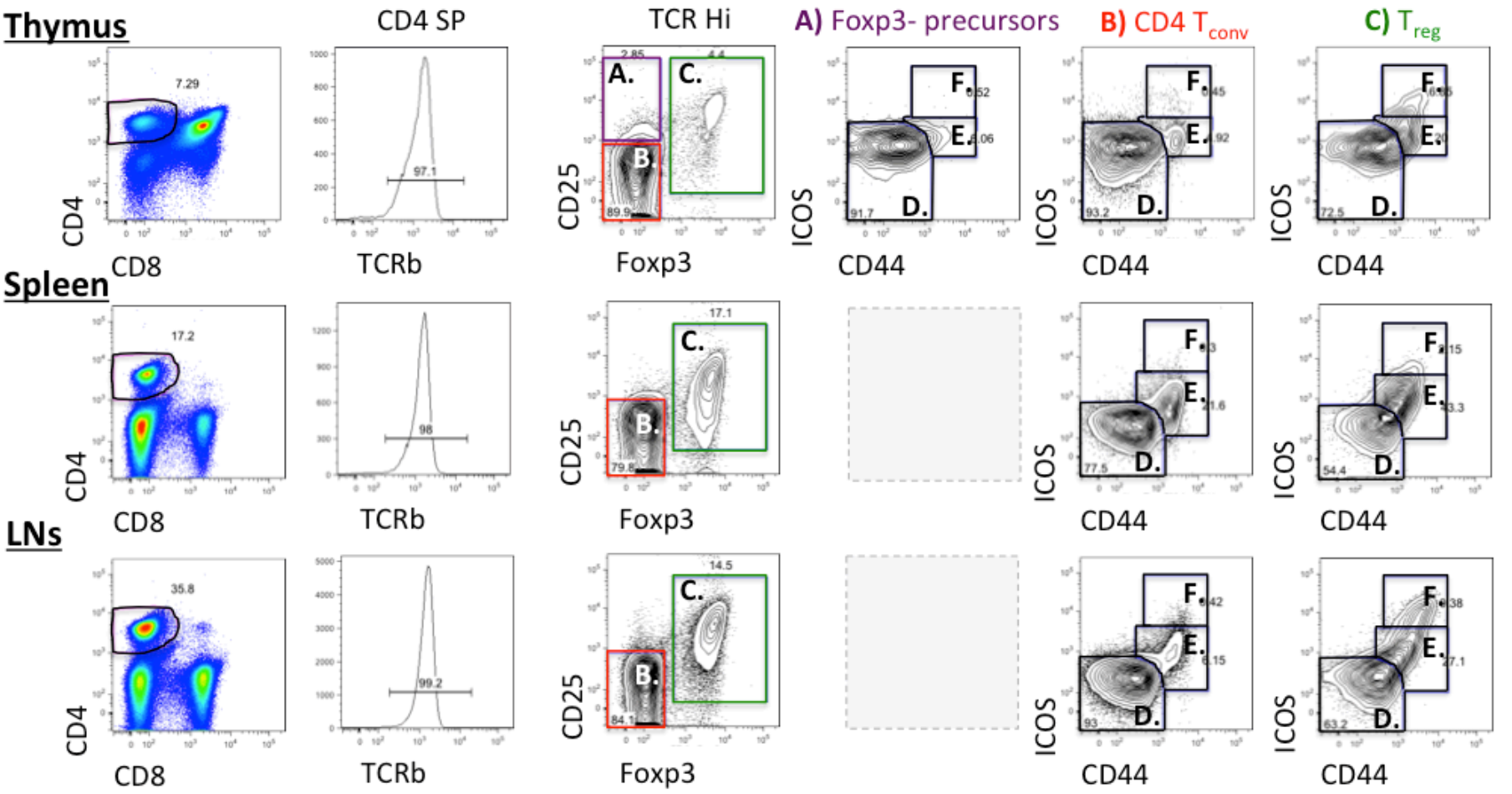
Section 4.0: Results

4.1: Adult thymus and lymph nodes are home to a resident population Treg with a highly activated, memory phenotype

Using ICOS and CD44 as activation markers, we were able to identify distinct populations of $CD25^{+}Foxp3^{+}$ regulatory T cells (Treg) in the thymus, spleen and inguinal

lymph nodes (iLNs) of adult wildtype C57/BL6 mice through flow cytometry. Although ICOS was expressed on all thymocytes, in each tissue we detected a population low for expression of both CD44 and ICOS (ICOS/$44^{lo/neg}$), as well as one with intermediate levels of expression (ICOS/44^{int}), equivalent to those seen on activated T-cells within the $CD25^{-}Foxp3^{-}$ conventional subset (***fig. 1***). However, we also observed a small population of $CD44^{+}$ Treg in the thymus, and substantial numbers in the iLNs, that had very high levels of ICOS

Figure 1 – Gating for ICOS/44 expression on Tregs, conventional CD4s and CD25+ precursors



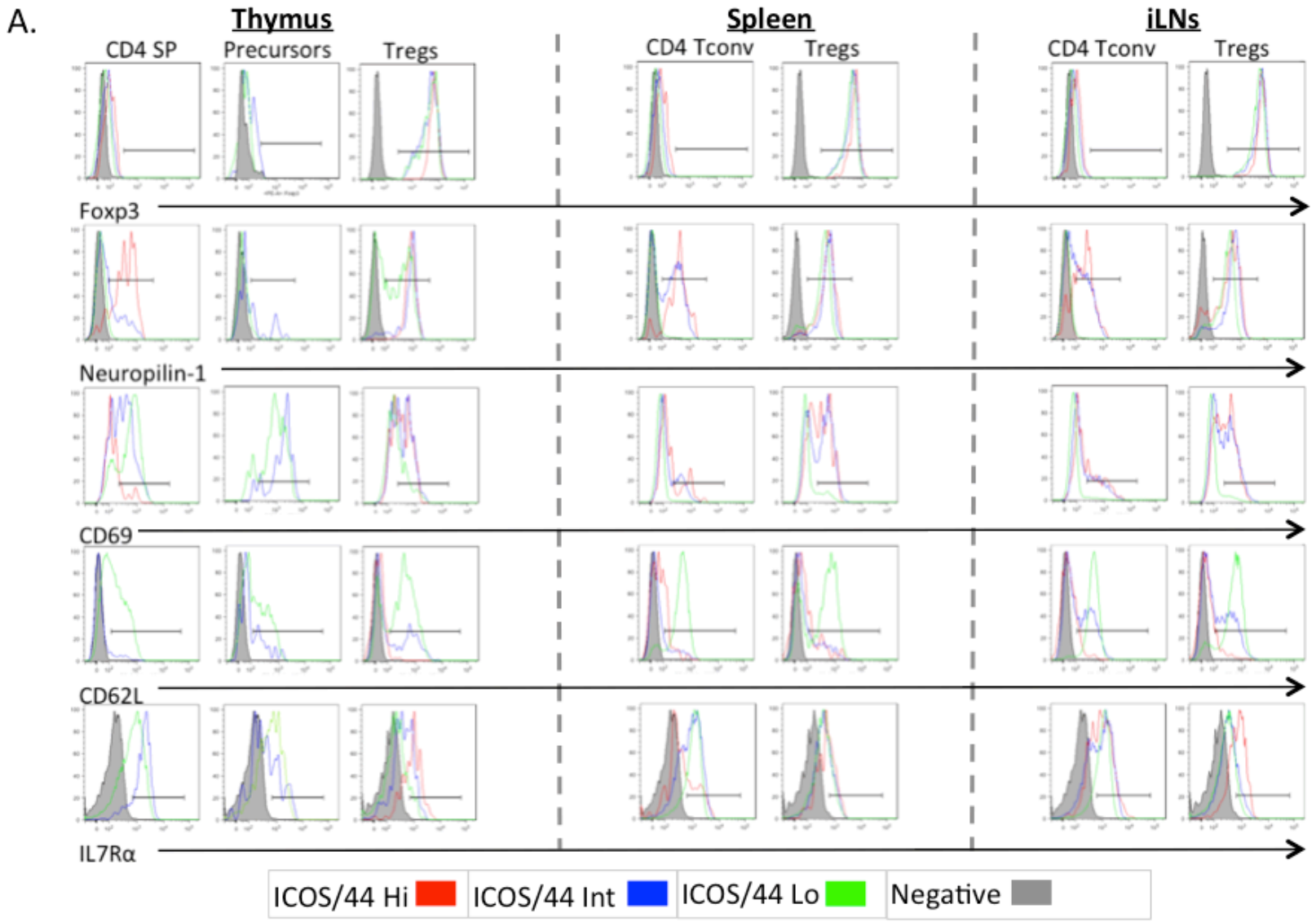
D) ICOS/44 low/negative (ICOS/44^{neg})
E) ICOS/44 intermediate (ICOS/44^{int})
F) ICOS/44 high (ICOS/44^{hi})

expression (ICOS/44^{hi}) (***fig. 1***). In the thymus, ICOS/44^{hi} cells were only detected in the CD25^{+}Foxp3^{+} population, amongst both CD4 SP (***fig. 1***) and DP thymocytes (not shown), and not in CD25^{+}Foxp3^{-} Treg precursors, and only very few conventional thymocytes with this level of expression were present (***fig. 1***).

Further phenotypic analysis of these 3 distinct ICOS/44 expressing subpopulations revealed expression of more markers typical of activated T cells, as summarized by the table in ***figure 3***. With increasing expression of ICOS and CD44, conventional T cells in the thymus lose CD69, and gain expression of Nrp-1, and all ICOS$^{int\text{-}hi}$ cells are CD62L^{negative} (***fig. 2-3***). Although these cells are likely a heterogenous population made up of; 1. mature iNKT which have lost CD69 expression as seen in early stage SP differentiation[27], as these are known to express Nrp-1, ICOS and CD44, and 2. re-circulating effector memory T-cells (which unlike central memory cells are CD62L^{neg}), as memory cells have similarly been seen to display activation markers such as ICOS and CD44, as well as Nrp-1, for simplicity these will still be referred to as “conventional T-cells”.

Although Nrp-1 is a known marker for all mature nTreg produced in the thymus, MFI for Nrp-1 was lower in ICOS/44^{lo} populations, which unlike ICOS/44$^{int\text{-}hi}$ Treg contained a subset absent of Nrp-1 expression (***fig. 2-3***). ICOS/44^{hi} Treg were also slightly but significantly higher in Foxp3 expression (***fig. 2-3***). ICOS/44^{neg} Treg in the thymus appeared to be enriched for CD69 expression relative to equivalent peripheral populations, whereas CD69 expression on ICOS/44$^{int\text{-}hi}$ Treg was homogenous across all tissues – the majority of cells were CD69^{+} (***fig. 2-3***). ICOS/44^{neg} Treg in all tissues expressed the naïve T-cell marker CD62L (***fig. 2-3***), which is required for entry into secondary lymphoid tissues, including the LNs and spleen. However, expression of this marker was lost upon up-regulation of ICOS and

Figure 2 – Expression profiles of ICOS/44 high, intermediate and negative T cell populations



Histograms showing expression of Treg associated (Foxp3, Nrp-1), naïve T cell (CD62L) and activation markers (CD69, IL7Rα) in ICOS/$44^{low/neg}$ (green), ICOS/44^{int} (blue) and ICOS/44^{hi} (red) subsets of conventional CD4s, Treg precursors and Foxp3^{+} Tregs. FACS data are shown for the thymus in the thymus (**left**), spleen (**middle**) and iLNs (**right**) of C57/BL6 wildtype mice. Histograms are representative of three mice (two CD62L and IL7Rα), and negative controls where shown are in grey.

