

1 **Title:**

2 A Circulating Subset of iNKT Cells Mediates Antitumor and Antiviral Immunity

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1 **Abstract:**

2 Invariant natural killer T (iNKT) cells are a group of innate-like T lymphocytes that recognize
3 lipid antigens. They are supposed to be tissue-resident and important for systemic and local
4 immune regulation. To investigate the heterogeneity of iNKT cells, we re-characterized iNKT
5 cells in the thymus and peripheral tissues. iNKT cells in the thymus were divided into three
6 subpopulations by the expression of the natural killer cell receptor CD244 and the chemokine
7 receptor CXCR6 and designated as C0 (CD244⁻CXCR6⁻), C1 (CD244⁻CXCR6⁺), or C2
8 (CD244⁺CXCR6⁺) iNKT cells. The development and maturation of C2 iNKT cells from C0
9 iNKT cells strictly depended on IL-15 produced by thymic epithelial cells. C2 iNKT cells
10 expressed high levels of IFN- γ and granzymes and exhibited more NK cell-like features, whereas
11 C1 iNKT cells showed more T cell-like characteristics. C2 iNKT cells were influenced by the
12 microbiome and aging and suppressed the expression of the autoimmune regulator AIRE in the
13 thymus. In peripheral tissues, C2 iNKT cells were circulating, which was distinct from
14 conventional tissue-resident C1 iNKT cells. Functionally, C2 iNKT cells protected mice from the
15 tumor metastasis of melanoma cells by enhancing anti-tumor immunity and promoted anti-viral
16 immune responses against influenza virus infection. Furthermore, we identified human
17 CD244⁺CXCR6⁺ iNKT cells with high cytotoxic properties as a counterpart of mouse C2 iNKT
18 cells. Thus, this study reveals a circulating subset of iNKT cells with NK cell-like properties
19 distinct from conventional tissue-resident iNKT cells.

20

21 **One Sentence Summary:**

22 A circulating subset of iNKT cells with NK cell-like properties drives effective anti-tumor and
23 anti-viral immunity.

1 INTRODUCTION

2 Invariant natural killer T (iNKT) cells are a group of innate-like T lymphocytes and have diverse
3 functions straddling innate and adaptive immune responses. Importantly, they are placed at the
4 frontline for protection against cancer and infection, and are involved in chronic inflammation.
5 iNKT cells express invariant T cell receptors (TCRs) and recognize lipid antigens presented on
6 CD1d, a non-polymorphic MHC class I-like molecule (1, 2). Upon stimulation by a potent
7 synthetic antigen, α -galactosylceramide (α -GalCer), or inflammatory cytokines, iNKT cells are
8 rapidly activated and produce large amounts of the cytokines IFN- γ and IL-4, which enhance
9 innate immune responses by activating antigen-presenting cells (APCs) and NK cells (3, 4).
10 Activated iNKT cells induce the maturation of APCs via CD40 ligand and augment adaptive
11 immune responses. They also express Fas ligand and TNF-related apoptosis-inducing ligand
12 (TRAIL) and exert cytolytic functions (2). Notably, iNKT cells are considered to be non-
13 circulating, tissue-resident cells. The regulation and function of iNKT cells differ between tissues,
14 which is important for tissue homeostasis and immunity (5, 6). However, how iNKT cells
15 acquire their heterogeneous homeostasis and function remains unclear.

16 Like conventional T cells, iNKT cells develop in the thymus from CD4⁺CD8⁺ double
17 positive (DP) thymocytes through positive selection (1, 7-11). Murine iNKT cells are separated
18 into four stages according to their intrathymic development. Stage 0 iNKT cell precursors
19 (defined as CD24^{high}CD44⁻NK1.1⁻) differentiate from DP thymocytes, then downregulate CD24
20 and become stage 1 immature iNKT cells (CD24^{low}CD44⁻NK1.1⁻). Subsequently, stage 1 iNKT
21 cells change into stage 2 immature iNKT cells (CD24^{low}CD44⁺NK1.1⁻) and finally differentiate
22 into stage 3 mature iNKT cells (CD24^{low}CD44⁺NK1.1⁺). iNKT cells can also be classified into
23 NKT1, NKT2, and NKT17 based on the expression of transcription factors and cytokines,

1 similar to helper T cell subsets (4). Besides their similarity with conventional T cells, iNKT cells
2 express a variety of NK cell receptors and the cytotoxic molecules perforin and granzymes.
3 However, how iNKT cells acquire NK-like or innate-like features during development needs to
4 be clarified.

5 Interleukin-15 (IL-15) is a pivotal cytokine for the differentiation, maintenance, and
6 response of immune cells (12, 13). It is constitutively produced by stromal and hematopoietic
7 cells in various organs, and its local expression is enhanced during inflammation and immune
8 responses (14, 15). IL-15 exerts its functions on lymphocytes expressing IL-2/15R β chain and
9 common γ -chain through trans-presentation with its high affinity receptor IL-15R α . Previous
10 studies in mice lacking IL-15 or IL-15R α demonstrate that the IL-15 signal plays an essential
11 role in the development and homeostasis of iNKT cells (16, 17). We have identified and
12 characterized IL-15-producing cells in each organ by using IL-15-CFP knock-in reporter mice
13 (15). However, it remains unclear whether different IL-15 niches play a role in the heterogeneity
14 of tissue iNKT cells.

15 To address these questions, we characterized iNKT cells in a series of mice bearing cell
16 type-specific IL-15 deletion. We identified an unrecognized iNKT cell population that depended
17 on IL-15 in the thymus and expressed both NK cell receptor CD244 (also known as 2B4) and
18 chemokine receptor CXCR6. CD244⁺CXCR6⁺ iNKT cells were circulating in peripheral tissues,
19 displayed more NK cell-like features, suppressed tumor dissemination, and enhanced anti-viral
20 immune responses, whereas CD244⁻CXCR6⁺ conventional iNKT cells were tissue-resident and
21 exhibited more T cell-like properties. Furthermore, we also identified CD244⁺CXCR6⁺ iNKT
22 cells that exhibited high cytotoxic properties in people. Thus, this study identifies two subsets of

- 1 iNKT cells, one circulating and the other tissue-resident, with distinct development and functions
- 2 in immunity.
- 3

1 RESULTS

2

3 **An iNKT cell subset produces high levels of IFN- γ in the thymus at steady state**

4 The majority of thymic iNKT cells are NK1.1⁺CD44⁺ mature cells (1, 18, 19). Herein, we
5 identified an iNKT cell subpopulation that expressed high levels of the natural killer cell receptor
6 CD244 (also known as 2B4) and chemokine receptor CXCR6 in the mature fraction of iNKT
7 cells. We designated the CD244⁺CXCR6⁺ double positive (DP) iNKT cells as C2 (because
8 expressing both CD244 and CXCR6) iNKT cells, CD244⁻CXCR6⁺ single positive (SP) iNKT as
9 C1 iNKT cells and CD244⁻CXCR6⁻ double negative iNKT as C0 iNKT cells. The C2 iNKT
10 cells made up 10-20% of mature iNKT cells in the thymus (Fig. 1A) and constantly produced
11 IFN- γ even at steady state, whereas C1 iNKT cells and C0 iNKT cells lacked this expression (Fig.
12 1, B and C). In addition, the expression of CD244 and CXCR6 was unchanged, but IFN- γ
13 production was enhanced, in C1 and C2 iNKT cells by stimulation with PMA and ionomycin (fig.
14 S1A). The transcription factor PLZF, which is required for the determination of iNKT cell
15 lineages but whose expression decreases with maturation (20), was downregulated in C1 and C2
16 iNKT cells compared to C0 iNKT cells (Fig. 1D and fig. S1B).

17 iNKT cells are classified into three functionally distinct subsets, IFN- γ -producing NKT1,
18 IL-4-producing NKT2, and IL-17-producing NKT17 cells, with the expression of different
19 transcription factors, T-bet, GATA3, and ROR γ t, respectively (4). The three iNKT cell subsets
20 were also separated by the expression of surface markers: NKT1 cells as IL-17RB⁻, NKT2 cells
21 as IL-17RB⁺CD4⁺, and NKT17 cells as IL-17RB⁺CD4⁻ (fig. S1C). We found that all C2 iNKT
22 cells were NKT1 cells in C57BL/6 mice, in line with their high IFN- γ expression at steady state
23 (Fig. 1E). We could also detect the CD244⁺CXCR6⁺ population similar to C2 iNKT cells in mice

1 on different genetic backgrounds such as BALB/c, although they completely lacked the
2 expression of NK1.1 (fig S1D). Most of these C2-like iNKT cells on a BALB/c background
3 appeared to be in the NKT1 and NKT17 fractions. However, we found that all C2-like iNKT
4 cells in BALB/c mice expressed IFN- γ but not IL-17A upon activation (fig. S1E). These results
5 suggest that C2 iNKT cells are NKT1 cells and may be important for type 1 immunity.

6 Because iNKT cells comprise CD4⁺ and CD4⁻ subpopulations, we next analyzed CD4
7 expression in C0, C1, and C2 iNKT cells. We found that less than 20% of C2 iNKT cells were
8 CD4⁺ cells, whereas more than 60% of C0 and C1 iNKT cells were CD4⁺ cells (Fig. 1F, and fig.
9 S1F), suggesting that C2 iNKT cells are more enriched in the CD4⁻ subpopulation. C2 iNKT
10 cells expressed higher levels of IFN- γ compared to both the CD4⁺ and CD4⁻ fractions of iNKT
11 cells (fig. S1G). However, the expression levels of developmental and functional molecules as
12 well as the production of IFN- γ were comparable in the CD4⁺ and CD4⁻ subpopulations of C2
13 iNKT cells, suggesting that there are almost no phenotypic or functional differences between
14 CD4⁺ and CD4⁻ C2 iNKT cells in mice (fig. S1, H and I).

15 To further characterize thymic C2 iNKT cells, digital RNA sequencing (dRNA-seq) was
16 performed for a comprehensive genomic profile. We identified a distinct cluster of C2 iNKT
17 cells that was clearly separated from other clusters of C0 and C1 iNKT cells in the thymus by
18 principal component analysis (PCA) (Fig. 1G). In contrast, the difference between CD4⁺ and
19 CD4⁻ C2 iNKT cells was much smaller than the differences among C2, C1, and C0 iNKT cells
20 (fig. S1J). Genes related to granzymes, chemokines, interferons, integrins, and killer cell lectin-
21 like receptors (KLRs) were highly expressed in C2 iNKT cells (Fig. 1H). Gene enrichment
22 analysis revealed the enhanced expression of genes correlated with cell cytotoxicity and
23 activation in C2 iNKT cells (Fig. 1I). Coincidentally, we found that Klf2, a transcription factor

1 important for natural killer (NK) cell homeostasis (21), was expressed at high levels in C2 iNKT
2 cells compared to C1 iNKT cells (Fig. 1, H and J). In contrast, the expression of genes related to
3 TCR signal strength or T cell activation, such as ICOS, Lef1 and CD5, was higher in C1 iNKT
4 cells (Fig. 1, H and K, and fig. S1K). We then performed PCA using the dRNA-seq data of C0,
5 C1, and C2 iNKT cells combined with CD4 SP T, CD8 SP T, and NK cells (Fig.1L). In addition,
6 PD-1, which is associated with the activation or exhaustion of T cells, showed comparable levels
7 between C1 and C2 iNKT cells (fig. S1L). Furthermore, we found that the frequency of C2
8 iNKT cells was higher in young mice but lower in aged mice (Fig. 1M). Besides existing in the
9 thymus, C2 iNKT cells were detected in the bone marrow, peripheral blood, and several
10 peripheral tissues, with a higher frequency in mucosal tissues such as the lung and lamina propria
11 of intestine than in the spleen or liver (Fig. 1N). Taken together, these results suggest that C2
12 iNKT cells exhibit a more NK cell- or innate-like identity, whereas C1 iNKT cells represent a
13 more T cell-like property.

14

15 **The development and maturation of C2 iNKT cells is dependent on the thymic epithelial** 16 **IL-15 niche**

17 IL-15, an essential cytokine for iNKT cells, is produced in the thymus by medullary thymic
18 epithelial cells (mTECs), blood vascular endothelial cells (BECs), and myeloid cells (15). To
19 elucidate the thymic IL-15 niche for C1 and C2 iNKT cell subsets, we generated and analyzed
20 FoxN1-Cre IL-15 conditional knockout (cKO) mice, which lack IL-15 expression in TECs (fig.
21 S2). Stage 3 mature iNKT cells and NKT1 cells significantly decreased in the thymus of FoxN1-
22 Cre IL-15 cKO mice, whereas other T cells remained unchanged (Fig. 2, A and B, and fig. S3, A
23 and B). The survival and proliferation of thymic iNKT cells were promoted by mTEC-derived

1 IL-15 (Fig. 2, C and D). Importantly, we found that C2 iNKT cells had disappeared in FoxN1-
2 Cre IL-15 cKO mice, whereas C1 iNKT cells were reduced by half and C0 iNKT cells were
3 unchanged (Fig. 2, E and F). The numbers of C1 and C2 iNKT cells were not recovered in
4 FoxN1-Cre IL-15 cKO mice in the presence of transgenic Bcl-2 expression, which elevates cell
5 survival, whereas C0 iNKT cells significantly increased (fig. S3C). In addition, besides iNKT
6 cells, we also found that $\gamma\delta$ T1 cells, which is a small population of thymocytes, were modestly
7 decreased in FoxN1-Cre IL-15 cKO mice (fig. S3, D and E), consistent with a recent report (22).
8 To exclude any influence from $\gamma\delta$ T1 cells, we analyzed FoxN1-Cre IL-15 cKO \times TCR δ KO
9 mice. We found that the three iNKT cell subsets changed very similarly compared with FoxN1-
10 Cre IL-15 cKO mice (fig. S3F), suggesting that $\gamma\delta$ T1 cells have minimal, if any, effect on iNKT
11 cells. In addition, some well-known IL-15-dependent lymphocytes such as NK cells, mucosa-
12 associated invariant T (MAIT) cells, and intraepithelial lymphocytes (IELs) were unchanged in
13 FoxN1-Cre IL-15 cKO mice (Fig. 2G and fig. S3, G to K).