Figure 3 – Summary of markers up- and down-regulated in ICOS/44^{+} Tregs (and conventional cells)

	ICOS/44 expression	Foxp3		(Foxp3)		Nrp-1		CD69		(CD69)		CD62L		IL7Ra		(IL7Ra)	
Conventional (CD25-Foxp3-)	*low/negative*	-		-		-		+		-		+		+/++		+	
	intermediate	-		-	↓	-/+	↓	-/+	↑	-/+	↓	-	↑	++	↓	-/+	↓
	high	-		-		+		-		-/+		-		++		-/+	
Precursors (CD25+Foxp3-)	*low/negative*	-				-		+				-/+		-/+			
	intermediate	-				-		+				-/+		+			
Tregs (CD25+Foxp3+)	*low/negative*	+/++		++		-/+		- -/+		-		+		-		- -/+	
	intermediate	+/++	↓	++	↓	+	↓	-/+	↓	-/+	↓	-/+	↑	-/+	↓	- -/+	↓
	high	++		++		+		-/+		-/+		-		+		-/++	

Key	
-	All cells negative
- -/+	Most negative, few positive
-/+	Even split of expression
+	All cells positive
++	High expression

Summary table of expression patterns of ICOS and CD44 co-expressing populations of conventional CD4s, Tregs and CD25^{+} precursors. Values are based on MFI and histogram distributions, showing patterns of up- and down-regulation of markers associated with Tregs, activated and naïve T cells in the thymus (and iLNs where different) of wildtype mice, where ↓ suggests up-regulation, and ↑down-regulation of expression with increasing ICOS/44 expression.

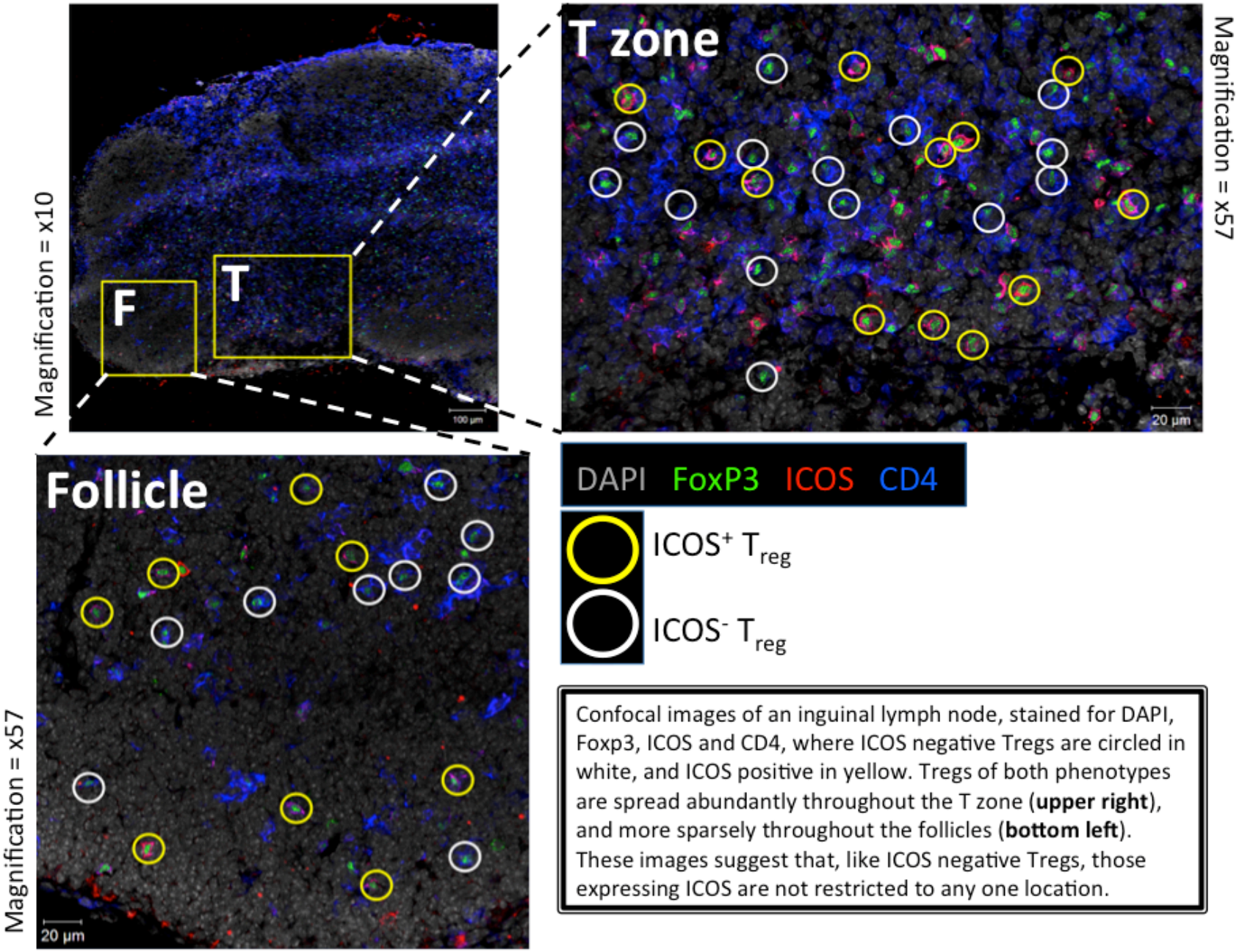
CD44 expression – few $CD62L^+$ cells were detected among $ICOS/44^{int}$, and no $CD62L^+$ cells were present in $ICOS/44^{hi}$ populations (***fig. 2-3***). Treg express lower IL-7Rα than conventional T-cells – IL-7 has been shown to play a role in homeostasis of naïve and memory T-cells, but has relatively minor function in Treg. Our data shows that IL-7Rα is significantly up-regulated by $ICOS/44^{hi}$ Treg in the thymus and iLNs, but not Treg in the spleen, nor ICOS/44 expressing conventional cells, which express the same, if not slightly reduced levels than $ICOS/44^{neg}$ cells (***fig. 2-3***).

4.2: ICOS expressing Treg are spread through the thymic medulla, and iLNs

Using confocal microscopy, it was possible to stain for ICOS and Foxp3 to distinguish $ICOS^+$ and $ICOS^-$ Treg (although levels of expression could not be defined) in both the thymus and the iLNs. ICOS is also expressed on $CD3^-4^+$ lymphoid tissue inducer cells (LTi), and hence CD3 staining was used in the thymus to detect conventional thymocytes expressing ICOS. $ICOS^+$ and $ICOS^-$ Treg were indistinguishable by location in both tissues; they were present in both the T zones and follicles of the iLNs (***fig. 4***), and were only observed in the thymic medulla (***fig. 5***), as marked by the presence of $ER\text{-}TR5^+$ mTECs. ICOS expressing cells as a whole were only observed in the medulla of the thymus (***fig. 5***), in keeping with the idea that a substantial proportion are iNKT, as the majority of thymic resident iNKT are thought to be retained in this location[49].

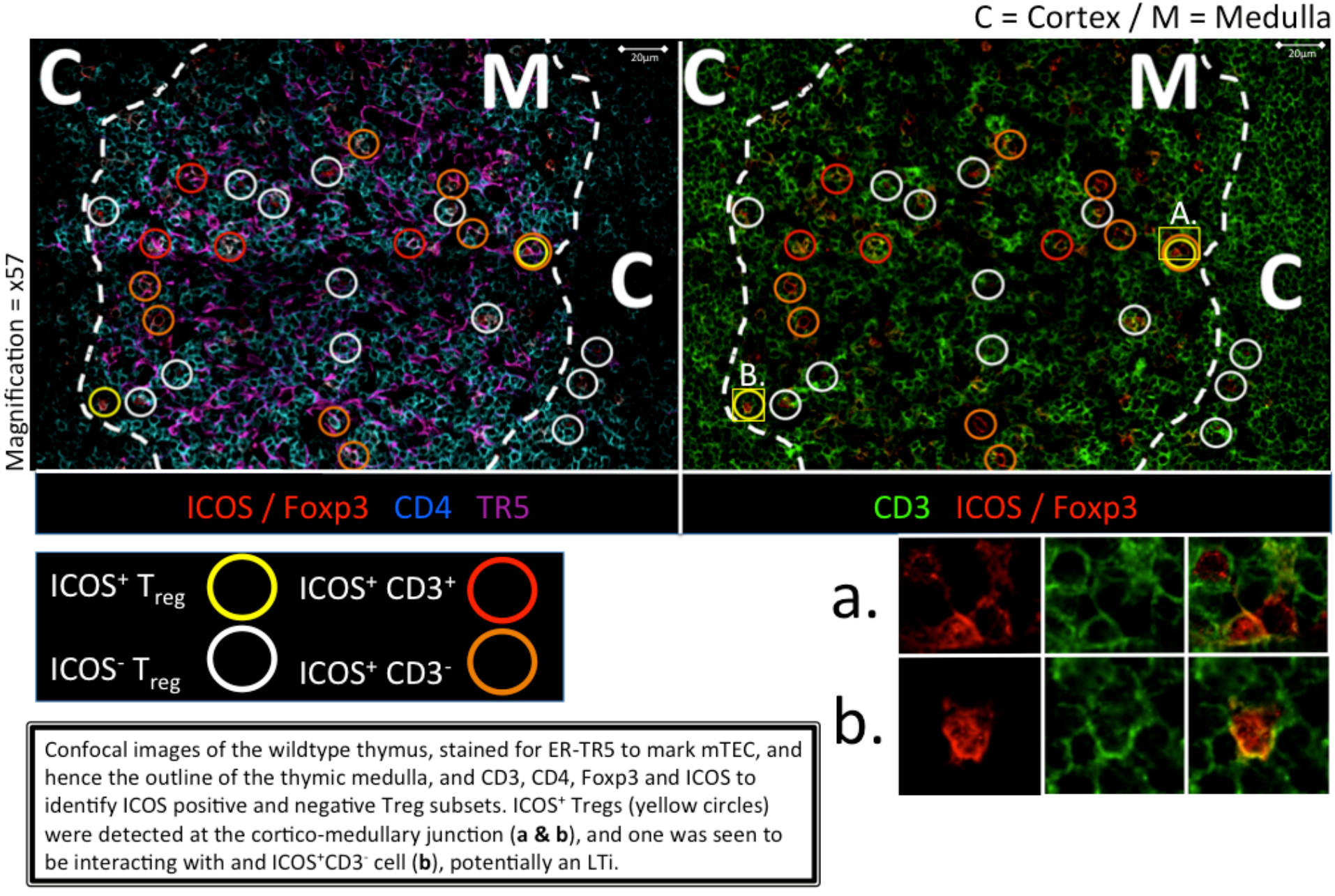
Consistent with the FACS data, $ICOS^+$ Treg were present in much greater abundance in the iLNs (***fig. 4***). The two thymic $ICOS^+$ Treg that were observed were located close to the cortico-medullary junction (***fig. 5***), and it is tempting to suggest that this is their specific place of residence in the medulla, however much more data is required for quantification

Figure 4 – ICOS positive and negative Treg subsets in the inguinal lymph nodes



Confocal images of an inguinal lymph node, stained for DAPI, Foxp3, ICOS and CD4, where ICOS negative Tregs are circled in white, and ICOS positive in yellow. Tregs of both phenotypes are spread abundantly throughout the T zone (**upper right**), and more sparsely throughout the follicles (**bottom left**). These images suggest that, like ICOS negative Tregs, those expressing ICOS are not restricted to any one location.

Figure 5 – location of ICOS expressing cells in the thymic medulla



Confocal images of the wildtype thymus, stained for ER-TR5 to mark mTEC, and hence the outline of the thymic medulla, and CD3, CD4, Foxp3 and ICOS to identify ICOS positive and negative Treg subsets. $ICOS^+$ Tregs (yellow circles) were detected at the cortico-medullary junction (**a & b**), and one was seen to be interacting with and $ICOS^+CD3^-$ cell (**b**), potentially an LTi.

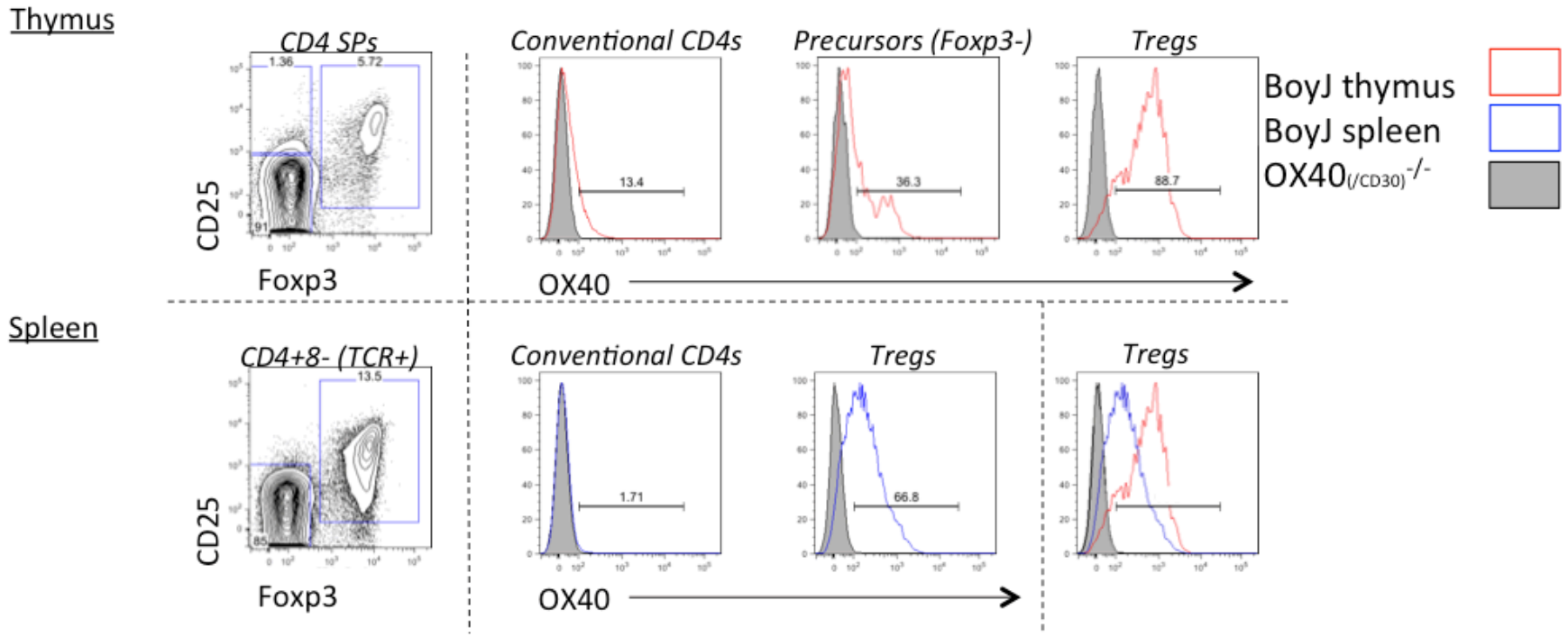
and confirmation of this theory. Similarly, one of the ICOS^{+} Treg observed appears to be in close proximity with a CD3^{-}4^{+}ICOS^{+} cell (***fig. 5***), but it is not possible to determine whether this is genuine interaction, or just co-incidental positioning without quantification. In the iLNs, neither ICOS$^{+/-}$ Treg were enriched at the marginal sinus, nor inter-follicular spaces associated with LTi (***fig. 4***).

4.3: ICOS/44^{high} Treg do not share homeostatic requirements of memory T cells

Given the shared phenotype of ICOS/44^{hi} Treg and memory cells, we were curious to see if they similarly shared requirements for homeostasis and survival. CD4^{+} memory cells are known to depend on OX40L and CD30L as survival signals provided by LTi in secondary lymphoid organs after induction by antigenic challenge. Using CD30$^{-/-}$, OX40$^{-/-}$ and CD30/OX40 double knockout (DKO) mice, we aimed to determine whether the same was true of our memory phenotype Treg. Firstly, flow cytometry was used to determine the expression of OX40 on Treg (***fig. 6***). We found OX40 to be expressed on some Treg right from the CD25^{+}Foxp3^{-} precursor stage, with the majority of thymic (88.7%) and splenic (66.8%) Treg expressing OX40 (***fig. 6***). By contrast, only a minority (13.4%) of SP CD4s were OX40^{+}, with even fewer in the spleen (1.71%) (***fig. 6***).

As evaluated by FACS, there was around a 50% reduction in both the proportion and number of Treg in the thymus of OX40$^{-/-}$ and DKO mice; however there was no reduction in precursor numbers in any of the KOs modeled (***fig. 7a***). There was also a significant increase in the proportion of Treg amongst CD4 SP cells in the CD30$^{-/-}$ thymus, although no increase in absolute numbers of Treg (***fig. 7a***). Both ICOS/44^{+} Treg populations were reduced (***fig. 7a***), but there did not appear to be a specific loss of ICOS/44^{+} effector / memory Treg as

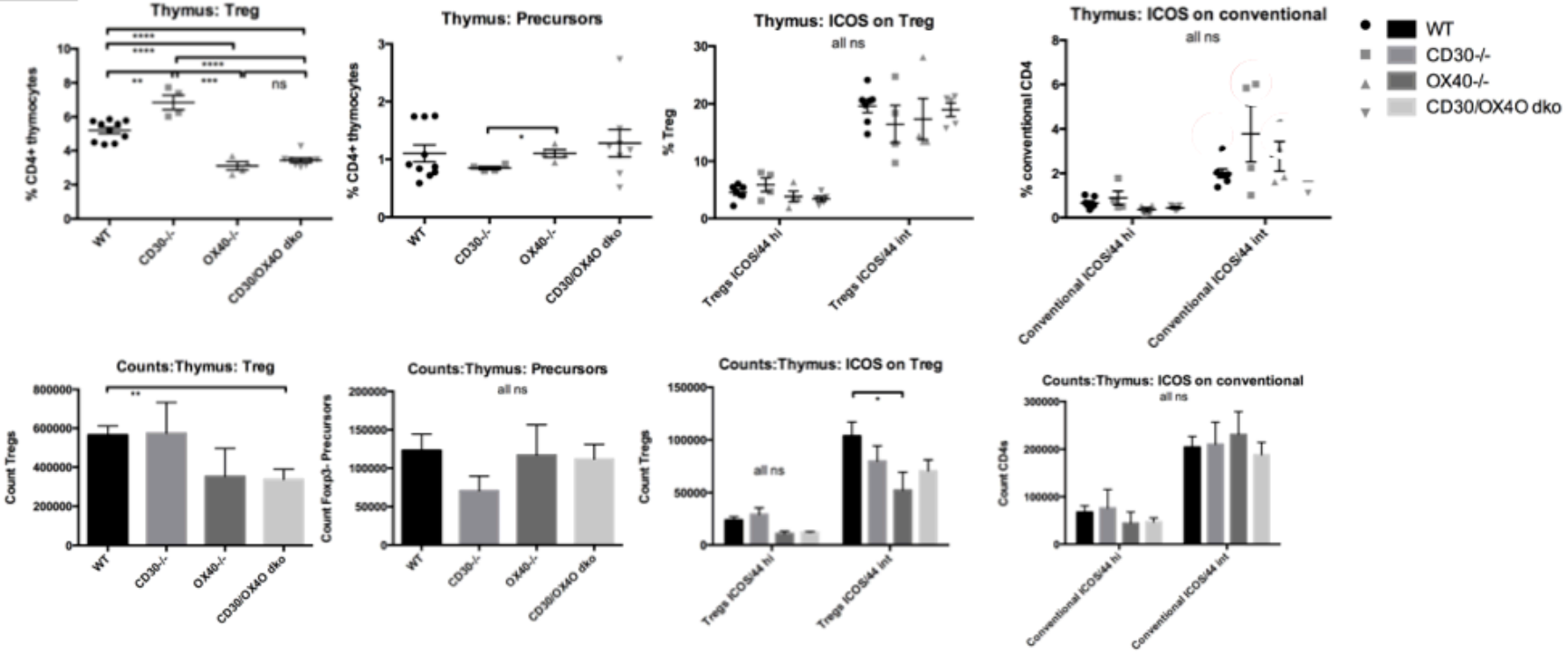
Figure 6 – OX40 is expressed on Tregs

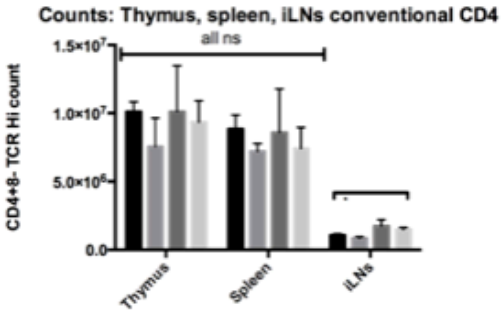


FACS plots showing OX40 expression on conventional CD4s, CD25⁺ Treg precursors (thymus only) and Foxp3⁺ Tregs in the thymus (**top row**) and spleen (**bottom**) of a wildtype mouse, where Tregs were selectively OX40⁺, with expression apparent on some cells from the precursor stage. Only a small population of conventional CD4s in the thymus, and not spleen, expressed OX40, and OX40 expression was higher on thymic Treg (**bottom far right**).