14 To assess the IL-15-dependent development and maturation of C0, C1, and C2 iNKT
15 cells, we differentiated each iNKT cell population *ex vivo* in the presence or absence of IL-15.
16 Almost all C0 iNKT cells differentiated into either C1 or C2 iNKT cells at equal frequency, and
17 a small fraction of C1 iNKT cells displayed the potential to mature into C2 iNKT cells in the
18 presence of IL-15 (Fig. 2, H and I). The phenotype of C2 iNKT cells remained unchanged after
19 stimulation, indicating that C2 iNKT cells are the most mature subset. In contrast, the
20 development from C0 or C1 iNKT cells into C2 iNKT cells was impaired in the absence of IL-15.
21 This IL-15 dependence was confirmed *in vivo*, as the administration of IL-15-IL-15R α
22 complexes (RLI) enhanced the frequency of C2 iNKT cells (Fig. 2J). Furthermore, we performed
23 an intrathymic injection of C0 iNKT cells from CD45.1 mice into CD45.2 recipients. We

1 observed that C0 iNKT cells differentiated into C1 and C2 iNKT cells in vivo (Fig. 2K).
2 However, stimulation with α -GalCer, a ligand for the TCR of iNKT cells, failed to promote C2
3 iNKT cells in the absence of IL-15 in FoxN1-Cre IL-15 cKO mice (Fig. 2L). Taken together,
4 these results suggest that the development and maturation of C2 iNKT cell depend on the thymic
5 epithelial IL-15 niche.

6

7 **C2 iNKT cells are controlled by the trans-presentation of IL-15–IL-15R α on mTECs**

8 Because IL-15–IL-15R α trans-presentation controls IL-15 signaling (13), we also employed IL-
9 15R α cKO mice to examine the precise IL-15 signal transduction for C2 iNKT cell development
10 in vivo. Interestingly, C2 iNKT cells were absent in the thymus of FoxN1-Cre IL-15R α cKO
11 mice, a phenotype similar to FoxN1-Cre IL-15 cKO mice (Fig. 3A), indicating that IL-15–IL-
12 15R α trans-presentation by mTECs is indispensable for C2 iNKT cell development. Additionally,
13 other T cells and NK cells remained unchanged in FoxN1-Cre IL-15R α cKO mice (fig. S4A).
14 Although BECs in the thymus expressed high levels of IL-15, a Tie-Cre IL-15 cKO mouse
15 model with bone marrow transplantation elucidated that BEC-derived IL-15 did not affect iNKT
16 cells or other T cells in the thymus (Fig. 3B and fig. S4B). We then asked whether iNKT cell
17 development or maintenance was also promoted by IL-15–IL-15R α trans-presentation between
18 TECs and myeloid cells, since some myeloid cells such as macrophages and dendritic cells
19 (DCs) express IL-15 and IL-15R α in the thymus. However, the numbers of iNKT cells and T
20 cells were unchanged in the thymus of LysM-Cre and CD11c-Cre IL-15 cKO mice (Fig. 3, C and
21 D, and fig. S4, C and D). Coincidentally, IL-15R α presented by myeloid cells and thymocytes
22 themselves did not affect iNKT cell development or maintenance in the thymic IL-15 niche (fig.

1 S4, E to G). Taken together, these results demonstrate that the IL-15–IL-15R α trans-presentation
2 by mTECs controls C2 iNKT cells in vivo.

3
4 **C2 iNKT cells are adjacent to mTECs and regulate self-antigen expression for immune**
5 **tolerance in the thymus**

6 To clarify the spatial relationship between IL-15-producing mTECs and iNKT cells in the
7 thymus, we carried out immunohistochemistry experiments on IL-15-CFP knock-in mice. Many
8 iNKT cells were distributed around IL-15-producing cells in the thymic medulla, and a
9 quantification of contacting thymocytes showed that iNKT cells were more adjacent to IL-15-
10 producing mTECs than CD4 and CD8 SP thymocytes (Fig. 3, E and F, and fig. S5, A and B).
11 Furthermore, we found that C2 iNKT cells constituted approximately 45% of CD244⁺TCR β ⁺
12 cells in the thymus (fig. S5C), and that the majority of these CD244⁺TCR β ⁺ cells were adjacent
13 to IL-15-CFP⁺ TECs in the thymic medulla (fig. S5, D and E). Collectively, these results suggest
14 that IL-15-producing mTECs may provide preferable sites for the development and maintenance
15 of C2 iNKT cells.

16 Because we found that C2 iNKT cells were the major source of IFN- γ in the thymus at
17 steady state (Fig. 3, G and H), we investigated the function of IFN- γ produced by C2 iNKT cells
18 in the thymic epithelial IL-15 niche. The expressions of the autoimmune regulator AIRE, a
19 transcription factor which induces the self-antigen expression in mTECs (23), and an AIRE-
20 dependent self-antigen, Ins2, were elevated in mTECs, and AIRE⁺ mTECs appeared to increase
21 in FoxN1-Cre IL-15 cKO mice (Fig. 3, I and J), consistent with a previous report that IFN- γ
22 influences the self-antigen expression by mTECs (24). By contrast, the expression of Fezf2,
23 another regulator of self-antigen expression in mTECs, remained unchanged (fig. S5F).

1 Furthermore, we employed a CD1d1/2 KO mouse line, which lacks iNKT cells, and found that
2 the expression levels of AIRE by mTECs were comparable between FoxN1-Cre IL-15 cKO ×
3 CD1d1/2 KO mice and control CD1d1/2 KO mice (Fig. 3K), suggesting that alteration of AIRE
4 expression was iNKT cell-dependent. Overall, C2 iNKT cells express high levels of IFN- γ ,
5 which may suppress the expression of AIRE and AIRE-dependent self-antigens in mTECs for
6 immune tolerance.

7

8 **C2 iNKT cells are influenced by the gut microbiome**

9 The gut microbiome alters with age and may influence iNKT cell homeostasis (25). Because
10 thymic C2 iNKT cells were reduced with aging (Fig. 1M), we compared thymic iNKT cells
11 between specific pathogen-free (SPF) and germ-free (GF) conditions. The frequency of thymic
12 C2 iNKT cells in GF mice was lower than in 4-week-old SPF mice (Fig. S6, A and B). However,
13 the frequency of C2 iNKT cells was unchanged by antibiotic treatment in 12-week-old mice (fig.
14 S6C), suggesting that microbiota influence C2 iNKT cells only in younger mice or that the
15 antibiotic treatment did not eliminate the microbiota that impact C2 iNKT cells. Together, these
16 results suggest that the frequency of thymic C2 iNKT cells may be influenced by the gut
17 microbiome.

18

19 **Heterogeneity of peripheral C1 and C2 iNKT cells**

20 We characterized C1 and C2 iNKT cells in the peripheral tissues of FoxN1-Cre IL-15 cKO mice.
21 Despite C1 iNKT cells being recovered, C2 iNKT cells remained severely reduced in the lung,
22 spleen, peripheral blood, and liver (Fig. 4, A to D and fig. S7A). In addition, we also confirmed
23 that the numbers of C1 and C2 iNKT cells in the lung of FoxN1-Cre IL-15 cKO mice × TCR δ

1 KO mice changed very similarly as in FoxN1-Cre IL-15 cKO mice (fig. S7B). Although the T
2 cell number in the peripheral tissues of FoxN1-Cre IL-15 cKO mice was comparable to control
3 mice, NK cells in FoxN1-Cre IL-15 cKO mice seemed to slightly increase in the lung and spleen
4 (fig. S7, C to F), unlike in the thymus where NK cells were unchanged (Fig. 2G), suggesting that
5 C2 iNKT cells may negatively influence NK cell maintenance in peripheral tissues at steady state.
6 These results suggest that the thymic epithelial IL-15 niche is important for peripheral C2 iNKT
7 cells and that FoxN1-Cre IL-15 cKO mice are a valuable tool for analyzing the functions of C2
8 iNKT cells in vivo.

9 To further investigate the differences between C1 and C2 iNKT cells in the peripheral
10 tissues, we isolated iNKT cells from the lung, where C2 iNKT cells were relatively abundant,
11 and analyzed their gene expression profile by dRNA-seq. Lung C1 and C2 iNKT cells were
12 separated into two distinct clusters by PCA (Fig. 4E). In comparison to C1 iNKT cells, C2 iNKT
13 cells in the lung expressed higher levels of chemokines, granzymes, integrins, and KLRs, but
14 lower levels of *Satb1*, *ICOS* and *Ly6a*, and the expression pattern of these genes was similar to
15 that in the thymus (Fig. 4F and fig. S7G). Furthermore, the transcription factor for NK cell
16 homeostasis, *Klf2*, was also higher in lung C2 iNKT cells (Fig. 4F). To compare the in vivo
17 distribution, we performed intravascular staining with anti-CD45 antibody. The majority of T
18 and NK cells were present within blood vessels of the lung, whereas C1 iNKT cells were
19 preferentially localized in the lung parenchyma (Fig. 4, G and H). Notably, the frequency of C2
20 iNKT cells was between these other cell types, indicating that C2 iNKT cells have more
21 transmigration activity between the lung parenchyma and blood vessels. To reveal the
22 dependency on the peripheral IL-15 niche, we analyzed lung C1 and C2 iNKT cells in *LysM-Cre*
23 and *CD11c-Cre* IL-15 cKO mice for myeloid cell-derived IL-15 and *SPC-Cre* IL-15 cKO mice

1 for pulmonary epithelial cell-derived IL-15. We found that the expansion or maturation of lung
2 C1 iNKT cells partially depended on IL-15 produced by peripheral myeloid cells, whereas C2
3 iNKT cells in the lung were independent of peripheral IL-15 (Fig. 4, I and J, and fig. S7H). In
4 addition, peripheral IL-15 also contributed to the maintenance of NK cells in the lung (Fig. 4, I
5 and J). Taken together, C2 iNKT cells display more innate-cell like properties than C1 iNKT
6 cells and depend on the thymic epithelial IL-15 niche even in the peripheral tissues, whereas C1
7 iNKT cells partially depend on the myeloid IL-15 niche in the peripheral tissues.

8

9 **Distinct cell dynamics of peripheral C1 and C2 iNKT cells**

10 iNKT cells are considered to be non-circulating, tissue-resident cells (5, 26). To further
11 investigate how C1 and C2 iNKT cells are maintained by different IL-15 niches and distributed
12 in distinct manners in the peripheral tissues, we performed parabiosis experiments between
13 CD45.1 and CD45.2 mice to assess the dynamics of C1 and C2 iNKT cells. Notably, we found
14 that C2 iNKT cells circulated in the peripheral tissues such as the lung and spleen, like NK cells
15 but contrary to C1 iNKT cells, which showed significantly higher tissue residency (Fig. 5, A to
16 D, and fig. S8, A and B). Consistent with recent reports that a purinergic receptor, P2X7R, is
17 related to the tissue-residency of lymphocytes and that tissue-resident iNKT cells are
18 preferentially controlled by P2X7R activation (27), P2X7R was expressed more in lung C1
19 iNKT cells than in C2 iNKT cells (Fig. 5, E and F). A similar pattern of iNKT cell residency was
20 observed in the bone marrow (Fig. 5, G and H), whereas C1 and C2 iNKT cell populations
21 mixed almost equally in peripheral blood according to the parabiosis experiments (fig. S8, C and
22 D). In the thymus, all C0, C1, and C2 iNKT cells were tissue-resident (Fig. 5I), consistent with a

1 previous report that NK1.1⁺ iNKT cells are retained in the thymus (18). The expression levels of
2 P2X7R were similar between C1 and C2 iNKT cell populations in the thymus (Fig. 5, J and K).

3 Because CXCR6, the receptor for CXCL16, is implicated in the tissue retention of iNKT
4 cells (28), we next analyzed CXCR6 KO mice. We found that C1 iNKT cells were significantly
5 reduced in the lungs of CXCR6 KO mice, whereas C2 iNKT cells were unaffected (Fig. 5L).
6 Because C2 iNKT cells expressed higher levels of integrin α 1 (CD49a), we administered anti-
7 CD49a blocking antibody into the mice. The number of C2 iNKT cells, but not C1 iNKT cells,
8 was partially reduced in the lung (Fig. 5M). Of note, neither CXCR6 deficiency nor CD49a
9 blocking impaired thymic iNKT cell development or maintenance (fig. S8, E and F). Klf2, a
10 transcription factor highly expressed in C2 iNKT cells, has been reported to regulate T cell
11 trafficking by promoting S1pr1 (29). We found that the S1pr1 expression was upregulated in C2
12 iNKT cells (fig. S8G). Furthermore, the frequency of intravascular C2 iNKT cells was modestly
13 decreased by FTY720 treatment, an antagonist of S1P1, similar to the alteration of intravascular
14 T cells (fig. S8H). Taken together, these results suggest that C2 iNKT cells are a circulating
15 iNKT cell subset and that their retention and migration in peripheral tissues are affected by
16 integrin α 1 and S1P1. In contrast, C1 iNKT cells represent a tissue-resident iNKT subset that
17 requires CXCR6 for its tissue residency.

18 19 **C2 iNKT cells exhibit high cytotoxicity and enhance anti-tumor immunity**

20 Because iNKT cells have potent anti-tumor activity by linking innate and adaptive immunity
21 (30-32), we employed a B16-F10 melanoma metastasis model to assess C2 iNKT cell function in
22 anti-tumor immunity. Metastatic nodules in the lung were increased in FoxN1-Cre IL-15 cKO
23 mice as well as FoxN1-Cre IL-15 cKO \times TCR δ KO mice when compared to control mice (Fig. 6,

1 A and B, and fig. S9A), which correlated with a significant decrease of C2 iNKT cells (Fig. 6C
2 and fig. S9B). In addition, NK cells, especially KLRG1⁺ mature NK cells, were fewer, and their
3 proliferation and activation were reduced following the B16-F10 melanoma inoculation in
4 FoxN1-Cre IL-15 cKO mice (Fig. 6, C to E). On the other hand, the numbers of C1 iNKT cells,
5 T cells, and B cells, which are also important for anti-tumor immunity, were unchanged in the
6 lungs of FoxN1-Cre IL-15 cKO mice after the inoculation (Fig. 6C and fig. S9C). Because CD8
7 T cells and NK cells exert their effector functions in anti-tumor immunity by producing IFN- γ ,
8 we analyzed the IFN- γ expression by using IFN- γ -Venus reporter mice. We found that CD8 T
9 cells and NK cells produced comparable levels of IFN- γ between FoxN1-Cre IL-15 cKO mice
10 and control mice after the inoculation of B16-F10 melanoma cells, suggesting that effector
11 functions of CD8 T cells and NK cells may be unchanged in FoxN1-Cre IL-15 cKO mice (fig.
12 S9D). Additionally, there was no difference in the number of metastatic nodules in the lungs of
13 FoxN1-Cre IL-15 cKO \times CD1d1/2 KO mice and control CD1d1/2 KO mice (Fig. 6F).
14 Collectively, these data suggest that C2 iNKT cells are involved in anti-tumor immunity.