Figure 7.1 – Frequency and ICOS/44 expression of thymic Tregs in OX40, CD30 and OX40/CD30 dko mice

a. Thymus





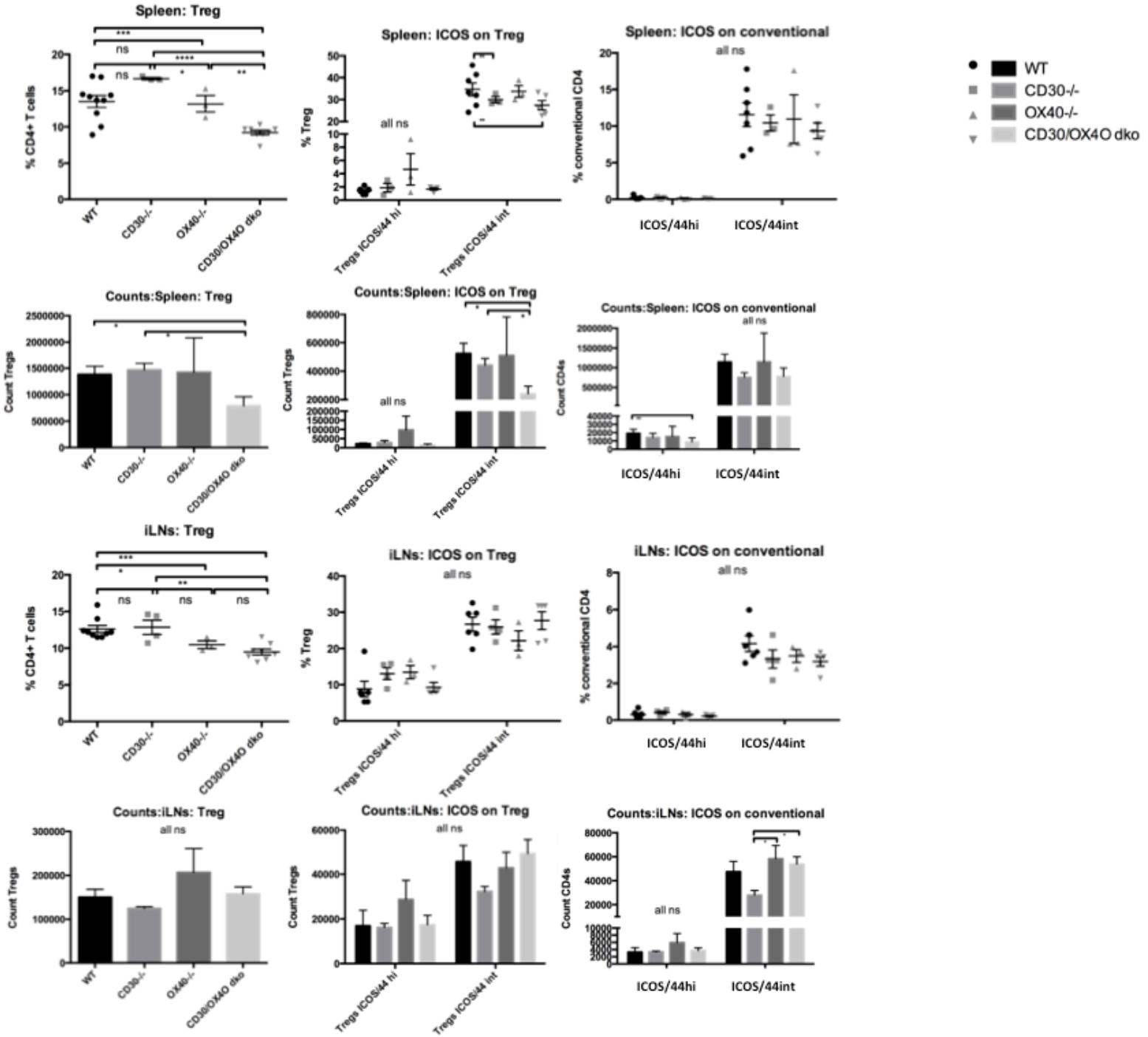
Analysis of FACS data from wildtype, OX40, CD30 and OX40/CD30 double knockout mouse thymus (**a**), spleen (**b**) and inguinal lymph nodes (**c**). **Top row**; proportions of Treg and CD25+ precursors from total CD4 SP thymocytes (T-cells in the periphery), and percentages of ICOS/44 intermediate and high cells within conventional CD4 and Foxp3^{+} Tregs are shown. **Middle row**; cell counts for the same populations of thymocytes or T cells, as calculated using FACS data, together with total lymphocyte counts in these organs, as determined using flow cytometric bead counting. **Bottom left (a only)**; total CD4 TCRhi counts for each organ, showing slight expansion in the OX40$^{-/-}$ and OX40$^{-/-}$/CD30$^{-/-}$ iLNs, where all other tissues had comparable cell numbers.

[Statistical significance is non-significant (ns) unless shown]

Figure 7.2 – Frequency and ICOS/44 expression of spleen and iLN Tregs in OX40, CD30 and OX40/CD30 dko mice

b. Spleen

c. iLNs



observed in our pilot experiments for the CD80/86$^{-/-}$ (not shown). A different story emerged in the spleen; Treg were depleted, but only in the DKO, and not the OX40$^{-/-}$ - this again was not restricted to one ICOS/44 phenotype (***fig. 7b***). Treg were also proportionally lowered in the iLNs of OX40$^{-/-}$ and DKO mice, however this may be accounted for by dilution caused by swelling of the lymph nodes, as absolute cell numbers were seen to be raised (***fig. 7c***). This effect was observed consistently in these KO models, and could imply expansion as a result of autoimmune responses.

4.4: ICOSL and CD80/86 are not required on mTEC for Treg selection

A wealth of data exists on the reliance on CD80/86 co-stimulation for generation of nTreg, and CD80/86 expression appears to be required for both selection of CD25^{+} progenitors[23,20,19], as well as maintenance of mature Foxp3^{+} cells in the thymus and periphery[18]. Indeed, our pilot experiment, and work by others in the lab have demonstrated a reduction in all Treg subpopulations in CD80/86$^{-/-}$ mice, with a specific loss of ICOS/44^{+} cells - large numbers of ICOS/44^{int} were lost, and ICOS/44^{hi} cells were notably absent (not shown). However, to our knowledge, no studies have currently determined whether CD80/86 expression is specifically required on thymic epithelial cells. ICOSL is another important immune co-stimulatory molecule expressed on mTEC, although a role in development of conventional thymocytes and Treg has yet to be established.

In order to investigate the requirement for CD80/86 and ICOSL expression on thymic stroma for selection of nTreg, we created bone marrow chimeras using wildtype CD45.1^{+} donor bone marrow transferred to an irradiated CD45.1^{-} host. Hosts were CD45.1^{-} wildtype, CD80/86$^{-/-}$ and ICOSL$^{-/-}$ mice, and Treg populations, split into donor (chimeric, CD45.1^{+}) and

Figure 8.1 – Bone marrow chimera FACS gating

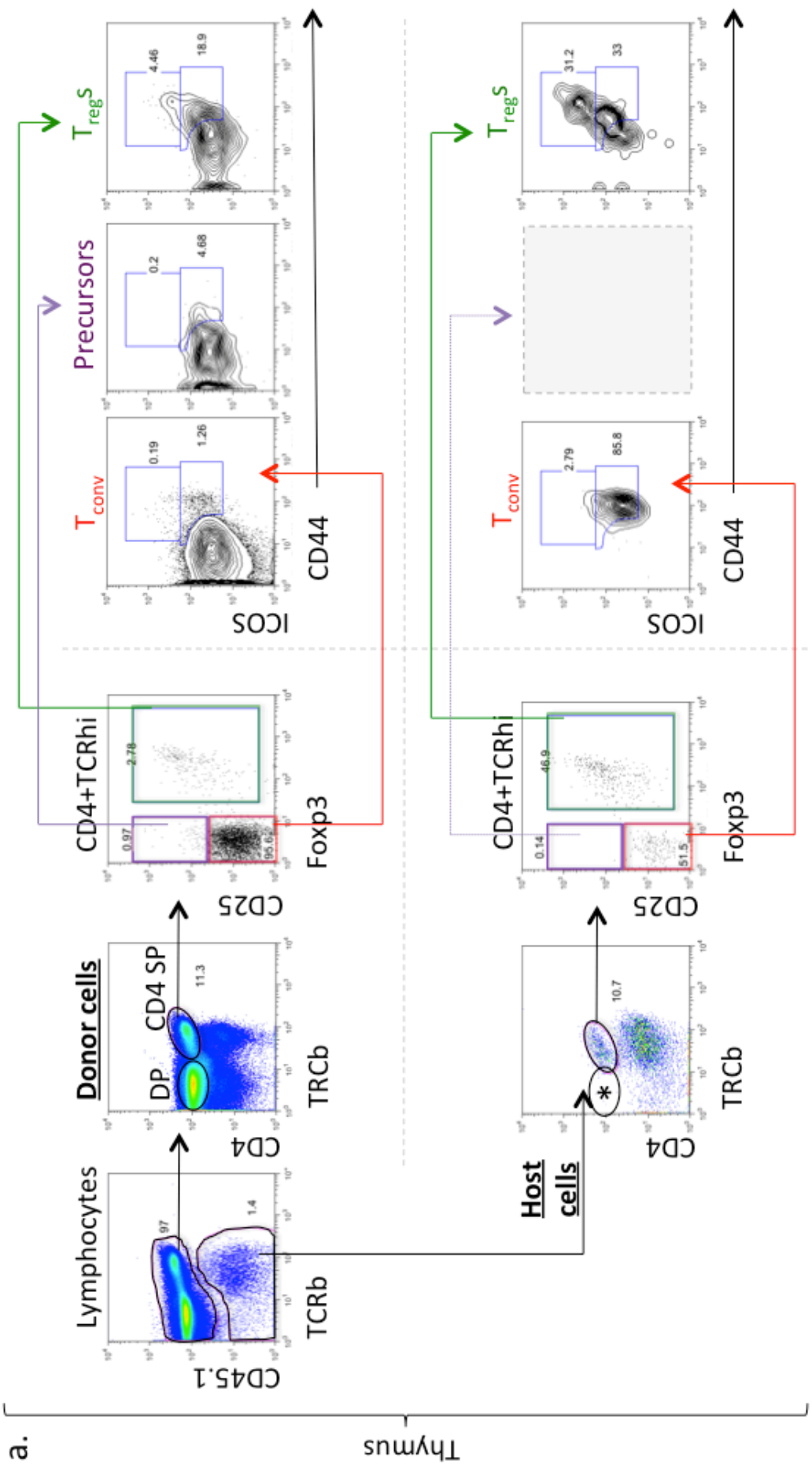
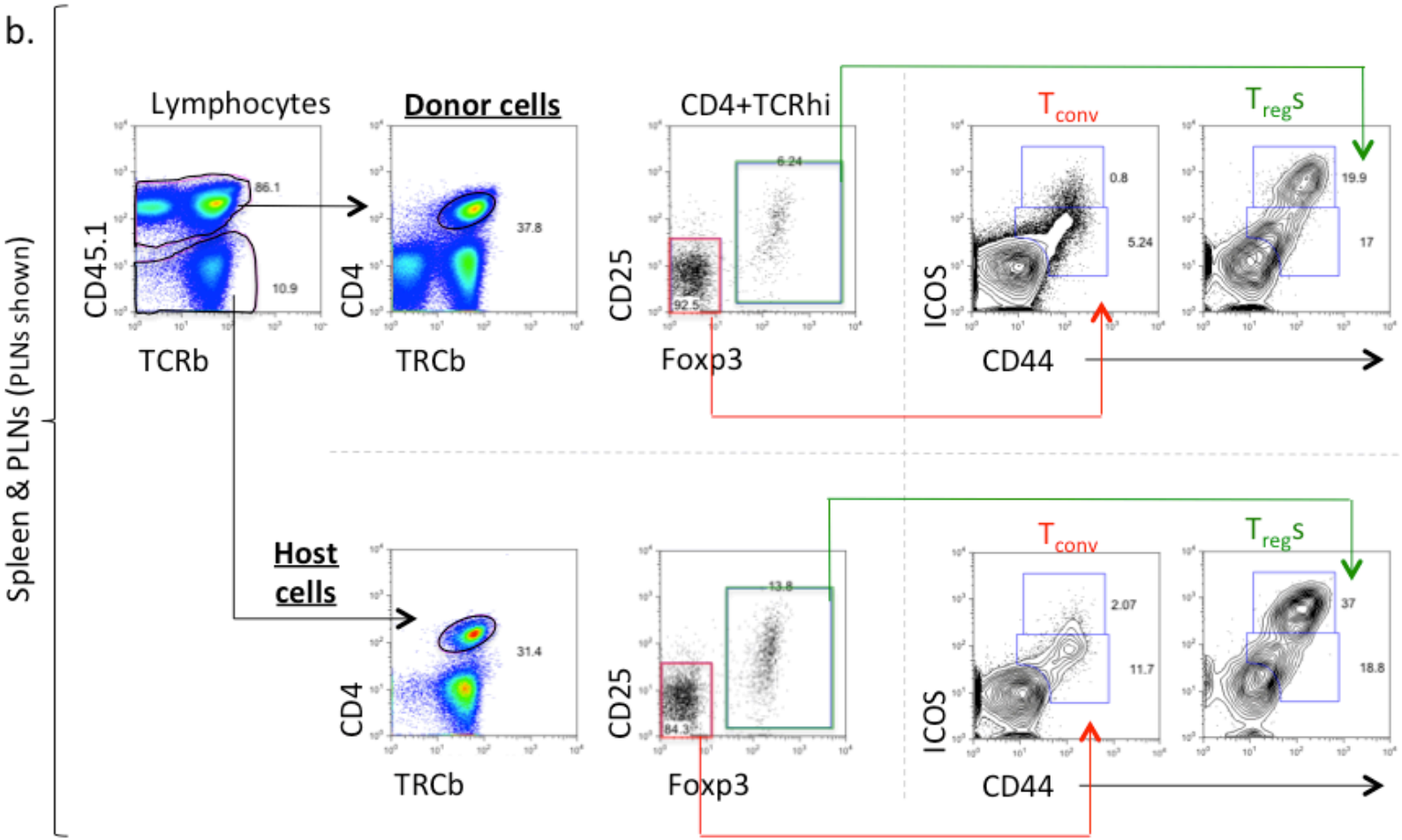


Figure 8.2 – Bone marrow chimera FACS gating



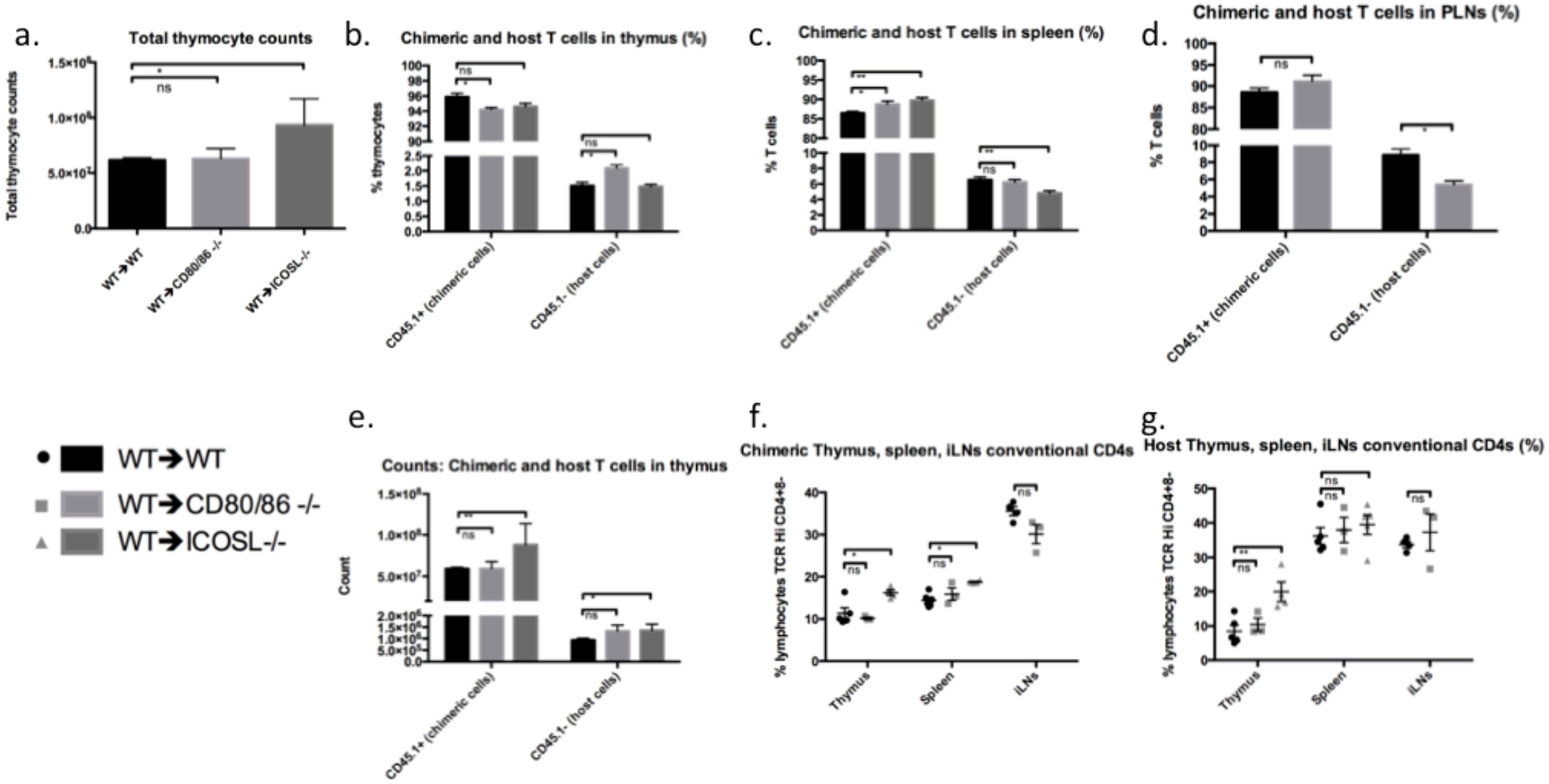
Representative FACS plots demonstrating the gating strategy for WT➔WT, WT➔ICOSL$^{-/-}$, and WT➔CD80/86$^{-/-}$ bone marrow chimeras in **a.** the thymus, and **b.** the spleen and PLNs. Cells were gated on CD45.1 expression, wherein CD45.1^{+} cells were chimeric (i.e. derived from donor bone marrow), and CD45.1^{-} cells were host bone marrow derived, and present mostly as a result of radio-resistance. Cells were subsequently gated on the CD4^{+} TCRhi population, to discount CD8 SP cells / cytotoxic T cells, as well as most DP T cells in the thymus (however, few DP cells up-regulating TCR expression will have been included). CD4^{+} TCRhi's were gated as described in figure 1, and separated into CD25^{-}Foxp3^{-} conventional CD4s, CD25^{+} Treg precursors – which were largely only present among donor derived thymocytes, and Foxp3^{+} Tregs. These classes were then further sub-categorized based on ICOS and CD44 expression.

host ($CD45.1^-$) cells, were analyzed by FACS (***fig. 8***). Repopulation of the thymus appeared to occur more efficiently in the $ICOSL^{-/-}$ hosts, where even host-derived thymocyte numbers were raised (***fig. 9***), providing difficulty in comparing populations numerically, as all $ICOSL^{-/-}$ host T-cell populations were numerically raised relative to wildtype (***fig. 9-10***). The proportion of our control population – $CD4^+8^-TCR^{hi}$ cells – was also significantly higher among donor-derived cells in the $ICOSL^{-/-}$ thymus and spleen, as well as host-derived thymocytes (***fig. 9***).

It was however still clear from our findings that there was no fault in thymic production of Treg of any phenotype from donor cells in either KO chimera – $CD25^+$ precursors and Treg were present at proportions (***fig. 10***) and in numbers (***fig. 11***) either comparable to or greater than those in the wildtype thymus. These findings were matched in the spleen ($ICOSL^{-/-}$ and $CD80/86^{-/-}$, ***fig. 12***) and PLNs ($CD80/86^{-/-}$, ***fig. 13***) - although splenic Treg were significantly reduced as a proportion in the $CD80/86^{-/-}$, the percentage change was only very slight. $ICOS/44^{int\text{-}hi}$ Treg, which we observed to be largely absent in the $CD80/86^{-/-}$ mouse (not shown) were similarly present in equivalent or higher numbers and percentages than wildtype hosts in the donor-derived Treg of both $CD80/86^{-/-}$ and $ICOSL^{-/-}$ chimeras (***figs. 10-13***). Interestingly, normal Treg proportions, complete with the $ICOS/44^+$ populations were also present among the host T-cells of $CD80/86^{-/-}$ mice (***fig. 11-13***), suggesting wildtype donor bone marrow has a restorative effect on both generation and homeostasis of $CD80/86^{-/-}$ T-cells.

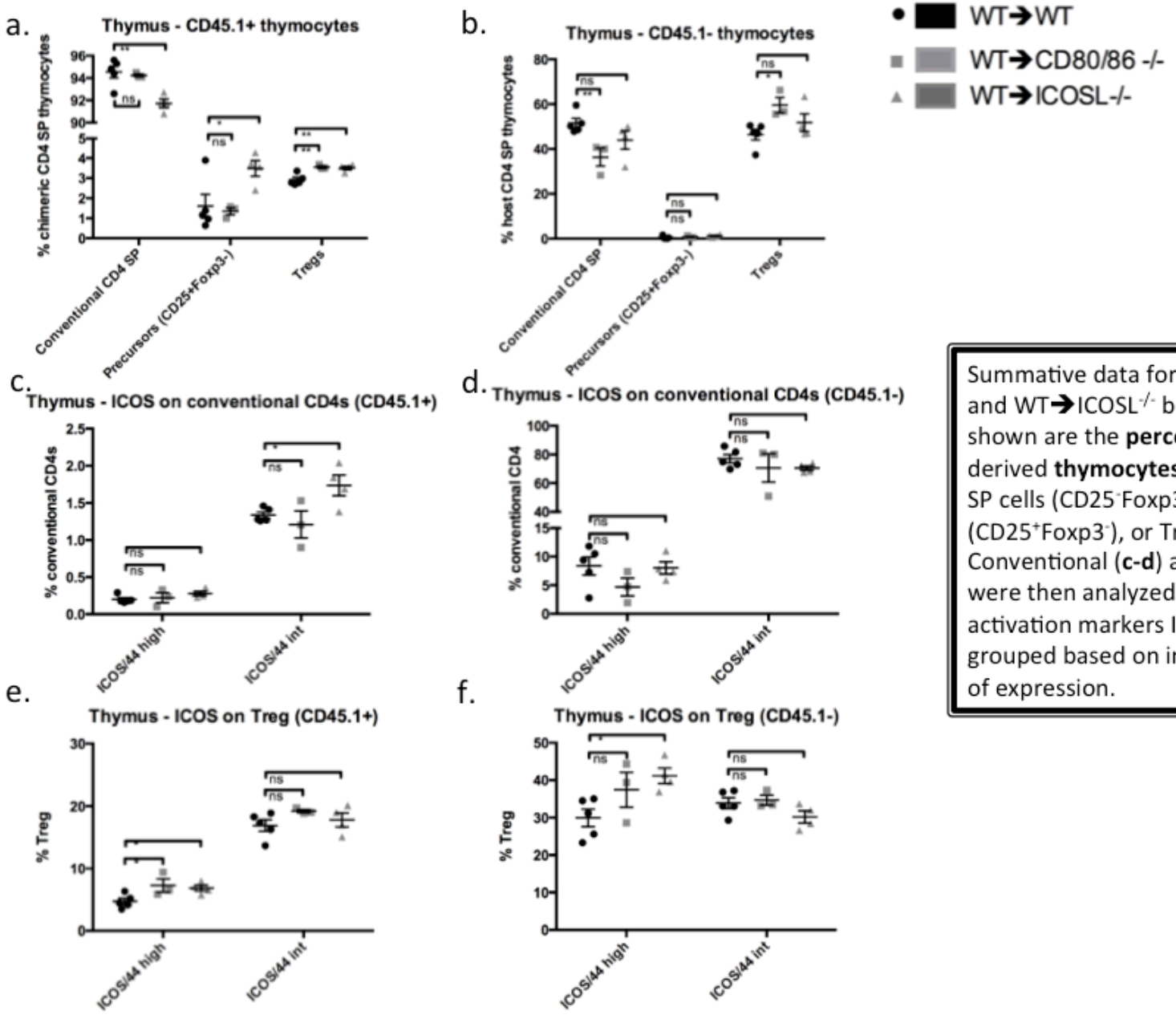
An overwhelming majority of $CD45.1^-$ host cells expressed ICOS and CD44 in all tissues of the bone marrow chimeras. DP thymocytes, which would represent a $CD4^+TCR^{low}$ population, were absent from host cells in each instance (***fig. 8a "*"***), suggesting that