15 Next, to assess the direct cytotoxicity of C2 iNKT cells, we checked the expression of
16 IFN- γ and the cytotoxic molecules such as granzyme B, perforin, and TRAIL, which mediate
17 anti-tumor immunity. C2 iNKT cells produced higher levels of IFN- γ and granzyme B than C1
18 iNKT cells after B16-F10 melanoma inoculation (Fig. 6, G and H). Notably, C2 iNKT cells
19 expressed extremely high levels of IFN- γ , like NK cells (Fig. 6G), whereas the levels were of
20 CD4⁺ and CD4⁻ C2 iNKT cells and were similar (fig. S9E). Upon co-culture with B16-F10
21 melanoma cells after TCR and IL-15 stimulation, C2 iNKT cells expressed higher levels of
22 granzyme B, perforin, and TRAIL than C1 iNKT cells (Fig. 6, I and J, and fig. S9F). Moreover, a

1 tumor inhibition assay showed that C2 iNKT cells had significantly higher cytotoxic activity
2 than C1 iNKT cells against B16-F10 melanoma cells (Fig. 6K).

3 To confirm the function of C2 iNKT cells in anti-tumor immunity, we adoptively
4 transferred C2 iNKT cells from CD45.1 mice into CD45.2 FoxN1-Cre IL-15 cKO recipient mice
5 one day after the inoculation of B16-F10 melanoma cells (fig. S9G). Metastatic nodules in the
6 lung were significantly decreased in mice with C2 iNKT cell transfer (Fig. 6L). The number of
7 NK cells, especially KLRG1⁺ mature NK cells, increased after the adoptive transfer of C2 iNKT
8 cells, whereas the numbers of T cells and B cells were unchanged (Fig. 6, M and N, and fig.
9 S9H). However, the IFN- γ expression by relevant cells was unaltered after the adoptive transfer
10 of C2 iNKT cells (Fig. 6O). α -GalCer injection in mice activates whole iNKT cells and enhances
11 anti-tumor immunity (33). We found that tumor metastases were also reduced in FoxN1-Cre IL-
12 15 cKO mice after stimulation with α -GalCer, maybe because the strong activation of C1 iNKT
13 cells compensated for the lack of C2 iNKT cells (fig. S9I). Moreover, we found that the tumor
14 cells suppressed the development and maturation of C2 iNKT cells from C0 and C1 iNKT cells
15 in ex vivo culture even in the presence of IL-15 (fig. S9, J and K). These results suggest that C2
16 iNKT cells seems to be inhibited within the tumor microenvironment, whereas the thymic IL-15
17 niche may provide a uniquely preferable site for them. Taken together, these findings suggest
18 that C2 iNKT cells are a highly effective tumor-suppressing iNKT cell subset and that they
19 enhance anti-tumor immunity against cancer metastasis in the lung. The function of C2 iNKT
20 cells in anti-tumor immunity may be ascribed to their high cytotoxic activity against tumor cells
21 and their supporting activity for the proliferation and maturation of NK cells.

22

23 **C2 iNKT cells promote the anti-viral immune response against influenza virus infection**

1 Although the consequences of IL-15 expression and iNKT cells in the control of pulmonary virus
2 infection have been well discussed (3, 4), the role of circulating iNKT cells in virus infection is
3 unknown. To address this question, FoxN1-Cre IL-15 cKO and control mice were infected
4 intranasally with a 0.5 LD₅₀ (50% lethal dose) of influenza A virus (IAV). In accordance with the
5 importance of IFN- γ production in anti-viral immunity mediated by lymphocytes, we found that
6 C2 iNKT cells highly expressed IFN- γ in response to IAV infection (Fig. 7A). In line with an
7 impaired virus clearance (Fig. 7B), C2 iNKT cells were significantly decreased in FoxN1-Cre
8 IL-15 cKO mice, whereas the number of C1 iNKT cells was unchanged compared to control
9 mice after IAV inoculation (Fig. 7C). Consistently, IAV-infected FoxN1-Cre IL-15 cKO mice
10 exhibited a reduced frequency and absolute number of IAV-specific CD8 T cells compared to
11 IAV-infected control mice (Fig. 7, D and E). The number of NK cells moderately decreased,
12 whereas the numbers of CD4 and CD8 T cells were unchanged (Fig. 7F). Furthermore, virus
13 clearance was unchanged between FoxN1-Cre IL-15 cKO \times CD1d1/2 KO mice and control
14 CD1d1/2 KO mice, suggesting that C2 iNKT cells were involved in anti-viral immunity (Fig.
15 7G). In addition, as CD11b⁺Gr1⁺ myeloid-derived suppressor cells (MDSCs) effectively
16 suppress anti-viral immunity (34), we next analyzed MDSCs and found that the frequency and
17 number of MDSCs increased in IAV-infected FoxN1-Cre IL-15 cKO mice (Fig. 7H and fig.
18 S10A), whereas the numbers of other myeloid cells such as DCs were unchanged (fig. S10, B
19 and C).

20 To confirm the function of C2 iNKT cells in anti-viral immunity, we adoptively
21 transferred C2 iNKT cells from CD45.1 mice into CD45.2 FoxN1-Cre IL-15 cKO recipient mice
22 and infected the mice with IAV on the same day. The virus clearance was significantly
23 accelerated in FoxN1-Cre IL-15 cKO mice following the C2 iNKT cell transfer (Fig. 7I). The

1 numbers of C1 iNKT cells, NK cells, and CD8 T cells, especially IAV-specific CD8 T cells,
2 were elevated in FoxN1-Cre IL-15 cKO recipient mice after the adoptive transfer of C2 iNKT
3 cells, whereas MDSCs slightly decreased, but not significantly, and CD4 T cells were unchanged
4 compared to FoxN1-Cre IL-15 cKO mice (Fig. 7, J to M, and fig. S10D). Furthermore, IFN- γ
5 production in C1 iNKT cells was upregulated, whereas IFN- γ production in other cells was not
6 altered after adoptive transfer of C2 iNKT cells (Fig. 7N), suggesting that C2 iNKT cells may
7 promote the effector function of C1 iNKT cells in anti-viral immunity. By contrast, when we
8 infected the mice with a lethal dose (2 LD₅₀) of IAV, the body weight loss became much milder,
9 and the survival rate was higher in FoxN1-Cre IL-15 cKO mice than control mice (fig. S10, E
10 and F), suggesting that an attenuated anti-viral immune response may result in a delay of virus
11 clearance, leading to sustained inflammation, tissue damage, and increased mortality in FoxN1-
12 Cre IL-15 cKO mice. Collectively, these results suggest that circulating C2 iNKT cells enhance
13 the immune response against IAV infection.

14

15 **Human C2 iNKT cells exhibit high cytotoxicity like mouse C2 iNKT cells**

16 Since CD244 and CXCR6 are expressed in human iNKT cells in peripheral blood (35) and the
17 expression levels correlate with disease progression in human immunodeficiency virus (HIV)
18 patients (36), we further investigated iNKT cells in human samples. CD244⁺CXCR6⁺ and
19 CD244⁻CXCR6⁺ iNKT cell subpopulations corresponding to mouse C2 and C1 iNKT cells,
20 respectively, were detectable in human peripheral blood and liver perfusates (Fig. 8A and fig.
21 S11A). Notably, all human C2 iNKT cells were CD4⁻ cells (Fig. 8B). We also found that the
22 frequency of human C2 iNKT cells was higher in young donors (18 to 32 years old) and lower in
23 aged donors (52 to 56 years old) (Fig. 8, A and C), suggesting the possibility that it is due to the

1 higher thymic dependency of C2 iNKT cells. Human C2 iNKT cells also highly expressed IFN- γ
2 compared to human C1 iNKT cells after stimulation, similar to mouse C2 iNKT cells, whereas
3 the production of IL-17 was undetectable (Fig. 8D and fig. S11B).

4 Finally, we analyzed gene expression of human C2 and C1 iNKT cells by dRNA-seq. We
5 found that human C2 and C1 iNKT cells were separated into two distinct clusters by PCA (Fig.
6 8E). Gene expression profiling revealed that human C2 iNKT cells expressed high levels of
7 genes related to granzymes, granulysin, and perforin for iNKT-cell mediated effector functions
8 and KLRs, like mouse C2 iNKT cells (Fig. 8F). Gene enrichment analysis also showed an
9 enhanced expression of genes correlated with cell cytotoxicity and activation in human C2 iNKT
10 cells (Fig. 8G). Furthermore, we checked whether developmental and functional molecules were
11 highly expressed on the cell surface of human C2 iNKT cells by flow cytometry. Human C2
12 iNKT cells expressed higher levels of CD161, CD94, and CCR5, and lower levels of ICOS,
13 compared to C1 iNKT cells (Fig. 8H and fig. S11C). Taken together, these data suggest that
14 human C2 iNKT cells exhibit high cytotoxicity and activity important for type 1 immunity,
15 similar to mouse C2 iNKT cells.

16

1 **DISCUSSION**

2 iNKT cells are a group of innate-like T lymphocytes and have been classified by their
3 maturational stages or lineage diversification with specific transcription factors (1, 2). They are
4 considered to be non-circulating, tissue-resident cells (5, 6, 26). In this study, however, we
5 identified circulating iNKT cells (C2 iNKT cells) in mice that were distinct from conventional
6 tissue-resident iNKT cells (C1 iNKT cells). We defined C2 iNKT cells as CD244⁺CXCR6⁺ DP
7 iNKT cells in NKT1 cells. In concert with the notion that conventional iNKT cells contribute to
8 local immunity such as tissue-specific homeostasis and protection, C1 iNKT cells exhibited
9 tissue-resident properties and probably play the same role. In contrast to tissue-resident
10 lymphocytes, circulating lymphocytes actively survey the body to encounter antigens and evoke
11 systemic immune responses. In this sense, C2 iNKT cells with circulating properties can
12 probably sense detrimental insults such as metastatic tumor cells or invading microorganisms
13 and orchestrate systemic immunity by their high cytotoxicity and by activating relevant immune
14 cells. C2 iNKT cells depended on the thymic IL-15 niche for their development and maturation,
15 whereas C1 iNKT cells were probably maintained by a distinct peripheral IL-15 niche.
16 Furthermore, we also identified CD244⁺CXCR6⁺ human C2 iNKT cells, a human counterpart of
17 mouse C2 iNKT cells, that exhibited high cytotoxicity. Thus, we propose a layered structure of
18 iNKT cells composed of tissue resident C1 and circulating C2 iNKT subsets. This structure is
19 analogous to that of group 1 innate lymphoid cells (ILCs) with tissue resident ILCs and
20 conventional circulating NK cells. These two subsets of iNKT cells together provide diverse
21 systemic and tissue-specific immune regulations.

22 We found that C2 iNKT cells were a highly IFN- γ -producing iNKT cell subset in the
23 thymus at steady state. C2 iNKT cells seemed to interact with IL-15-producing mTECs and

1 received trans-presented IL-15-IL-15R α for their development and maturation. Of interest, we
2 found that C2 iNKT cells were the major source of IFN- γ in the thymus. Similar to IFN- γ -
3 deficient mice (24), FoxN1-Cre IL-15 cKO mice, which lack C2 iNKT cells, exhibited an
4 elevated expression of AIRE, suggesting a crosstalk between C2 iNKT cells and mTECs. It is
5 known that the crosstalk between thymocytes and mTECs is crucial for the development of the
6 thymic medulla (37, 38), and we found that iNKT cells in the medulla were adjacent to mTECs.
7 Thus, IL-15-producing mTECs may provide a preferred site for C2 iNKT cell development and
8 maturation from C0 iNKT cells, and, conversely, C2 iNKT cells expressing IFN- γ might regulate
9 immune tolerance through the expression of AIRE-dependent tissue-specific antigens.

10 Despite C1 iNKT cells being recovered, C2 iNKT cells remained severely reduced in
11 peripheral tissues such as the lung, spleen, peripheral blood, and liver. These results suggested
12 that C1 iNKT cells may have undergone expansion or maturation in peripheral tissues, whereas
13 the development and maturation of C2 iNKT cells by IL-15 was limited to the thymic medulla.
14 Yet, there remains another possibility that the recovery in the peripheral tissues may be due to
15 the different emigration rates between C2 and C1 iNKT cells.

16 In the lungs, C2 iNKT cells were associated with the lung parenchyma by integrin α 1,
17 similar to memory CD8 T cells (39), whereas C1 iNKT cells required the chemokine receptor
18 CXCR6, as previously reported for iNKT cell localization (28). C2 iNKT cells in the peripheral
19 tissues showed a similar gene expression pattern to those in the thymus, with more NK cell- or
20 innate cell-like properties. In accordance with their circulating properties, C2 iNKT cells
21 expressed S1PR1 at higher levels, suggesting that C2 iNKT cells may recirculate in a S1P1-
22 dependent manner (29). Besides the higher expression of genes related to cell activity, mobility,
23 and cytotoxicity, C2 iNKT cells also highly expressed several NK cell receptors including

1 CD244 (2B4) and killer cell lectin-like receptors, which have both positive and negative
2 functions (40). In contrast to the marked decrease of C2 iNKT cells in mice with IL-15
3 deficiency in the thymus, NK cells, another IL-15-dependent innate-like lymphocyte cell type,
4 were slightly increased in the peripheral tissues. Because C2 iNKT cells were reduced in the
5 peripheral tissues and did not depend on peripheral IL-15, we speculate that C2 iNKT cells may
6 have suppressive effects on peripheral NK cells at steady state, however, the precise mechanism
7 needs to be investigated further.

8 iNKT cells are influenced by the microbiota in intestines and involved in inflammatory
9 bowel diseases, and iNKT cells from GF mice are hypo-responsive compared with those from
10 SPF mice (25). Another study showed that the number of iNKT cells increases after the
11 monocolonization of neonatal GF mice with *Bacteroides fragilis* (41). We found a reduction of
12 thymic C2 in parallel with aging, and that C2 iNKT cells were decreased in 4-week-old GF mice.
13 Because the gut microbiome alters with weaning, these data suggest that dietary changes may
14 have influenced C2 iNKT cell development by the microbiota. However, the frequency of C2
15 iNKT cells was unchanged in antibiotic-treated 12-week-old mice, maybe due to the high
16 antibiotics resistance of *Bacteroides fragilis* (42). Our data also may explain the notion that
17 alterations of the gut microbiome in young mice have more profound effects on iNKT cells than
18 in aged mice.