Figure 9 – Summative figure for bone marrow chimera FACS analysis: chimerism and conventional CD4s



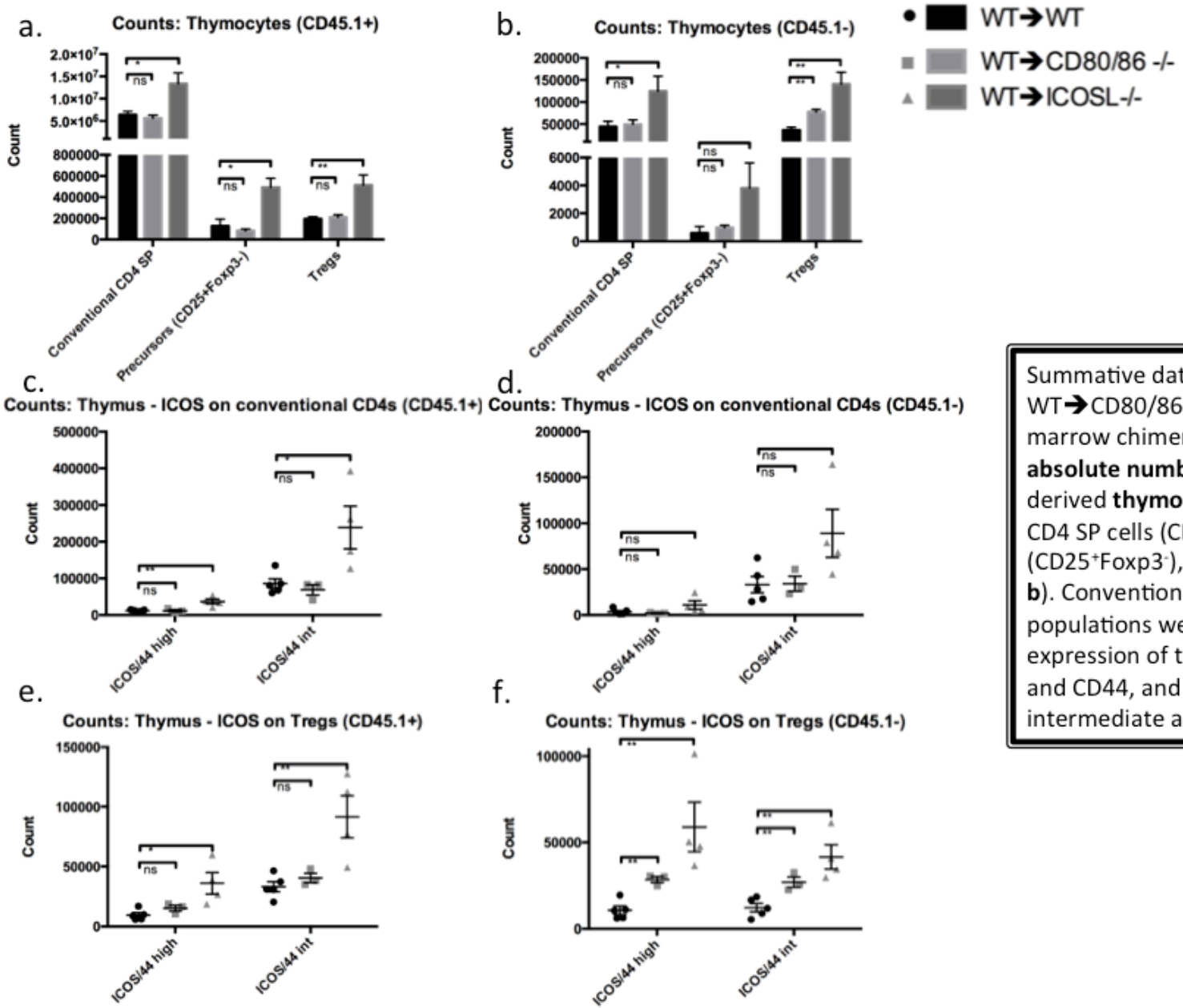
Summative data for WT➔WT, WT➔CD80/86$^{-/-}$ and WT➔ICOSL$^{-/-}$ bone marrow chimeras, analyzing the comparability of chimeras based on relative cellularity and size of CD4^{+}8^{-}TCR^{hi} population. Figures show **a.** thymic cellularity, **b-e.** relative chimerism between CD45.1^{+} donor-derived and CD45.1^{-} host derived cells, **f-g.** proportions of CD4^{+}8^{-}TCR^{hi} cells among host and donor populations in the thymus, spleen and PLNs.

Figure 10 – Summative figure for bone marrow chimera FACS analysis: thymus percentages



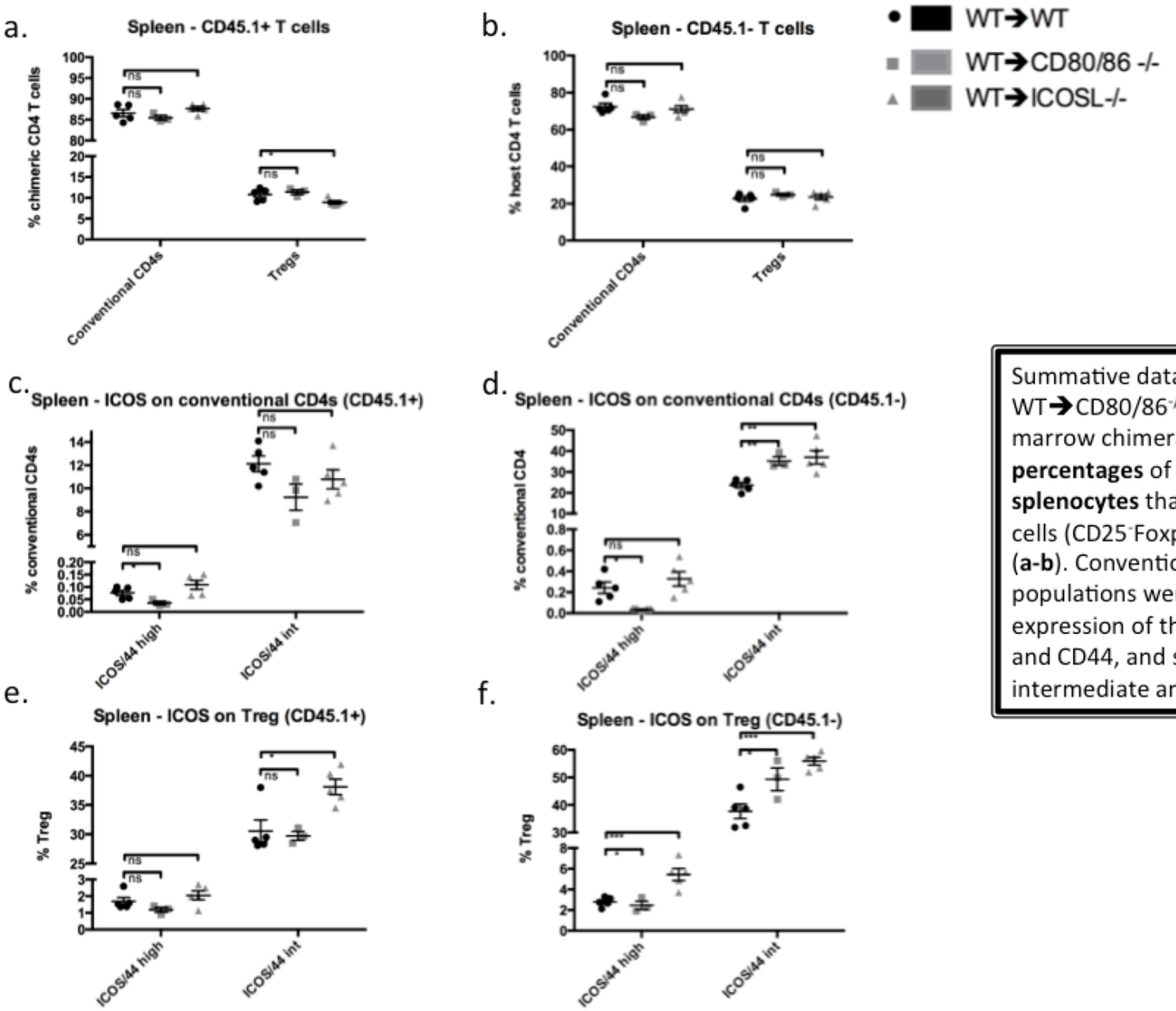
Summative data for WT→WT, WT→CD80/86$^{-/-}$ and WT→ICOSL$^{-/-}$ bone marrow chimeras. Data shown are the **percentages** of donor- and host-derived **thymocytes** that were conventional CD4 SP cells (CD25^{-}Foxp3^{-}), Treg precursors (CD25^{+}Foxp3^{-}), or Tregs (CD25^{+}Foxp3^{+}) (**a-b**). Conventional (**c-d**) and Treg (**e-f**) populations were then analyzed for expression of the activation markers ICOS and CD44, and sub-grouped based on intermediate and high levels of expression.

Figure 11 – Summative figure for bone marrow chimera FACS analysis: thymus counts



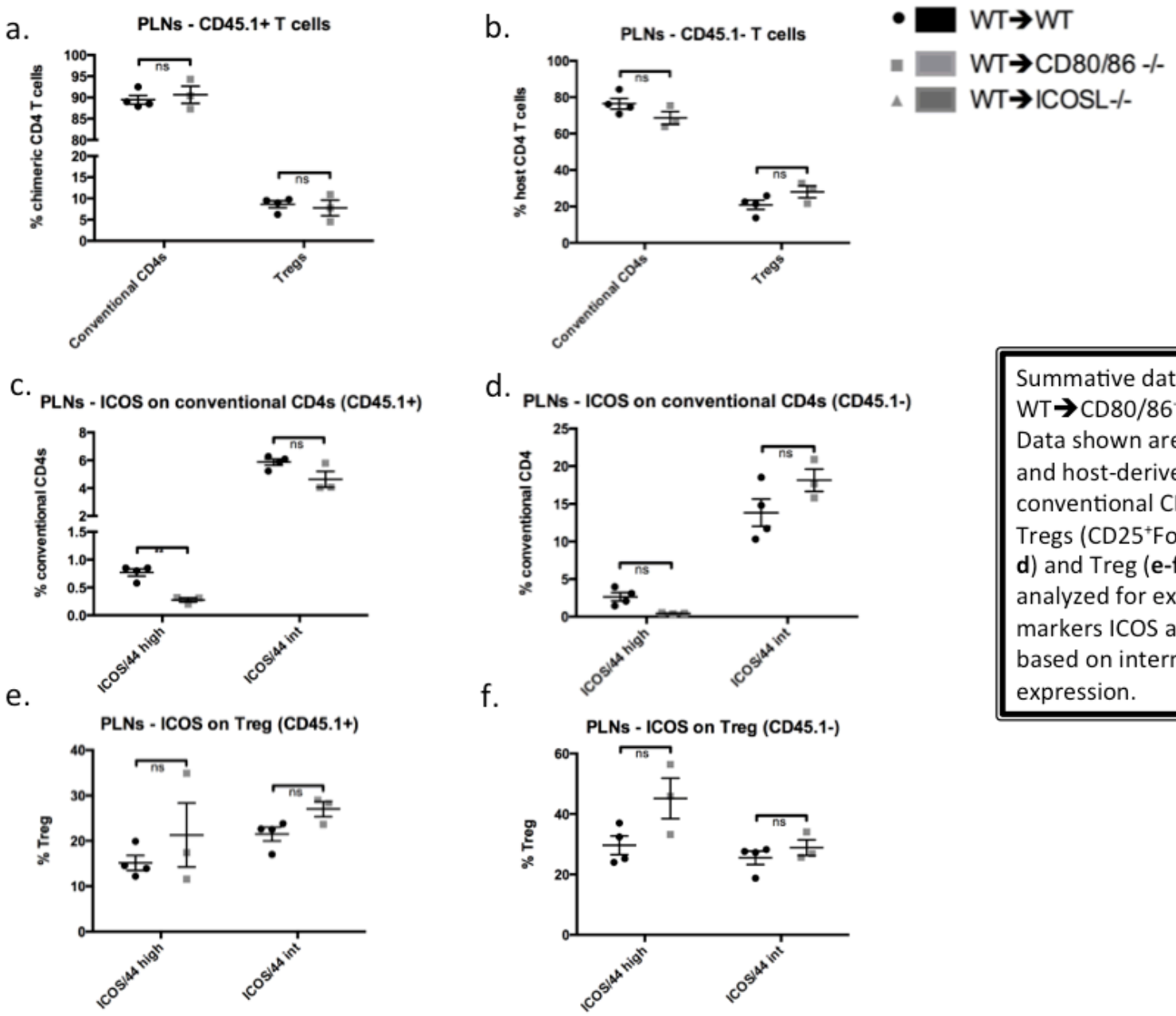
Summative data for WT➔WT, WT➔CD80/86$^{-/-}$ and WT➔ICOSL$^{-/-}$ bone marrow chimeras. Data shown are the **absolute numbers** of donor- and host-derived **thymocytes** that were conventional CD4 SP cells (CD25^{-}Foxp3^{-}), Treg precursors (CD25^{+}Foxp3^{-}), or Tregs (CD25^{+}Foxp3^{+}) (**a-b**). Conventional (**c-d**) and Treg (**e-f**) populations were then analyzed for expression of the activation markers ICOS and CD44, and sub-grouped based on intermediate and high levels of expression.

Figure 12 – Summative figures for BM chimera FACS analysis – spleen



Summative data for WT➔WT, WT➔CD80/86$^{-/-}$ and WT➔ICOSL$^{-/-}$ bone marrow chimeras. Data shown are the **percentages** of donor- and host-derived **splenocytes** that were conventional CD4 SP cells (CD25^{-}Foxp3^{-}) or Tregs (CD25^{+}Foxp3^{+}) (**a-b**). Conventional (**c-d**) and Treg (**e-f**) populations were then analyzed for expression of the activation markers ICOS and CD44, and sub-grouped based on intermediate and high levels of expression.

Figure 13 – Summative figures for BM chimera FACS analysis – PLNs



Summative data for WT➔WT and WT➔CD80/86$^{-/-}$ bone marrow chimeras. Data shown are the **percentages** of donor- and host-derived **iLN T-cells** that were conventional CD4 SP cells (CD25^{-}Foxp3^{-}) or Tregs (CD25^{+}Foxp3^{+}) (**a-b**). Conventional (**c-d**) and Treg (**e-f**) populations were then analyzed for expression of the activation markers ICOS and CD44, and sub-grouped based on intermediate and high levels of expression.

ablation of host bone marrow was complete upon irradiation, and the host cells remaining are radio-resistant rather than newly generated. This is supported by the absence of $CD25^{+}$ Treg precursors (***fig. 10***) – although these did appear to be present in the $ICOSL^{-/-}$ thymus (***fig. 10***), this can likely be attributed to stress-induced activation of conventional cells, which up-regulate CD25, as there was a delay of 24hrs between harvesting and staining. $CD45.1^{-}$ host-derived conventional cells were overwhelmingly of the $ICOS/44^{int}$ phenotype (***figs. 10-13***), whereas Treg were split evenly between $ICOS/44^{int}$ and $ICOS/44^{hi}$ populations (***figs. 10-13***). Among donor-derived cells, significantly higher proportions of $ICOS/44^{int}$ Treg were observed in the $ICOSL^{-/-}$ (***fig. 10-11***), although this could be the result of receptor up-regulation in the face of ligand deletion.

Lastly, we observed notable reductions or total absence of the small numbers of host- and donor-derived $ICOS/44^{hi}$ conventional cells in the spleen and PLNs of $CD80/86^{-/-}$ hosts (***figs. 12-13***). CD80/86 is known to be an important factor in development of iNKT[50] and memory cells[51], both of which express ICOS and CD44. Our pilot study showed a similar reduction in conventional $ICOS/44^{int}$ cells (not shown), and we observed a loss of iNKT numbers in the thymus, but not spleen and iLNs (not shown), although these numbers were not sufficient to explain the loss of $ICOS/44^{int}$ cells. Hence the results from the bone marrow chimera show either a likely persisting defect in the formation of iNKT and/or memory cells as a result of deletion of CD80/86 from stromal cells.

Section 5.0: Discussion

5.1: Co-stimulatory requirements for Treg generation

Treg possessing a highly activated phenotype, including expression of the markers ICOS and CD44, have previously been demonstrated to be antigen-specific effector / memory populations[32,31]. In preliminary experiments, we have observed these cells to have a specific dependence on co-stimulation by the CD28 ligands CD80 and CD86 - ICOS/44 expressing Treg were absent in CD80/86$^{-/-}$ mice, although a total reduction in Treg numbers was not exclusive to this subset (data not shown). Studies have suggested multiple factors contribute to the loss of Treg in these mice, including failure in initial selection, as few CD25^{+} precursors are present[20,22,52], as well as reduced conventional T-cell production of the Treg trophic factor IL-2, which is required for homeostatic proliferation of mature populations[18,23,41]. We observed normal Treg populations to be restored in mice when the deletion of CD80/86 was restricted to the stromal cell compartment through reconstitution with wildtype bone marrow (BM).

That equivalent numbers of CD25^{+} precursors were detected in the thymi of both WT➔WT and WT➔CD80/86$^{-/-}$ mice suggests that selection of Treg - requiring strong TCR ligation together with CD80/86 co-stimulation - was normal. As this process has been shown to be largely mediated by both mTEC and medullary DCs[14], this finding suggests that mTEC selection is either redundant in the presence of wildtype DCs, or relies on alternative co-stimulatory pathways. However, it is difficult to be certain that mTEC CD80/86 expression is redundant physiologically, as the cellularity of the thymus in our BM chimeras was greatly reduced, and TCR transgenic models have shown the selective niche for Treg precursors to be limited[53]. Hence, if more thymocytes were present, the absence of selecting mTECs could

prove to be a limiting factor, reducing nTreg generation. That Foxp3^{+} DP cells were similarly present in normal numbers in our CD80/86$^{-/-}$ host BM chimeras (data not shown) suggests that cortical DCs are likely sufficient for their selection. Redundancy of DC expression of CD80/86 with respect to mTECs could be elucidated using a conditional knockout model, wherein the gene for CD80 or CD86 is flanked by Lox-P sites, and can be excised on expression of CRE-recombinase under control of the DC-specific promoter for CD11c.

Importantly, ICOS/44$^{int\text{-}hi}$ Treg were also found among CD45.1^{-} host cells of CD80/86$^{-/-}$ BM chimeras. As thymic host precursors were not present, and ICOS/44^{+} cells were mostly absent in the CD80/86$^{-/-}$, it appears that ICOS/44^{+} populations can be formed from ICOS/44^{-} in an environment sufficient for CD80/86. ICOS/44^{hi} conventional cells were, however, conspicuously absent from the spleen and PLNs of WT➔CD80/86$^{-/-}$ BM chimeras. Our preliminary experiments confirmed a reduction in conventional ICOS/44^{+} cells in CD80/86$^{-/-}$ mice, some of which were explained by reduced thymic output of iNKT (data not shown). It is therefore possible that this defect in thymic iNKT production is to some degree still present, even after reconstitution with wildtype BM.

Our phenotyping of the thymic ICOS/44^{hi} Treg population suggests maturity; they uniformly express high levels of both Foxp3 and Nrp-1, and have lost expression of the naïve T cell marker CD62L. This is consistent with the idea that ICOS^{+} and CD44^{+} Treg constitute an effector / memory population, arising from naïve precursors upon peripheral antigen encounter[31,32,41]. This is supported from the opposite perspective by the enrichment of CD69^{+} cells among thymic ICOS/44^{neg} Treg relative to the periphery. In the thymus CD69 is a marker for immature SP populations, and is expressed immediately following positive selection, then down-regulated prior to emigration[27] – however, peripheral T-cells express

CD69 upon activation[54]. These patterns of expression are hence consistent with Treg maturing as ICOS/44^{neg} cells before exiting to the periphery, then up-regulating ICOS and CD44 upon antigen encounter, and re-circulating to the thymus.

RAG-GFP mice have revealed that up to 60% of thymic Treg represent an older, either re-circulating or resident population (GFP$^-$), rather than those recently generated within the thymus (GFP$^+$)[23]. The mature phenotype of ICOS/44$^+$ Treg suggests that they represent a significant proportion of, although not all, thymic GFP$^-$ Treg. Although this system cannot differentiate between cells that undergo prolonged thymic retention after generation, and those that are re-circulating from the periphery, it would prove useful in confirming the maturity of ICOS/44$^+$ Treg. Interestingly, a recent study on RAG-GFP mice deficient in CD40 or CD80/86 has shown the GFP$^-$ subset to be particularly susceptible to a loss of IL-2 relative to GFP$^+$ cells and precursors[23]. It was suggested that the cause of thymic Treg loss in CD40$^{-/-}$ mice was down to failure in homeostasis of GFP$^-$ cells, with no observable defect in thymic generation[23]. Although this paper found equivalent defects in both GFP$^{+/-}$ Treg subsets in CD80/86$^{-/-}$ thymi[23], as the GFP$^-$ subset is too large to consist solely of ICOS/44$^+$ cells, the loss of ICOS/44$^+$ Treg from the GFP$^-$ subset may have been diluted by the inclusion of GFP$^-$ ICOS/44$^-$ Treg, which are relatively retained. In any case, it would be useful to introduce exogenous IL-2:α-IL-2 conjugates to observe whether the proportion of ICOS/44$^+$ cells is restored in CD80/86$^{-/-}$ mice, and confirming selective sensitivity to IL-2 depletion. If this were the case, the loss of ICOS/44$^+$ Treg would likely be the result of disrupted homeostasis of a precursor population, rather than ICOS/44^{hi} Treg themselves, as these cells likely depend on IL-7 for turnover[41].