19 In contrast to the influence of the microbiota, iNKT cells provide protection against
20 various pathogens such as bacteria and viruses (43). iNKT cells play an important role in host
21 defense at the early phase of infection through the recognition of microbial glycolipids. C2 iNKT
22 cells, which were highly cytotoxic and highly express IFN- γ , may contribute to this process more
23 efficiently with their circulating properties. In addition, peripheral C2 iNKT cells highly

1 expressed TLR2 and IL-1 β , which are involved in immune responses against influenza virus (44).
2 They also secreted abundantly chemokines such as CCL2, CCL5, and CXCL2, which are related
3 to immune cell recruitment and elevated in influenza virus infection (45). Although viral
4 infections may not induce the synthesis of lipid antigens, iNKT cells undergo activation, show
5 protective effects in the lung and liver, and expand virus-specific memory CD8 T cells (34).
6 Notably, we found that virus clearance was impaired in FoxN1-Cre IL-15 cKO mice after
7 infection with a 0.5 LD₅₀ dose of IAV, with a concomitant decrease of IAV-specific CD8 T and
8 NK cells and increase of MDSCs, whereas the adoptive transfer of C2 iNKT cells rescued the
9 attenuated immune response. These results suggest that C2 iNKT cells enhance anti-viral
10 immunity against lung IAV infection. It is possible that the expansion and activation of IAV-
11 specific CD8 T cells and NK cells may be due to both direct activation by C2 iNKT cells and the
12 indirect effect of MDSC suppression by C2 iNKT cells. Because iNKT cells reduce IAV-induced
13 MDSCs (34) and apolipoprotein E (ApoE) restricts MDSC maintenance (46), C2 iNKT cells
14 probably function as an effective MDSCs-suppressing iNKT subset in IAV infection by their
15 high expression of ApoE (Fig. 4F). Furthermore, it is reported that microbiota regulate immune
16 defenses against IVA infection (47), and our results suggest that C2 iNKT cells may be involved
17 in this process. Nevertheless, similar to the results of IL-15 knockout mice (48), FoxN1-Cre IL-
18 15 cKO mice showed milder body weight loss and decreased mortality risk after a lethal dose of
19 IAV infection, suggesting that circulating C2 iNKT cells may also be a key regulator for the
20 pathogenesis of IAV-induced acute lung injury, which leads to the progression of symptoms and
21 mortality.

22 Besides their direct effector functions, iNKT cells activate NK cells and CTLs by IFN- γ
23 and induce the maturation of DCs via cognate TCR-CD1d interactions (2). The lung metastasis

1 of B16-F10 melanoma cells was exacerbated in mice in the absence of C2 iNKT cells, and the
2 adoptive transfer of C2 iNKT cells into FoxN1-Cre IL-15 cKO mice rescued the impairment.
3 Thus, these results suggest that besides tissue-resident C1 iNKT cells, circulating C2 iNKT cells
4 also contribute to iNKT cell-mediated antitumor immunity. Consistent with the high expression
5 of cytotoxic molecules, such as IFN- γ , granzyme B, perforin, and TRAIL, C2 iNKT cells had
6 direct tumor inhibitory activity. Our data also suggest the possibility that C2 iNKT cells may
7 promote NK cell activation and maturation in the B16-F10 melanoma metastasis model. Taken
8 together, circulating C2 iNKT cells serve as a highly effective tumor-suppressing iNKT cell
9 subset in vivo. Because conventional tissue-resident iNKT cells may trigger a tissue-repair
10 program that in turn may promote tumor progression (5), the rapid and direct suppression of
11 cancer metastasis by C2 iNKT cells may be important for anti-tumor immunity. We propose that
12 C2 iNKT cells circulate in the peripheral tissues, contributing to cancer immunosurveillance.
13 Furthermore, because of their high mobility and effector functions, C2 iNKT cells may become a
14 useful tool to trigger local immune responses against cancers.

15 It is reported that human CD4⁺ and CD4⁻ iNKT cells in peripheral blood have distinct
16 features. Human CD4⁻ iNKT cells express genes associated with the activation of effector
17 functions and cytolysis, which resembles NK cells with Th1 cytokine expression (35, 49). In this
18 study, we identified human C2 iNKT cells as human CD4⁻ iNKT cells with high cell activation
19 and expression of cytotoxic molecules, such as granzymes, granulysin, and perforin, related to
20 iNKT cell-mediated effector functions in anti-tumor and anti-viral immunity. In addition, CCR5,
21 a chemokine receptor important for human T cell trafficking (50), was highly expressed in
22 human C2 iNKT cells. A functional enrichment analysis showed that genes enhanced in human
23 C2 iNKT cells may be involved in the SARS-CoV-2 signaling pathway, and it is also reported

1 that the expression of CD244 in human iNKT cells is correlated with the disease progression of
2 HIV patients (36). Thus, the precise roles of human C2 iNKT cells in anti-viral or anti-tumor
3 immunity need future investigations.

4 In this study, we identified a circulating iNKT cell subset that enhanced anti-tumor and
5 anti-viral immune responses. These cells were distinct from the conventional tissue-resident
6 iNKT cell subset. Our findings lead to the novel idea of a layered structure of iNKT cells with
7 distinct development, dynamics, and functions. Furthermore, they may help advance iNKT cell-
8 targeted immunotherapies against cancers and infections.

9

1 MATERIALS AND METHODS

2

3 **Study design**

4 The aim of this study was to identify and characterize the developmental and functional
5 heterogeneity of circulating and tissue-resident iNKT cells. We performed multimodal analysis
6 with a series of IL-15 reporter mouse and conditional knock-out mouse models, and human
7 samples. Parabiosis was used to classify circulating and tissue-resident iNKT cells. B16-F10
8 melanoma lung metastasis and influenza A virus (IAV) infection models were used to investigate
9 the function of circulating iNKT cells. The sample size of all the experiments was determined on
10 the basis of our previous studies and prior experiments that were sufficient for statistical analysis
11 and on the consideration of animal welfare. The number of independent experiments is indicated
12 in the figure legends. The mouse experiments were performed unblinded, as mice of different
13 genotypes were identified and kept track of.

14

15 **Mice and generation of IL-15-floxed mice**

16 One- to 16-week-old male or female IL-15-floxed (generated in this study), IL-15-CFP knock-in
17 (*15*), IFN- γ -Venus reporter (*51*), FoxN1-Cre (*52*), Tie2-Cre (*53*), CD4-Cre (*54*), CD11c-Cre (*55*),
18 LysM-Cre (*56*), CXCR6-GFP/GFP (KO) (*57*), CD1d1/2 knock-out (KO) (*58*), TCR δ KO (*59*),
19 SPC-Cre mice (*60*), and IL-15R α -floxed mice on a C57BL/6 background were used. For more
20 details, see the Supplementary Materials. All mice were maintained under specific pathogen-free
21 (SPF) conditions at the Experimental Research Center for Infectious Diseases, Institute for Life
22 and Medical Sciences, Kyoto University. All procedures were carried out under Sevoflurane

1 anesthesia to minimize animal suffering. All mouse protocols were approved by the Animal
2 Experimentation Committee of the Institute for Life and Medical Sciences, Kyoto University.

3

4 **Human samples**

5 The human peripheral blood samples were collected from 18- to 52-year-old male or female
6 donors who provided informed consent for the study, which was approved by the Institutional
7 Review Board (IRB) at Kyoto University (IRB number: G1212). The human peripheral blood
8 samples from 20- to 56-year old male or female donors were obtained from HemaCare Corp.
9 (Northridge, CA). The human liver perfusates samples were collected from donors who provided
10 informed consent for the study, which was approved by the IRB at Kyoto University (IRB
11 number: G1240).

12

13 **Cell isolation**

14 Lymphocytes from the indicated tissues were prepared as described previously (61, 62). After
15 perfusion with PBS, the lung was minced with scissors and incubated at 37°C for 1 hour in
16 RPMI 1640 medium containing 10% FBS, 1 mg/mL collagenase D and 50 µg/mL Dnase
17 (Roche). Digested tissue was suspended and passed through a 40-µm strainer (Greiner Bio-One).
18 Leukocytes were separated by centrifugation through 30% Percoll. The isolation of mTECs was
19 performed as described previously (15).

20

21 **Flow cytometry**

22 Cells were prepared from the indicated organs and surface-stained for 20 minutes at 4°C in PBS
23 containing 0.2% BSA and 0.05% NaN₃ with fluorescent dye- or biotin-conjugated antibodies.

1 Detailed antibody information is shown in Supplementary table S1. Biotinylated monoclonal
2 antibodies were detected with PE- or Brilliant Violet 421-conjugated streptavidin (Thermo
3 Fisher Scientific). PBS57-loaded mouse and human CD1d tetramer or PBS57-unloaded control,
4 and 5-OP-RU-loaded MR1 tetramer were kindly provided by the NIH Tetramer Core Facility.
5 For more details, see the Supplementary Materials.

6

7 **Cell culture**

8 iNKT cells were sorted using a FACSAria II cell sorter (BD Biosciences) into a tube with RPMI
9 1640 medium containing 10% FBS, 50 μ M 2-mercaptoethanol and 10 mM HEPES (pH7.4). For
10 iNKT cell differentiation or maturation, each iNKT cell subset was cultured with 2 μ g/ml plate-
11 bound anti-CD3 antibody (145-2C11) with or without 50 ng/mL IL-15 and with or without $1 \times$
12 10^4 B16-F10 melanoma cells at 37°C in 5% CO₂. After 3 days, all cells were stained with the
13 relevant antibodies for flow cytometry. For the expression of cytotoxic molecules in iNKT cells,
14 lung bulk lymphocytes were cultured with 2 μ g/mL plate-bound anti-CD3 antibody, 100 ng/mL
15 IL-15 and 1×10^4 B16-F10 melanoma cells for 12 hours.

16

17 **Immunohistochemistry**

18 IL-15-CFP knock-in mice were used (15), and PBS-57-loaded CD1d tetramer or PBS-57-
19 unloaded control (NIH Tetramer Core Facility) immunofluorescence was performed as described
20 previously (63). For more details, see the Supplementary Materials.

21

22 **Digital RNA-seq and data analysis**

1 Mouse iNKT cells were freshly isolated from the thymus and lungs of C57BL/6 mice. Human
2 iNKT cells were isolated from peripheral blood. Two hundred C1 or C2 iNKT cells were sorted
3 into a tube using a FACS Aria II. Digital RNA-sequencing (dRNA-seq) (64, 65) was performed
4 as described previously (66). Differential gene expression and principal component analysis
5 (PCA) were performed using rlog-normalized dRNA-seq data by DESeq2 (67), and 95%
6 confidence ellipses for each group are shown in the plot of PCA. Heat maps were drawn on the
7 gene counts, which were normalized by the total counts of all genes using R package ggplots2.
8 To identify genes differentially expressed, \log_2 fold changes were calculated between C1 and C2
9 iNKT cells. Genes were considered differentially expressed when they had \log_2 fold changes of
10 ≥ 1 or ≤ -1 , and p values ≤ 0.01 . Gene enrichment analysis was performed using Metascape (68).

11

12 **Parabiosis**

13 Female 8-week-old congenic CD45.1 and CD45.2 congenic C57BL/6J mice were surgically
14 connected in parabiosis (69). Lateral skin from the elbow to knee in each mouse was sutured, the
15 forelimbs and hindlimbs were tied together, and the skin incisions were closed using surgical
16 adhesive. After surgery, the mice were maintained on water containing antibiotics for about 2
17 weeks to prevent infection and analyzed 60 days later. Partner-derived cells in both parabionts
18 (CD45.1 and CD45.2) were analyzed as independent data points.

19

20 **Intrathymic injection**

21 Intrathymic injection was performed as previously reported (18). For more details, see the
22 Supplementary Materials.

23

1 **In vivo stimulation of iNKT cells**

2 Mice were administered with 1 $\mu\text{g}/\text{mouse}$ α -galactosylceramide (α -Gal) or with IL-15/IL-15R α
3 complex (RLI). For more details, see the Supplementary Materials.

4
5 **Integrin α 1 blockade**

6 Functional blocking hamster anti-mouse CD49a antibody (Ha31/8) (BD Biosciences) and
7 isotype-matched control antibody (Ha4/8) were used for integrin α 1 (CD49a) blocking
8 experiments as described previously (70). For each experiment, wild-type mice were
9 administered 150 $\mu\text{g}/\text{mouse}$ blocking antibody or isotype-matched control by intravenous (i.v.)
10 injection every two days and analyzed 6 days after the first antibody administration.

11
12 **Adoptive transfer of C2 iNKT cells**

13 Adoptive transfer experiments were performed as previously reported (71). C2 iNKT cells were
14 isolated from the thymus of CD45.1 WT mice and cultured for 1 day with 2 $\mu\text{g}/\text{mL}$ plate-bound
15 anti-CD3 antibody (145-2C11) and 50 ng/mL IL-15 for expansion. Approximately 1×10^5 C2
16 iNKT cells from CD45.1 mice were transferred into CD45.2 FoxN1-Cre IL-15 cKO recipients or
17 CD45.2 FoxN1-Cre IL-15 cKO \times IFN- γ -Venus reporter mice by i.v. injection. The mice were
18 intravenously inoculated with B16-F10 melanoma cells one day before the transfer or
19 intranasally infected with IAV (PR8) on the same day.

20
21 **B16-F10 melanoma lung metastasis**

22 The B16-F10 melanoma cell line was cultured in high glucose-containing Dulbecco's modified
23 Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$

1 *streptomycin* and 2 mmol/L L-glutamine. For the lung metastasis assay, a single cell suspension
2 of B16-F10 cell was prepared in PBS, and 4×10^4 cells were injected intravenously into recipient
3 mice. The lungs were harvested, tumor nodules were counted on day 14, and lymphocytes were
4 isolated and analyzed.

5

6 **Tumor inhibition assay**

7 The ex vivo tumor inhibition assay was performed according to a previously report (72) with
8 minor modifications. For more details, see the Supplementary Materials.

9

10 **Intravascular staining**

11 The intravascular staining of CD45 and FTY720 treatment was described previously (73, 74).
12 For more details, see the Supplementary Materials.

13

14 **Influenza A virus (IAV) infection**

15 The influenza A virus (IAV) used in this study was A/Puerto Rico/8/34 (PR8; H1N1). For IAV
16 infection, 6–8-week-old male or female mice were intranasally administered 50 μ L PBS
17 containing 0.5 or 2 LD₅₀ dose of IAV/Puerto Rico/8/34 (IAV/PR8, H1N1). For the flow
18 cytometry analysis, mice were sacrificed at day 7 post-infection, and immune cells were isolated
19 from the whole lung. To assess virus-specific CD8 T cells, BV421-conjugated H-2D^b IAV/PR8
20 NP₃₆₆₋₃₇₄ tetramer-ASNENMETM, kindly provided by NIH Tetramer Core Facility, was used.
21 For virus titrations, a 50% tissue culture infectious dose (TCID₅₀) assay was performed. The lung
22 from mice 4 days post infection was homogenized with 4 volumes of PBS and assayed for virus
23 infectivity on MDCK cells for the cytopathic effect with a 10-fold dilution series. Mice were

1 monitored daily for body weight loss and survival until death occurred or euthanasia was
2 performed during a period of 14 days. Mice that lost 20% of their body weight were considered
3 to have reached humane endpoints and were euthanized within a day according to the animal
4 experiment protocol.

5

6 **Real-time RT-PCR**

7 Total RNA was extracted from the sorted cells and reverse-transcribed using random primers.
8 cDNA was analyzed by real-time RT-PCR using SYBR Green PCR Master Mix (Qiagen). For
9 more details, see the Supplementary Materials.

10

11 **Bone marrow transplantation**

12 Tie2-Cre IL-15 cKO host mice were irradiated and injected with 5×10^5 bone marrow cells. For
13 more details, see the Supplementary Materials.