That ICOS/44^{+} Treg in the thymus can simply be explained as recirculating antigen-experienced cells is complicated by our observation of ICOS/44^{hi} cells among TCR^{hi} DP Foxp3^{+} thymocytes (data not shown). Our pilot experiment shows that these are again lost in CD80/86$^{-/-}$ mice, suggesting either that ICOS/44^{hi} Treg can be generated within the thymus as a phenotypically unique subset, or instead ICOS and CD44 are transiently up-regulated on some, if not all developing Treg (although the latter seems unlikely due to the mature phenotype of ICOS/44^{hi} Treg). Initial phenotyping of 7-day old newborn mice suggests that ICOS/44^{hi} Treg are not present at this early stage, although some cells were ICOS/44^{int} (data not shown). Given the delayed appearance of Treg in the newborn thymus, which coincides with medullary development[26], it seems plausible that extended maturation of the thymic micro-environment is required for generation of ICOS/44^{hi} Treg. Several approaches would be effective for determining the ontogeny of ICOS/44^{hi} Treg; Treg could be phenotyped in mice of increasing age to determine the first point of emergence, the development of sorted ICOS/44$^{+/-}$ Treg could be tracked on introduction into fetal thymic organ cultures (FTOC)[55], or Treg could be selectively deleted in RAG-GFP mice using α-CD25 antibodies[4], and re-population of GFP^{+} Treg examined.

No defects in Treg generation or homeostasis were observed in either pilot experiments involving ICOSL$^{-/-}$ mice (data not shown), or WT➔ICOSL$^{-/-}$ BM chimeras. This is surprising, as previous reports suggest that ICOS signaling is crucial in the maintenance of effector / memory Treg[34,56], although these studies were performed in the context of the immune response to allo-antigen challenge, and may not represent the homeostatic requirements of these cells. Currently, no role has been ascribed to mTEC expression of ICOSL[57].

5.2: Homeostatic requirements of T_{EM} and memory Treg are not shared

Having analyzed Treg populations by FACS in a variety of primary and secondary lymphoid organs, we detected ICOS/44$^{neg\text{-}lo}$ Treg in each of the thymus, spleen, BM, and all PLNs, but ICOS/44^{hi} Treg were markedly absent from the spleen and BM, and significantly reduced in the mesenteric lymph nodes (mLN) relative to other PLNs (PLNs and BM not shown). This is a curious finding, as the spleen, and particularly the bone marrow are known to provide IL-7 to support homeostasis of resident memory cell populations[58,59], and ICOS/44^{hi} Treg have up-regulated IL-7Rα expression, likely sharing this requirement for IL-7[41]. The reduced representation in the MLNs may imply that ICOS/44^{hi} cells are solely nTreg, as iTreg are enriched in the MLN through induction in response to food and microbiotal antigens in the gut[60]. Antigen expression may instead dictate the location of ICOS/44^{hi} Treg, as they are specifically enriched at lymph nodes draining the tissues, and in the thymus, where TSAs are promiscuously expressed.

As conventional memory cells, iNKT and Treg are known to be relatively radio-resistant populations[5,61], the CD45.1^{-} host cells of our BM chimeras presented a unique opportunity to observe the phenotype of these cells in isolation. Conventional T-cells were seen to possess an ICOS/44^{int} phenotype, suggesting we're correct in our assumption that these represent T_{EM} and iNKT in the wildtype. Because ICOS/44^{int} Treg appear to share both a phenotype, and tissue distribution with conventional CD4 memory cells, it is tempting to assume that they possess the same homeostatic requirements, including the provision of OX40L and CD30L by LTi[44,48]. A shared dependence on IL-7 seems unlikely, as ICOS/44^{int} Treg fail to up-regulate IL-7Rα, but this could easily be confirmed in a survival assay wherein Treg

are transferred to an IL-$7^{-/-}$ host. Staining of $ICOS^{+}$ Treg in sections of iLN for confocal analysis demonstrated localization indistinguishable from their $ICOS^{-}$ counterparts, with no sign of enrichment at sites associated with LTi[48]. $ICOS^{+}$ cells in the thymus were restricted to the medulla, as were Treg, but although we only observed two ICOS+ Treg, they were both located at the cortico-medullary junction, suggesting these cells are re-circulating from the periphery, and one was caught in interaction with an $ICOS^{+}CD3^{-}CD4^{+}$ cell – a phenotype associated with LTi, although the markers RORgt and IL-7Rα would be required for confirmation.

We detected ICOS/44+ Treg in a RORgt-/- pilot experiment (not shown), but $ICOS/44^{+}$ conventional cells in these mice do not possess genuine memory function – to better evaluated the dependence of these cells on LTi, we utilized mice deleted of OX40 and/or CD30. $CD30^{-/-}$ Treg seemed phenotypically normal, were present in normal numbers, and were if anything proportionally raised in the thymus. We detected expression of OX40 on Treg right from the precursor stage – expression was highest in thymic Treg, but the majority of thymic and splenic Treg were $OX40^{+}$, unlike conventional T cells, which were almost entirely $OX40^{-}$. When OX40 was deleted, thymic Treg were significantly reduced – surprising, as OX40 ligation has previously been shown to down-regulate Foxp3 expression[62] - although precursors were present in normal proportions. Treg in the spleen were reduced significantly to a lesser extent, but only in the OX40/CD30 DKO. This suggests that OX40 plays an important role after initial selection in thymic generation of Treg, which can be made up in the periphery, presumably by expansion of existing populations, except in DKO mice. OX40 and CD30 may hence share redundancy in providing homeostatic signals to Treg in the spleen that are non-essential, but required for optimal Treg survival. As lost numbers of Treg

in these knockout models included, but was not restricted to, ICOS/44^{+} cells, memory Treg and conventional memory cells cannot share signaling requirements for survival.

Interestingly, LNs from OX40 and DKO mice were consistently enlarged, and contained reduced proportions, but normal numbers, of Treg, thanks to the expansion of conventional CD4s. This may suggest expansion in response to either infection or recognition of self-antigen, although this cannot lead to pathogenicity in OX40$^{-/-}$ mice, as T-cells lack the capacity to produce inflammatory cytokines[63]. T-cell expansion could be diagnostic of defective Treg suppression, and hence it would be interesting to find the role played by OX40 in their generation and homeostasis - whether deletion limits the TCR repertoire or suppressive capacity of those Treg that are produced – and indeed where OX40L is expressed in the thymus. The ability of OX40$^{-/-}$ Treg to regulate can easily be assessed by co-transfer with total Foxp3$^{-/-}$ lymphocytes into a mouse lacking T-cells (e.g. RAG$^{-/-}$) – failure to regulate will present as massive auto-immunity, detectable by effector cell expression of IFNγ and tissue invasion, and the production of auto-antibodies[45].

5.3: Conclusion

Although we have shed some light on the factors required for the development of memory–phenotype Treg, and indeed nTreg as a whole, further studies are required to elucidate the origin of these cells – whether they assume their activated phenotype solely after peripheral antigen encounter, or whether they are a heterogenous population, some of which are phenotypically distinct from thymic generation. Although some studies have attributed high suppressor function, proliferative capability and resistance to apoptosis specifically to ICOS^{+} Treg[36,37], we have found the distribution and phenotype of ICOS/44 co-

expressing populations to vary. Hence we would be interested to divide ICOS^{+} Treg into intermediate and high sub-populations, and re-evaluate these capabilities, together with assessing the expression of suppressive cytokines (TGFβ, IL-10) and surface molecules (CTLA-4) upon in vitro activation.

Another preliminary study on Treg populations in two Aire$^{-/-}$ mice also yielded an interesting result worth pursuing. We confirmed previous findings that Aire$^{-/-}$ Treg generation is inhibited[17], as thymic Treg numbers were ~50% of wildtype, although precursor formation is unaffected[17]. ICOS/44 expression was normal, suggesting that selection on TSAs and medullary DCs does not differentiate ICOS^{+} cells, with TSA selection redundant in generation of CD25^{+} precursors. Aire$^{-/-}$ mice are known to be prone to tissue-specific auto-immunity, which we confirmed through autoantibody screens, revealing reactivity against gut and glomerular tissue (not shown). In one of these mice we observed enlargement of the iLNs, accompanied by an expansion of Treg, specifically ICOS/44^{hi} cells. It would hence be of interest to observe and compare the roles of ICOS/44$^{neg/int/hi}$ Treg populations in controlling autoimmune responses, particularly whether ICOS/44^{hi} cells take a central role, which could be achieved using the clever inducible skin-inflammation model of Rosenblum et al[33].

Section 6.0: References

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Table 1:
Antibodies for FACS

Antibody	Staining panel	Surface (S) / Intracellular (IC)	Concentration	Supplier / Clone
CD4 V500	Treg / iNKT / BM chimera	S	1 : 200	BD Horizon, RM4-5
CD8 PB	Treg	S	1 : 200	eBioscience, 53-6.7
CD25 APC	Treg / BM chimera	S	1 : 500	eBioscience, PC61.5
CD44 A700	Treg / iNKT / BM chimera	S	1 : 100	eBioscience, IM7
CD69 FITC	Treg	S	1 : 200	eBioscience, HI 2F3
TCRb 780	Treg / iNKT / BM chimera	S	1 : 100	eBioscience, H57-597
ICOS PerCP Cy5.5	Treg / BM chimera	S	1 : 200	eBioscience, 15F9
Neuropilin-1 Biotin	Treg / BM chimera	S	1 : 500	R&D systems, BAF566
Foxp3 PE	Treg / BM chimera	IC	1 : 100	eBioscience, FJK-16s
CD8 APC	iNKT	S	1 : 800	eBioscience, 53-6.7
NK1.1 PE	iNKT	S	1 : 200	eBioscience, PK136
CD24 PerCP Cy5.5	iNKT	S	1 : 1000	eBioscience, M1/69
CD45.1 PB	BM chimera	S	1 : 150	eBioscience, A20

Table 2:
Antibodies for confocal

Step	Antibody	Concentration	Supplier / Clone
iLN staining protocol			
1	Foxp3 FITC	1 : 100	eBioscience, FJK-16s
2	Rabbit α-FITC 488	1 : 200	Invitrogen
3	Donkey α-Rabbit 488	1 : 100	Invitrogen
1	ICOS biotin	1 : 25	eBioscience, 15-F9
2	SA 555	1 : 500	eBioscience
1	CD4 647	1 : 150	Withers lab
Thymus staining protocol			
1	CD3 FITC	1 : 25	eBioscience, 145-2C11
2	Rabbit α-FITC 488	1 : 200	Invitrogen
3	Donkey α-Rabbit 488	1 : 100	Invitrogen
1	Foxp3 biotin	1 : 100	eBioscience, FJK-16s
1	ICOS biotin	1 : 25	eBioscience, 15F9
2	SA 555	1 : 500	eBioscience
1	Rat IgM anti-TR5	1 : 100	Invitrogen
2	Goat α-rat IgM 594	1 : 400	Invitrogen