14

15 **Antibiotic treatment**

16 Mice 8 weeks of age were orally administered with an antibiotics (ABX) cocktail (1 g/L
17 ampicillin, 0.5 g/L vancomycin, 1 g/L metronidazole, and 1 g/L neomycin) (Sigma) in drinking
18 water. Mice were analyzed at 4 weeks of treatment.

19

20 **Statistical analysis**

21 All statistical analyses were performed using GraphPad Prism 7 or 8 software. All data are
22 presented as means \pm SD or \pm SEM. Comparisons between two samples were performed using
23 an unpaired two-tailed Student's *t*-test or unpaired *t*-test with Welch's correction. For multiple

1 group comparisons, one-way or two-way ANOVA analyses with a multiple-comparison test
2 were used. **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

3

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14
15 **Author contributions:**

16 G.C. and K.I. designed the research and wrote the manuscript. G.C., A.S., J.J., T.O., Y.M., H.M.,
17 S.A., T.A., S.T., Y.Z., and K.S. performed the experiments and analyses. H.M., J.M.D., Y.I.,
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21
22 **Competing interests:**

23 The authors declare no competing interests.

1

2 **Data and materials availability:**

3 All data needed to evaluate the conclusions in the paper are present in the paper or the
4 Supplementary Materials. The gene expression data of dRNA-seq have been deposited into the
5 Gene Expression Omnibus (GEO) public data set with accession number GSE 150813, GSE
6 206553 and GSE 206627.

7

1 **SUPPLEMENTARY MATERIALS**

2

3 Supplementary Materials and Methods

4

5 Figs. S1 to S10

6 fig. S1. Characterization of mouse iNKT cells.

7 fig. S2. Generation of IL-15-floxed mice.

8 fig. S3. Effects of TEC-derived IL-15 on lymphocytes.

9 fig. S4. IL-15 and IL-15R α trans-presentation in thymocyte development.

10 fig. S5. Distribution of thymocytes and IL-15-producing mTECs in thymic medulla.

11 fig. S6. Effects of microbiome on C2 iNKT cells.

12 fig. S7. iNKT cells and relevant lymphocytes in peripheral tissues of IL-15 cKO mice.

13 fig. S8. Circulation and retention of iNKT cells and NK cells.

14 fig. S9. Alteration of relevant lymphocytes in a B16-F10 melanoma cell metastasis model.

15 fig. S10. Alteration of relevant lymphocytes in an influenza virus infection model.

16 fig. S11. Characterization of human iNKT cells.

17

18 Table S1. List of antibodies used for flow cytometry

19 Data file S1

20

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21

22

1 **FIGURE captions**

2

3 **Fig. 1. Characterization of mouse C2 iNKT cells.**

4 **(A)** Gating strategy for C2 iNKT (CD244⁺CXCR6⁺), C1 iNKT (CD244⁻CXCR6⁺), and C0 iNKT
5 (CD244⁻CXCR6⁻) cells in mature iNKT cells in the thymus. **(B)** Flow cytometric analysis of the
6 IFN- γ /Venus expression in thymic iNKT cell populations of IFN- γ -Venus reporter mice at steady
7 state. **(C)** *IFN- γ* mRNA levels relative to *Hprt* in C2 and C1 iNKT cells were measured by qPCR
8 (n = 3). **(D)** Flow cytometric analysis of PLZF expression in the indicated thymic iNKT cell
9 subsets. **(E)** Flow cytometric analysis of NKT1, NKT2, and NKT17 populations in thymic C2
10 and C1 iNKT cells in mice. **(F)** Flow cytometric analysis of CD4 expression in thymic C2 and
11 C1 iNKT cells. **(G)** Principal component analysis (PCA) performed on thymic C2, C1, and C0
12 iNKT cells. Expression data were measured by dRNA-seq. **(H)** Heat map of selected genes that
13 were differentially expressed between thymic C2 and C1 iNKT cells ($p \leq 0.01$) from three
14 biological replicates. **(I)** Bar chart depicting the functional analysis of differentially expressed
15 genes between thymic C2 and C1 iNKT cells. Normalized enrichment scores were calculated
16 using Metascape. **(J)** The mRNA expression levels of transcription factor *Klf2* relative to *Hprt* in
17 C2 and C1 iNKT cells measured by qPCR (n = 3). **(K)** Flow cytometric analysis of ICOS
18 expression in C2 and C1 iNKT cells. **(L)** PCA performed on C2, C1, and C0 iNKT cells, and
19 CD4 SP, CD8 SP, and NK cells. Expression data were measured by dRNA-seq. **(M)** Frequency
20 of C2, C1, and C0 iNKT cells in the mature NK1.1⁺ iNKT population in the thymus of 1- to 48-
21 week-old (wks) specific pathogen-free (SPF) mice (n = 4 per each time point). **(N)** Frequency of
22 C2 iNKT cells in the mature NK1.1⁺ iNKT population of the indicated peripheral tissues (n = 5
23 per each tissue). BM, bone marrow; Adipo, adipose tissue; LP, lamina propria; PBL, peripheral

1 blood lymphocytes. Data represent at least three independent experiments with similar results (A,
2 B, D, E, F, and K). Data are means \pm SEM and pooled from three separate mice with duplicate
3 measurement per mouse (C and J); Student's *t*-test. Data are means \pm SD and pooled from 3-4
4 independent experiments (M and N). **, $p < 0.01$.

5

1 **Fig. 2. The thymic IL-15 niche is essential for the development and maturation of C2 iNKT**
2 **cells.**

3 (A) Number of iNKT cells at stages 1, 2, and 3 in the thymus of FoxN1-Cre IL-15 cKO mice
4 (FoxN1; IL-15 cKO) and littermate control mice (Control) (n = 5). (B) Number of NKT1, NKT2,
5 and NKT17 cells in the thymus of FoxN1-Cre IL-15 cKO mice and littermate control mice (n =
6 4). (C) Flow cytometric analysis for anti-apoptotic factor Bcl-2 and Bcl-xL expression in mature
7 NK1.1⁺ iNKT cells in FoxN1-Cre IL-15 cKO mice and littermate control mice. (D) Frequency of
8 Ki-67⁺ cells in mature iNKT cells of FoxN1-Cre IL-15 cKO mice and littermate control mice (n
9 = 4). (E and F) Flow cytometric analysis (E) and number (F) of C2, C1, and C0 iNKT cells in
10 the thymus of FoxN1-Cre IL-15 cKO mice or littermate control mice (n = 10). (G) Number of
11 NK cells (n = 4) and MAIT cells (n = 5) in the thymus of FoxN1-Cre IL-15 cKO mice and
12 littermate control mice. (H and I) Flow cytometric analysis (H) and frequency (I) of iNKT cells
13 4 days after the culture of freshly isolated thymic C2, C1, and C0 iNKT cells with (+) or without
14 (-) IL-15 (n = 4). (J) Wild-type mice were administered IL-15/IL-15R α complex (RLI) or
15 control PBS (Control) by intravenous injection every two days. Frequency of thymic C2, C1, and
16 C0 iNKT cells in whole iNKT cells 4 days after the first administration (n = 4). (K) CD45.1 C0
17 iNKT cells were injected into the thymus of CD45.2 wild-type mice, and the frequency of C2
18 and C1 iNKT cells in CD45.1 iNKT cells was assessed 4 days later (n = 4). (L) FoxN1-Cre IL-
19 15 cKO (cKO) mice or littermate control mice (Con) were administered α -galactosylceramide
20 (α -Gal) (+) or control PBS (-) by intraperitoneal injection every two days. The frequency of
21 thymic C2, C1, and C0 iNKT cells in thymocytes 4 days after the first administration (n = 4).
22 Data are mean \pm SD and pooled from 3-5 independent experiments; Student's *t*-test or *t*-test with
23 Welch's correction (A, B, D, F, G and J) and one-way or two-way ANOVA (I and L). Data

1 represent at least three independent experiments with similar results (C, E, H and K). **, $p <$

2 0.01; *, $p < 0.05$; n.s., not significant.

3

1 **Fig. 3. Developmental and functional regulation of C2 iNKT cells in the thymus.**
2 **(A)** Number of thymic C2, C1, and C0 iNKT cells in FoxN1-Cre IL-15R α cKO mice (FoxN1;
3 IL-15R α cKO) and littermate control mice (Control) (n = 5). **(B)** Lethally irradiated hosts (Tie2-
4 Cre IL-15 cKO mice or littermate control mice, CD45.2) were reconstituted with bone marrow
5 cells in wild-type mice (CD45.1). Number of donor-derived thymic C2, C1, and C0 iNKT cells
6 in hosts after bone marrow transplantation (BMT) (n = 5). **(C and D)** Number of thymic C2 and
7 C1 iNKT cells in LysM-Cre IL-15 cKO mice (LysM; IL-15 cKO) (n = 4) (C) and CD11c-Cre
8 IL-15 cKO mice (CD11c; IL-15 cKO) (n = 5) (D) with littermate control mice (Control). **(E)**
9 Immunohistochemistry of iNKT cells in the thymus of IL-15-CFP knock-in (KI) mice. White
10 arrows indicate iNKT cells (PBS57-loaded CD1d-tetramer⁺TCR β ⁺). Boxed areas in the upper
11 panels are enlarged in the lower panels (n = 3). M, thymic medulla. Scale bars, 50 μ m. **(F)**
12 Frequency of CD4 SP, CD8 SP, and iNKT cells in contact with IL-15-expressing cells in the
13 thymic medulla detected by immunohistochemistry as shown in (E) and fig. S5B (n = 3). **(G and**
14 **H)** Flow cytometric analysis of IFN- γ /Venus expression in the indicated thymocytes of IFN- γ -
15 Venus reporter mice. **(I)** *Aire* and *Ins2* mRNA levels relative to *Hprt* in freshly isolated
16 medullary thymic epithelia cells (mTECs) in FoxN1-Cre IL-15 cKO mice (FoxN1; IL-15 cKO)
17 or littermate control mice (Control) measured by qPCR (n = 3). **(J)** Immunohistochemistry for
18 *Aire*⁺ mTECs in FoxN1-Cre IL-15 cKO mice and littermate control mice (n = 3). Scale bars, 50
19 μ m. **(K)** *Aire* mRNA levels relative to *Hprt* in freshly isolated medullary thymic epithelia cells
20 (mTECs) from FoxN1-Cre IL-15 cKO \times CD1d1/2 KO mice (FoxN1; IL-15 cKO; CD1d1/2 KO)
21 or littermate control CD1d1/2 KO mice were measured by qPCR (n = 3). Data are means \pm SD
22 and pooled from 4-5 independent experiments (A, B, C, and D); Student's *t*-test or *t*-test with
23 Welch's correction. Data represent at least three independent experiments with similar results (G

1 and H). Data represent three separate mice with similar results (E and J). Data are means \pm SEM
2 and pooled from three separate mice with duplicate measurements per mouse; one-way ANOVA
3 (F) and Student's *t*-test (I and K). **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

4

1 **Fig. 4. Characterization of peripheral C2 and C1 iNKT cells.**

2 **(A and B)** Flow cytometric analysis (A) and number (B) of C2 and C1 iNKT cells in the lung of
3 FoxN1-Cre IL-15 cKO mice (FoxN1; IL-15 cKO) or littermate control mice (Control) (n = 8). **(C**
4 **and D)** Number of C2 and C1 iNKT cells in the spleen (C) (n = 6) and peripheral blood
5 lymphocytes (PBL) (D) in FoxN1-Cre IL-15 cKO mice and littermate control mice (n = 4). **(E)**
6 PCA between lung C2 and C1 iNKT cells. Expression data were measured by dRNA-seq. **(F)**
7 Heat map of selected genes that were differentially expressed between lung C2 and C1 iNKT
8 cells ($p \leq 0.01$) from three biological replicates. Gene names shown in blue displayed similar
9 expression patterns with those in the thymus. **(G)** Intravascular staining with anti-CD45 antibody
10 administered intravenously 3 min before the isolation of the indicated lymphocyte populations.
11 Frequency of CD45⁺ intravascular cells in each population is shown. **(H)** Frequency of CD45⁺
12 intravascular cells among the indicated lymphocytes by intravascular staining of CD45, as shown
13 in (G) (n = 4). **(I and J)** Number of lung C2 and C1 iNKT cells and NK cells in LysM-Cre IL-15
14 cKO mice (LysM; IL-15 cKO) (I) and SPC-Cre IL-15 cKO mice (SPC; IL-15 cKO) (J) with
15 littermate control mice (n = 5). Data represent at least three independent experiments with
16 similar results (A and G). Data are means \pm SD and pooled from 3-5 independent experiments;
17 Student's *t*-test or *t*-test with Welch's correction (B, C, D, I, and J) and one-way ANOVA (H). **,
18 $p < 0.01$; *, $p < 0.05$; n.s., not significant.

19

1 **Fig. 5. Distinct cell dynamics of C2 and C1 iNKT cells.**

2 **(A to D)** Genetically marked CD45.1 and CD45.2 mice of the same age underwent parabiosis
3 surgery and were analyzed after 60 days. Flow cytometric analysis for partner-derived and host-
4 derived cells in peripheral C2 and C1 iNKT cells of the lung (A) and spleen (C). Frequency of
5 partner-derived cells in C2 and C1 iNKT cells in the lung (B) (n = 9) and spleen (D) (n = 8). **(E**
6 **and F)** Flow cytometric analysis (E) and mean fluorescence intensities (MFI) (F) of P2X7R
7 expression between lung C2 and C1 iNKT cells (n = 3). **(G and H)** Flow cytometric analysis for
8 partner-derived and host-derived cells in C2 and C1 iNKT cells of the bone marrow (BM) of
9 parabionts (G). Frequency of partner-derived cells in BM C2 and C1 iNKT cells (H) (n = 5). **(I)**
10 Flow cytometric analysis of partner-derived and host-derived cells in the indicated thymic iNKT
11 cells 60 days after parabiosis surgery using same-aged CD45.1 and CD45.2 mice. **(J and K)**
12 Flow cytometric analysis (J) and MFI (K) of P2X7R expression between thymic C2 and C1
13 iNKT cells (n = 3). **(L)** Number of lung C2 and C1 iNKT cells in CXCR6-GFP/GFP mice
14 (CXCR6 KO) and wild-type (WT) mice (n = 4). **(M)** WT mice were administered anti-integrin
15 $\alpha 1$ (α -CD49a) blocking antibody or isotype control (Iso) by intravenous injection every two
16 days. Number of lung C2 and C1 iNKT cells 6 days after the first antibody administration (n = 4).
17 Data represent at least two independent experiments with similar results (A, C, E, G, I, and J).
18 Data are means \pm SD (B, D, H, L, and M) or \pm SEM (F and K); Student's *t*-test and pooled from
19 2-4 independent experiments. **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

20

1 **Fig. 6. C2 iNKT cells enhance anti-tumor immunity.**
2 **(A and B)** FoxN1-Cre IL-15 cKO mice (FoxN1; IL-15 cKO) or littermate control mice (Control)
3 were administered 4×10^4 B16-F10 melanoma cells by intravenous injection. The lung
4 metastasis (A) and number of metastatic nodules (B) in the lung after 14 days of inoculation with
5 melanoma cells (n = 9). **(C)** FoxN1-Cre IL-15 cKO (cKO) mice or littermate control mice (Con)
6 were administered 4×10^4 B16-F10 melanoma cells (+) or control PBS (-) 14 days before the
7 analysis. Number of C2 and C1 iNKT cells and NK cells in the lung are shown. **(D)** Frequency
8 of KLRG1⁺ NK cells in the total NK population in the lungs of FoxN1-Cre IL-15 cKO mice and
9 littermate control mice after 14 days of inoculation with B16-F10 melanoma cells (n = 5). **(E)**
10 Frequency of Ki-67⁺ cells in NK cells in FoxN1-Cre IL-15 cKO mice and littermate control mice
11 after 14 days of inoculation with B16-F10 melanoma cells (n = 5). **(F)** The number of metastatic
12 nodules in the lung of FoxN1-Cre IL-15 cKO \times CD1d1/2 KO mice (FoxN1; IL-15 cKO;
13 CD1d1/2 KO) or littermate control CD1d1/2 KO mice after 14 days of inoculation with B16-F10
14 melanoma cells (n = 5). **(G)** Flow cytometric analysis of IFN- γ expression in lung CD8 T cells,
15 NK cells, and C2 and C1 iNKT cells in IFN- γ -Venus reporter mice after 14 days of inoculation
16 with B16-F10 melanoma cells. **(H)** Flow cytometric analysis of granzyme B expression in lung
17 C2 and C1 iNKT cells after 14 days of inoculation with B16-F10 melanoma cells. **(I and J)** Flow
18 cytometric analysis for granzyme B (I) and perforin (J) expression in the indicated lung
19 lymphocytes after 12 hours culture with B16-F10 melanoma cells in the presence of IL-15. **(K)**
20 Target tumor cell viability in the tumor inhibition assay. Effector cells (C2 and C1 iNKT cells or
21 NK cells) were isolated from B16-F10-inoculated and α -galactosylceramide-stimulated wild-
22 type mice. The effector cells and B16-F10 tumor target cells were co-cultured at the indicated
23 E:T ratios (n = 6). **(L and M)** CD45.1 C2 iNKT cells were adoptively transferred into CD45.2

1 FoxN1-Cre IL-15 cKO mice. Number of metastatic nodules (L) and C1 iNKT cells and NK cells
2 (M) in recipients after 14 days of inoculation with B16-F10 melanoma cells (n = 5). (N)
3 Frequency of KLRG1⁺ NK cells in total NK cells in the lungs of FoxN1-Cre IL-15 cKO mice
4 with or without the adoptive transfer of C2 iNKT cells after 14 days of inoculation with B16-F10
5 melanoma cells (n = 5). (O) Flow cytometric analysis of the IFN- γ expression in the indicated
6 lung lymphocytes in CD45.2 FoxN1-Cre IL-15 cKO \times IFN- γ -Venus reporter mice with or
7 without the adoptive transfer of C2 iNKT cells after 14 days of inoculation with B16-F10
8 melanoma cells. Data represent at least three independent experiments with similar results (A, G,
9 H, I, J, and O). Data are means \pm SD and pooled from 2-5 independent experiments; Student's *t*-
10 test (B, D, E, F, L, M, and N) or one-way ANOVA (C and K). **, $p < 0.01$; *, $p < 0.05$; n.s., not
11 significant.

12

1 **Fig. 7. C2 iNKT cells enhance anti-viral immune responses.**

2 (A) FoxN1-Cre IL-15 cKO × IFN- γ -Venus reporter mice were intranasally administered with a
3 0.5 LD₅₀ dose of IAV PR8. IFN- γ expression in lung CD8 T cells, NK cells, and C2 and C1
4 iNKT cells 7 days later. (B) Virus titers in the lung of FoxN1-Cre IL-15 cKO mice (FoxN1; IL-
5 15 cKO) or littermate control mice (Control) after 4 days of infection with the 0.5 LD₅₀ dose of
6 IAV assessed by the TCID₅₀ assay (n = 5). (C) Number of C2 and C1 iNKT cells in the lung of
7 FoxN1-Cre IL-15 cKO mice and control mice after 7 days of infection with the 0.5 LD₅₀ dose of
8 PR8 (n = 6). (D and E) Flow cytometric analysis (D) and frequency or number (E) of influenza
9 NP₃₆₆₋₃₇₄-specific CD8 T cells in the lung of FoxN1-Cre IL-15 cKO or control mice on day 7
10 post infection (n = 6). (F) Number of NK cells, CD8 and CD4 T cells in the lung of FoxN1-Cre
11 IL-15 cKO or control mice on day 7 post infection (n = 6). (G) Virus titers in the lung of FoxN1-
12 Cre IL-15 cKO × CD1d1/2 KO mice (FoxN1; IL-15 cKO; CD1d1/2 KO) or control CD1d1/2
13 KO mice after 4 days of IAV infection assessed by the TCID₅₀ assay (n = 4). (H) Frequency and
14 number of myeloid-derived suppressor cells (MDSCs) in the lung myeloid cells of FoxN1-Cre
15 IL-15 cKO or control mice on day 7 post infection (n = 6). (I to M) CD45.1 C2 iNKT cells were
16 adoptively transferred into CD45.2 FoxN1-Cre IL-15 cKO mice by intravenous injection. Virus
17 titers in the lung of CD45.2 FoxN1-Cre IL-15 cKO recipients after 4 days of infection with the
18 0.5 LD₅₀ dose of IAV assessed by TCID₅₀ assay (I) (n = 6). Number of C1 iNKT cells (J),
19 influenza NP₃₆₆₋₃₇₄-specific CD8 T cells (K), CD8 T cells (K), NK cells (L), and MDSCs (M) in
20 the lung after 7 days of infection (n = 5-6). (N) Flow cytometric analysis of IFN- γ expression in
21 the indicated lung lymphocytes of FoxN1-Cre IL-15 cKO × IFN- γ -Venus reporter mice with or
22 without the adoptive transfer of C2 iNKT cells after 7 days of infection with the 0.5 LD₅₀ dose of
23 IAV. Data represent at least three independent experiments with similar results (A, D, and N).

1 Data are means \pm SD and pooled from 2-4 independent experiments; with Student's *t*-test (B, C,
2 and E-M); Student's *t*-test. **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

3

1 **Fig. 8. Characterization of human C2 iNKT cells.**

2 (A) Flow cytometric analysis of human C2 (CD244⁺CXCR6⁺) and C1 (CD244⁻CXCR6⁺) iNKT
3 cells in the peripheral blood of young and aged donors. (B) Flow cytometric analysis of CD4
4 expression in human C2 and C1 iNKT cells. (C) Frequency of human C2 iNKT cells in the
5 peripheral blood of young donors (n = 5) and aged donors (n = 2), as shown in (A). (D)
6 Frequency of IFN- γ ⁺ cells in human C2 and C1 iNKT cells after stimulation with 25 ng/mL
7 PMA and 1 μ g/mL ionomycin for 4 hours (n = 4). (E) Principal component analysis (PCA)
8 performed on human C2 and C1 iNKT cells. Expression data were measured by dRNA-seq. (F)
9 Heat map of selected genes that were differentially expressed between human C2 and C1 iNKT
10 cells ($p \leq 0.01$) from four biological replicates. (G) Bar chart depicting the functional analysis of
11 differentially expressed genes between human C2 and C1 iNKT cells. Normalized enrichment
12 scores were calculated using Metascape. (H) Flow cytometric analysis of CD161, CD94, CCR5,
13 and ICOS expression in human C2 and C1 iNKT cells. Data represent at least two independent
14 experiments with similar results (A, B, and H). Data are means \pm SD and pooled from 2-3
15 independent experiments (C and D); with Student's *t*-test. *, $p < 0.05$.

16

Fig. 1

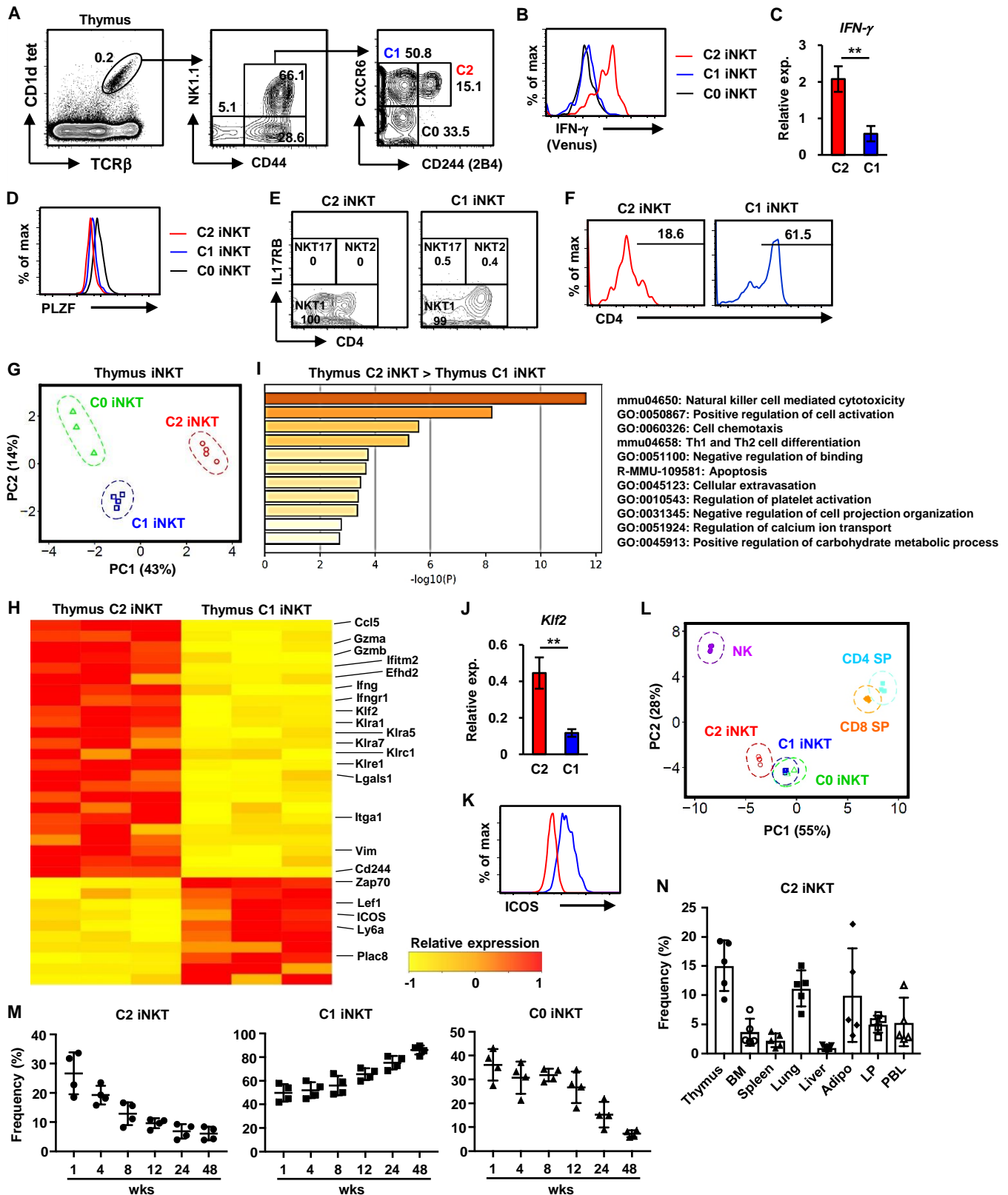


Fig. 2

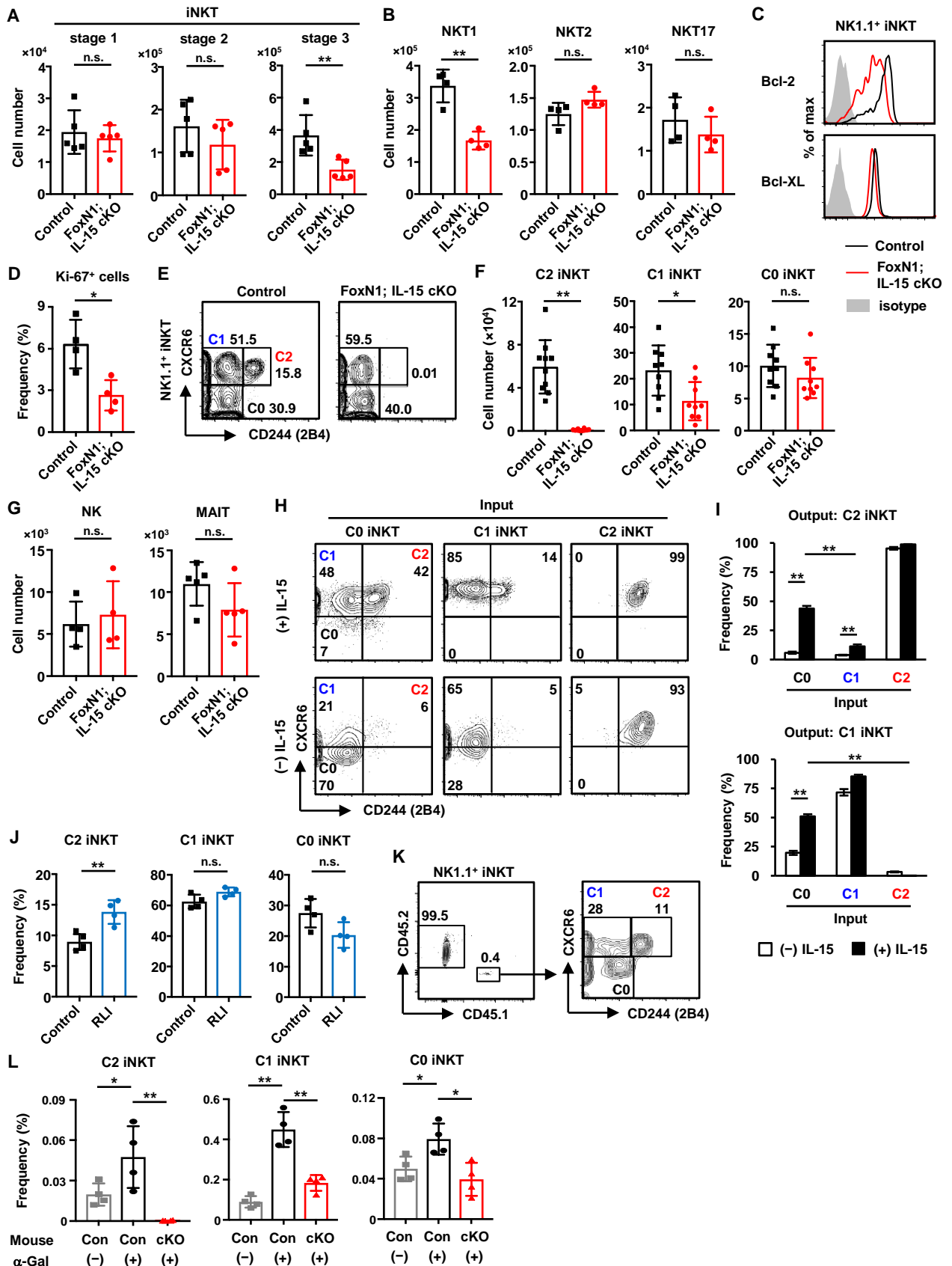


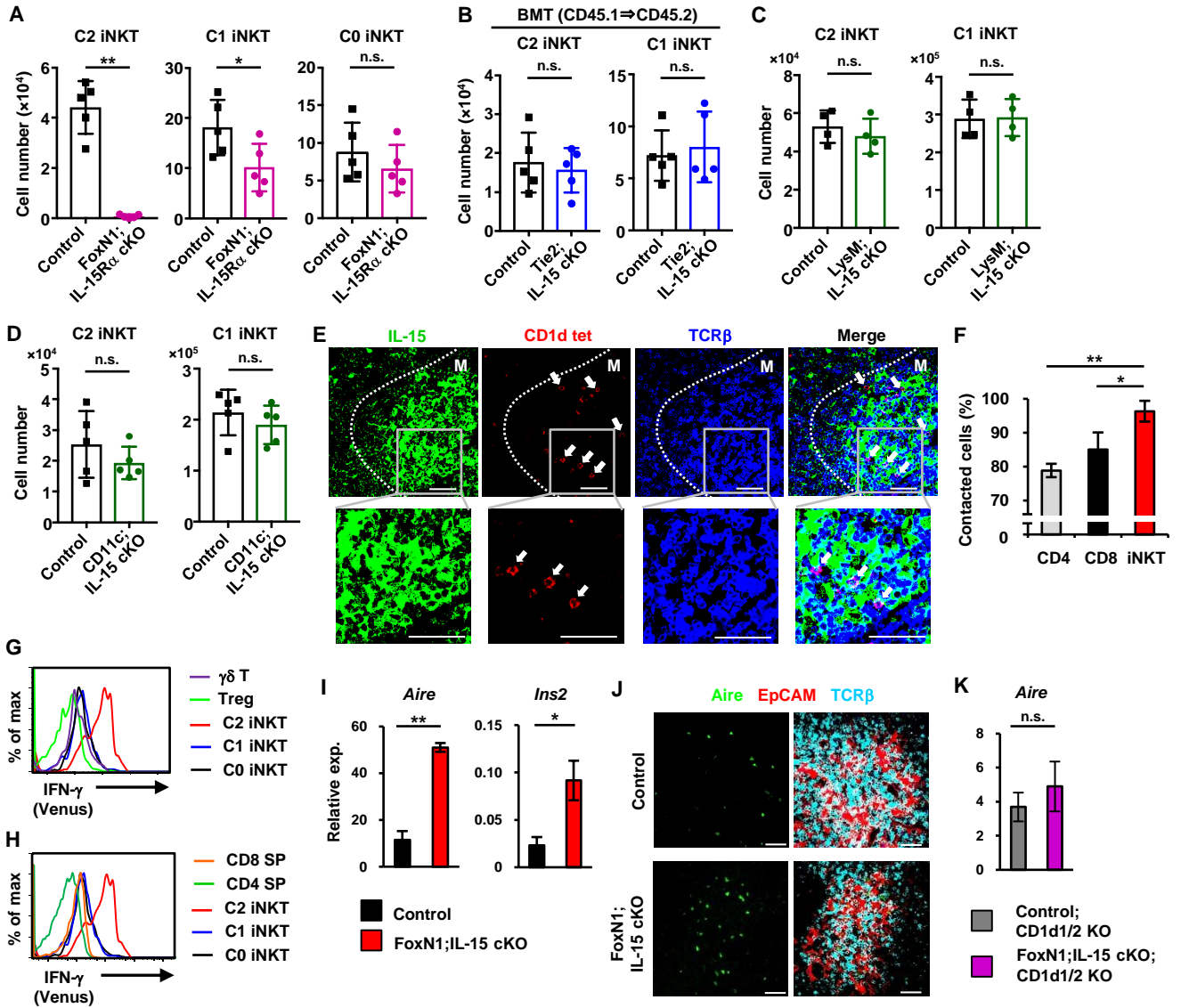
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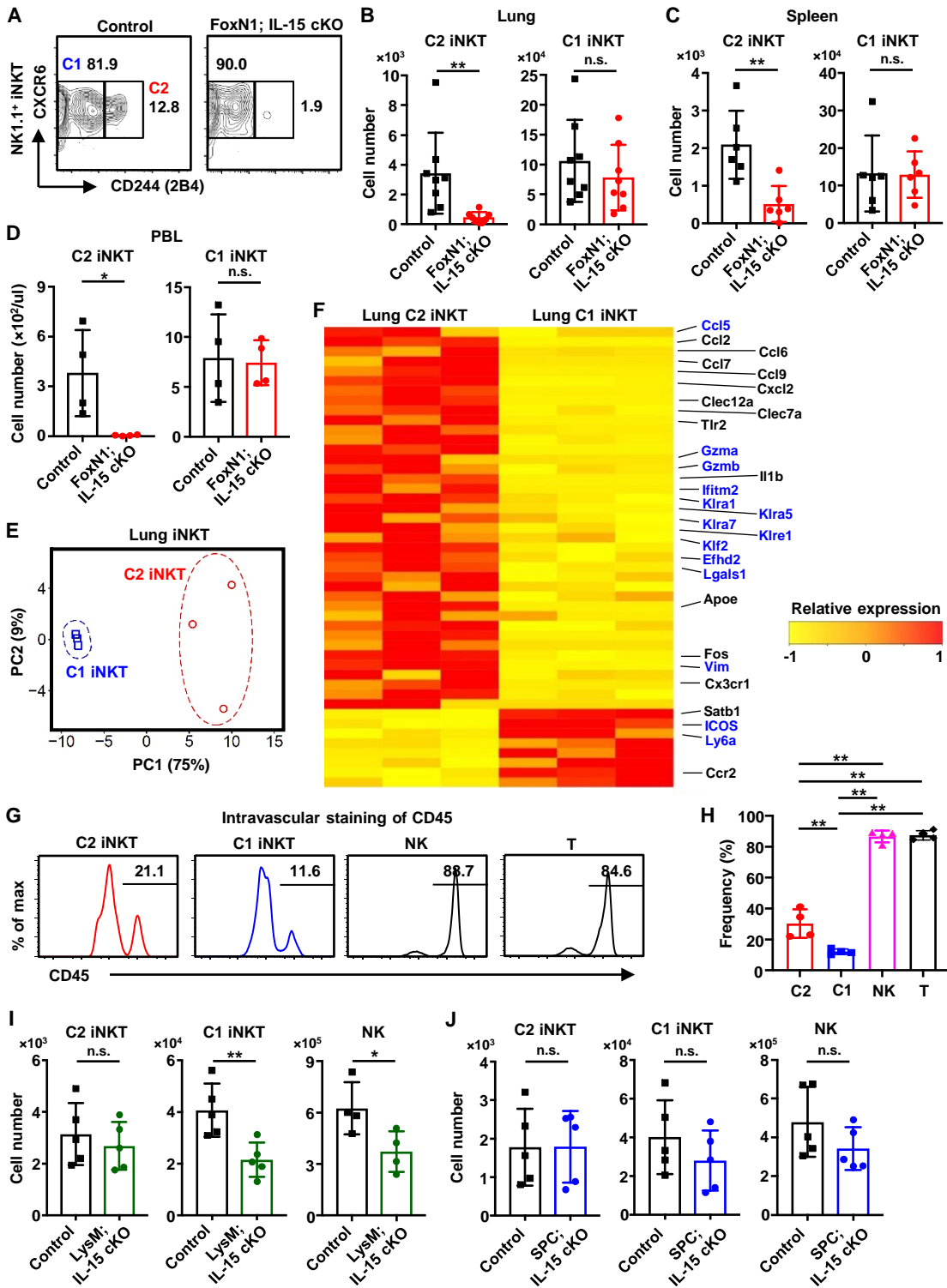
Fig. 4

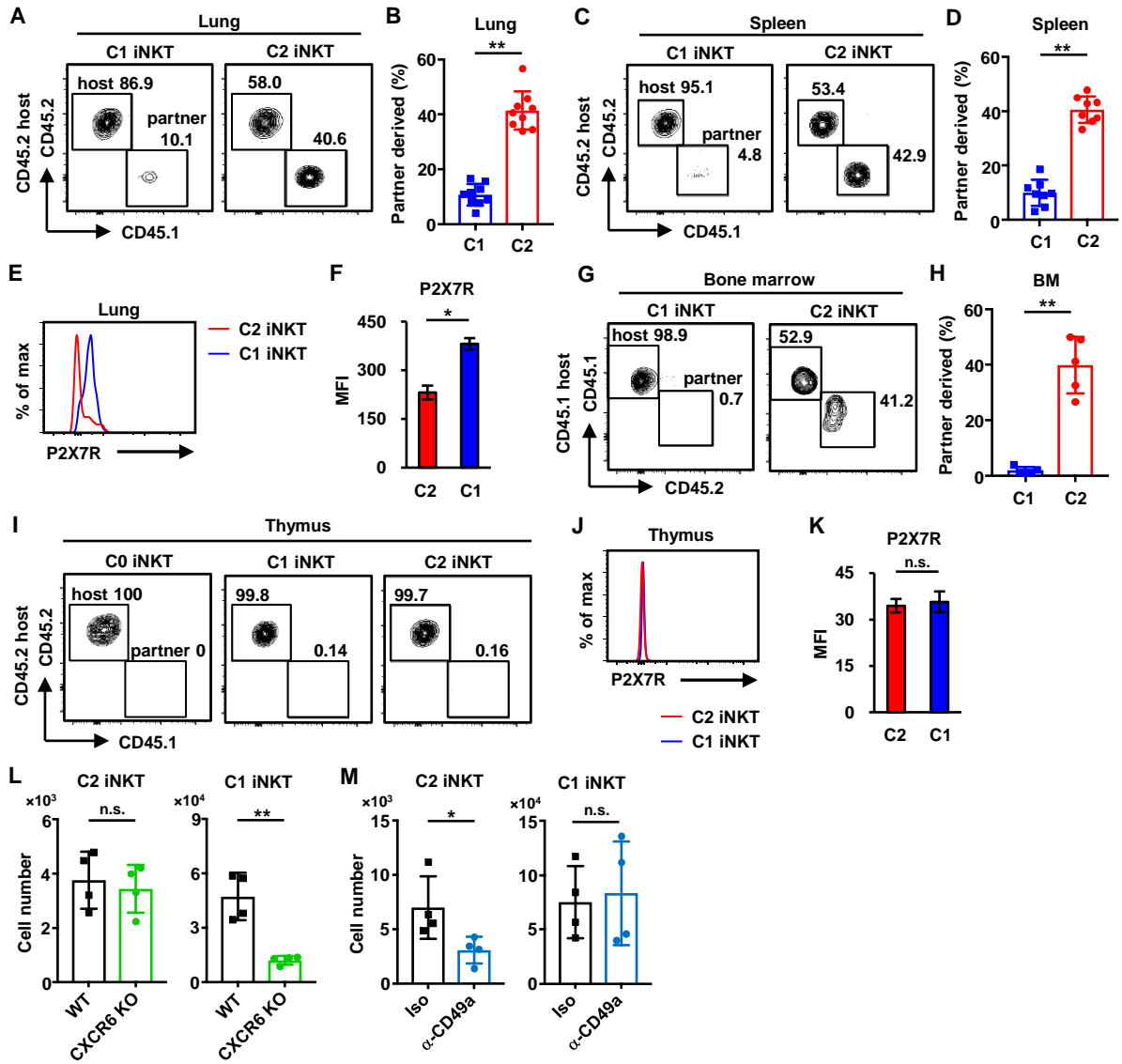
Fig. 5

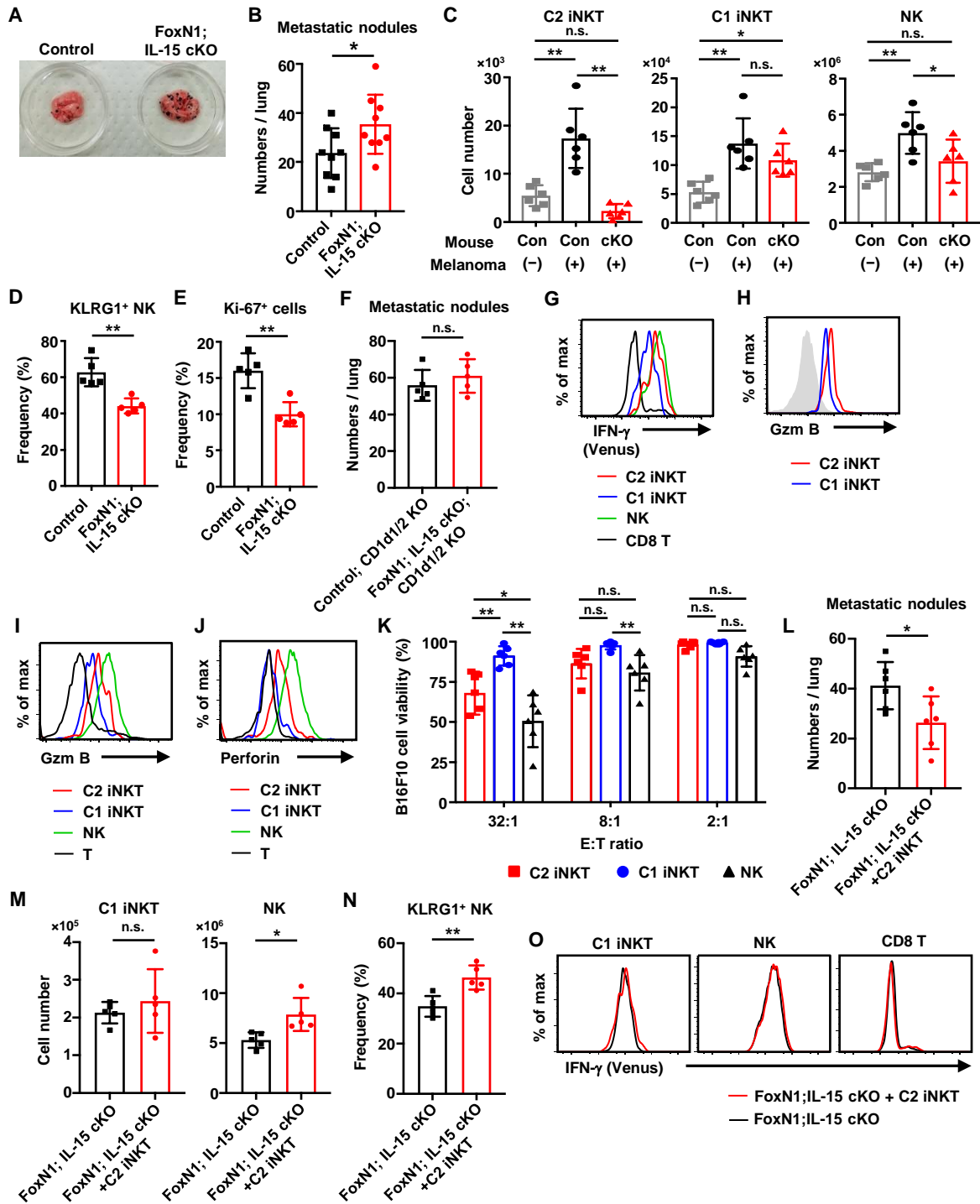
Fig. 6

Fig. 7

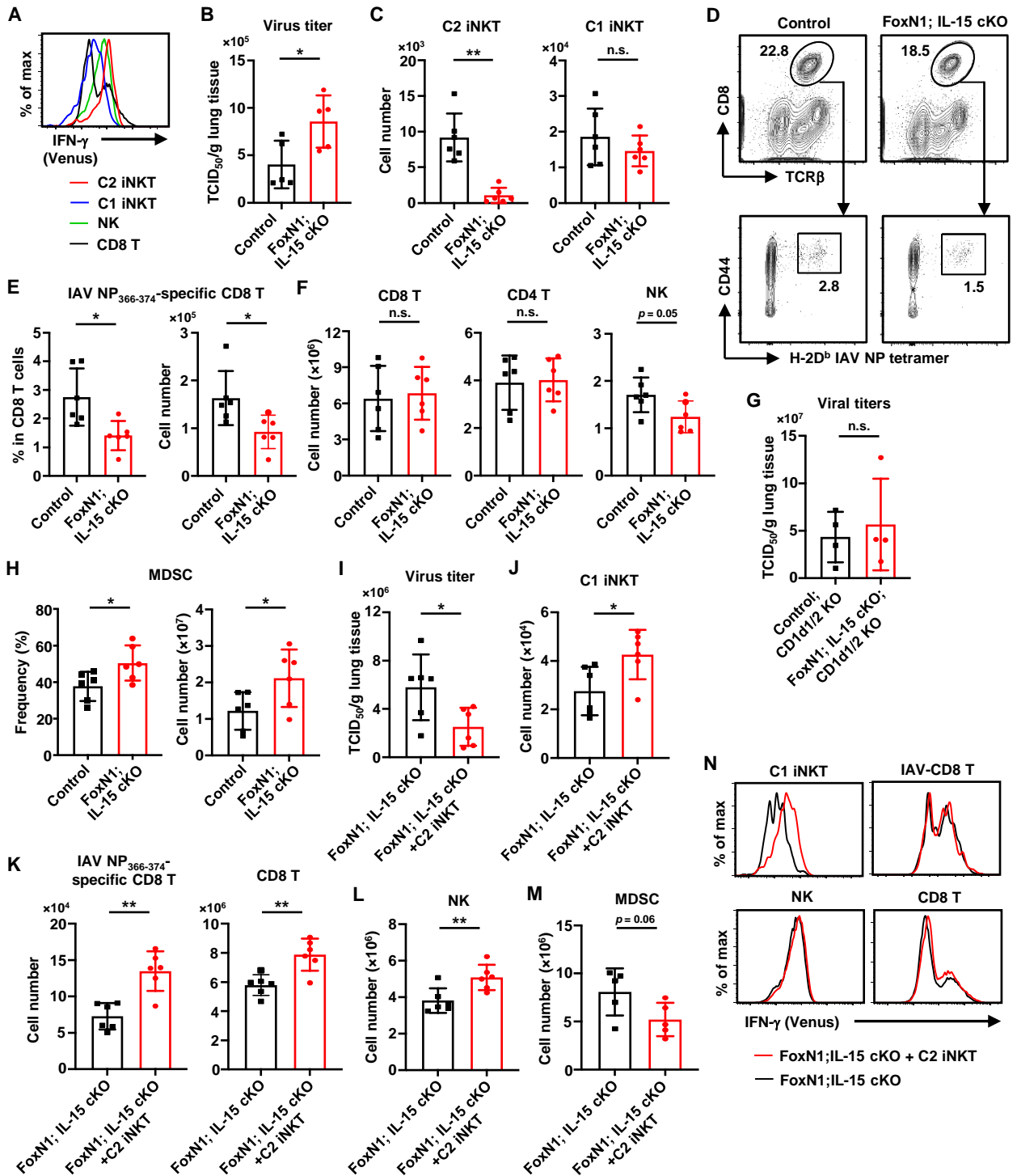
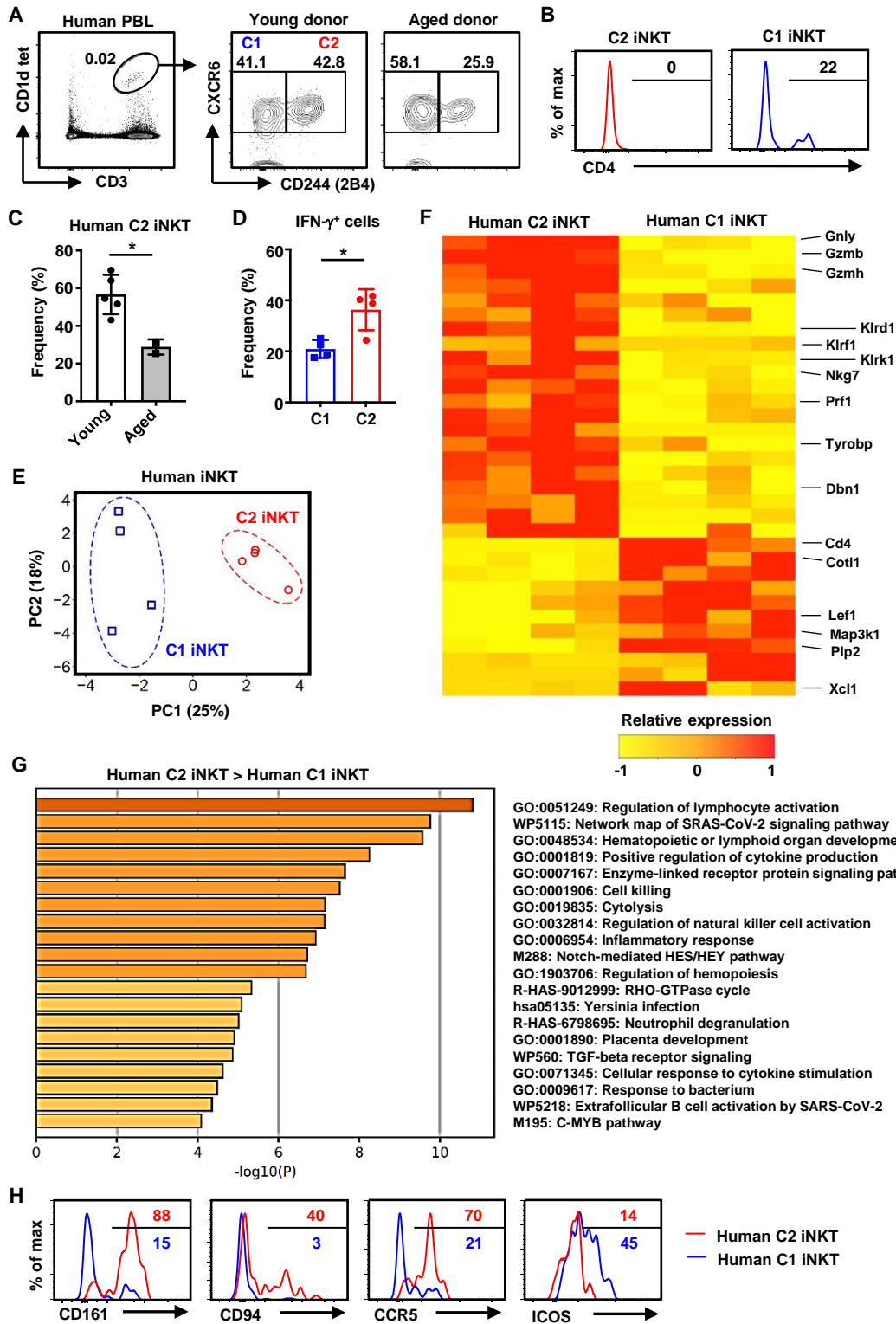


Fig. 8



1 **SUPPLEMENTARY MATERIALS**

2

3 **Supplementary Materials and Methods**

4

5 **Mice and generation of IL-15-floxed mice**

6 IL-15-floxed mice were generated by targeting the insertion of the loxP sites into the endogenous
7 IL-15 gene, as shown in fig. S2. To construct the targeting vector, we subcloned genomic DNA
8 fragments encoding exons 3 to 6 of endogenous IL-15 gene from the C57BL/6 genome
9 containing the 2.5-kb upstream and 6.0-kb downstream fragments of exon 5 by PCR. Two loxP
10 sites were inserted 507 bp upstream and 243 bp downstream from the end of exon 5, respectively.
11 The targeting construct was transfected by electroporation into the KY1.1 embryonic stem cell
12 (ESC) line derived from C57BL/6J x 129S6/SvEvTac mouse embryos (a kind gift from Dr. J.
13 Takeda, Osaka University), and targeted clones were screened by DTA/G418-resistance and
14 PCR. Established ESC clones were injected into ICR eight-cell embryos to produce chimeric
15 mice. Chimeric mice were bred with Flpe transgenic mice to remove the FRT-flanked neomycin
16 resistance gene cassette (a kind gift of Dr. M. Mishina, Ritsumeikan University). IL-15-
17 floxed mice were confirmed by PCR and DNA sequencing, backcrossed into C57BL/6 mice nine
18 times, and then bred to Cre-driver mice to obtain conditional knockout strains.

19 SPC-Cre mice were obtained from Dr. Y. Oike and the Center for Animal Resources and
20 Development (CARD), Kumamoto University. BALB/c mice were purchased from Japan SLC,
21 and germ-free mice on a C57BL/6 background were purchased from CLEA Japan. IL-15R α -
22 floxed mice were purchased from the Jackson Laboratories.

23

24 **Flow cytometry**

1 Detailed antibody information is shown in Supplementary table S1. For intracellular staining, T
2 cells were stained for surface antigens, fixed, permeabilized, and stained using the Foxp3
3 Staining Buffer Set or IC Fixation Buffer (Thermo Fisher Scientific). Cells were analyzed on a
4 FACSVerse or a LSRFortessa X-20 flow cytometer (BD Biosciences) using FlowJo software. In
5 the figures, values in gated areas indicate the percentages in each population.

6

7 **Immunohistochemistry**

8 For CD244 staining, thymic lobes were incubated overnight with biotin-labeled anti-CD244
9 antibody or isotype control. Then, the thymic lobes were washed and fixed with 4%
10 paraformaldehyde for five hours and snap frozen. Tissue sections of 10 μ m thickness were
11 stained with anti-GFP, Alexa Flour 546-anti-rabbit IgG and FITC-anti-Aire (5H12) antibodies
12 from Invitrogen, and DyLight488 anti-rabbit IgG (Poly4064), streptavidin-APC, FITC- or PE-
13 anti-EpCAM (G8.8), and Brilliant Violet 421-anti-TCR β (H57-589) antibodies from BioLegend.
14 Confocal microscopy was performed with a TSC-SP8 microscope (Leica Microsystems).

15

16 **Intrathymic injection**

17 C0 iNKT cells were freshly isolated from 4-week-old CD45.1 mice, and 1.5×10^3 C0 iNKT cells
18 in 10 μ L PBS were injected into the thymus of CD45.2 recipient mice of the same age. Then, we
19 analyzed CD45.1⁺ thymocytes after enrichment by negative selection with MACS CD8
20 microbeads and LD columns (Miltenyi Biotec) 4 days after the injection.

21

22 **Intravascular staining**

1 Anti-CD45 antibody (3 $\mu\text{g}/\text{mouse}$, 30-F11, BioLegend) was administered intravenously 3 min
2 before the isolation of lymphocytes from the lung. The frequency of CD45⁺ intravascular cells
3 and CD45⁻ extravascular cells was calculated. For FTY720 treatment, mice were treated with 25
4 $\mu\text{g}/\text{mouse}$ FTY720 (Calbiochem) injected intraperitoneally every day and assessed by
5 intravascular staining with CD45 antibody on day 3.

6

7 **In vivo stimulation of iNKT cells**

8 Mice were administered with 1 $\mu\text{g}/\text{mouse}$ α -galactosylceramide (α -Gal) every two days by
9 intraperitoneal injection or with IL-15/IL-15R α complex (RLI) (provided by Dr. J. M. Dijkstra)
10 once a day by intravenous injection. Thymocytes were isolated and analyzed 4 days after the first
11 administration. In the B16-F10 melanoma lung metastasis experiment, mice were administered
12 intravenously 200 ng/mouse α -GalCer 2 days before the inoculation of B16-F10 melanoma cells
13 as described previously (33). Then, the mice were intraperitoneally injected with 1 $\mu\text{g}/\text{mouse}$ α -
14 GalCer every two days. After 14 days, the lungs were checked for tumor metastasis.

15

16 **Real-time RT-PCR**

17 The results were analyzed by real-time RT-PCR using a StepOnePlus real time PCR system
18 (Applied Biosystems) and normalized to the corresponding levels of *Hprt* mRNA in thymocyte
19 cDNA from wild-type mice. The primer sequences were as follows: *Hprt*, 5'-
20 GTTGGATACAGGCCAGACTTTGTTG-3' and 5'-GATTCAACTTGCGCTCATCTTAGGC-
21 3'; *Ifng*, 5'-CTGCAGAGCCAGATTATCTC-3' and 5'-CATTGAATGCTTGGCGCTGG-3';
22 *Klf2*, 5'-GACCCACAAGTGGCACAA-3' and 5'-CTTTCGGTAGTGGCGGGTAA-3'; *Aire*, 5'-
23 TGCATAGCATCCTGGACGGCTTCC-3' and 5'-CCTGGGCTGGAGACGCTCTTTGAG-3';

1 *Ins2*, 5'-GCGGCAAGACCTACACCAAG-3' and 5'-TCTACAATGCCACGCTTCT-3'; and
2 *Fezf2*, 5'-ACCCAGCTTCCTATCCCCAT-3' and 5'-GAGCATTGAACACCTTGCCG-3'.

3

4 **Bone marrow transplantation**

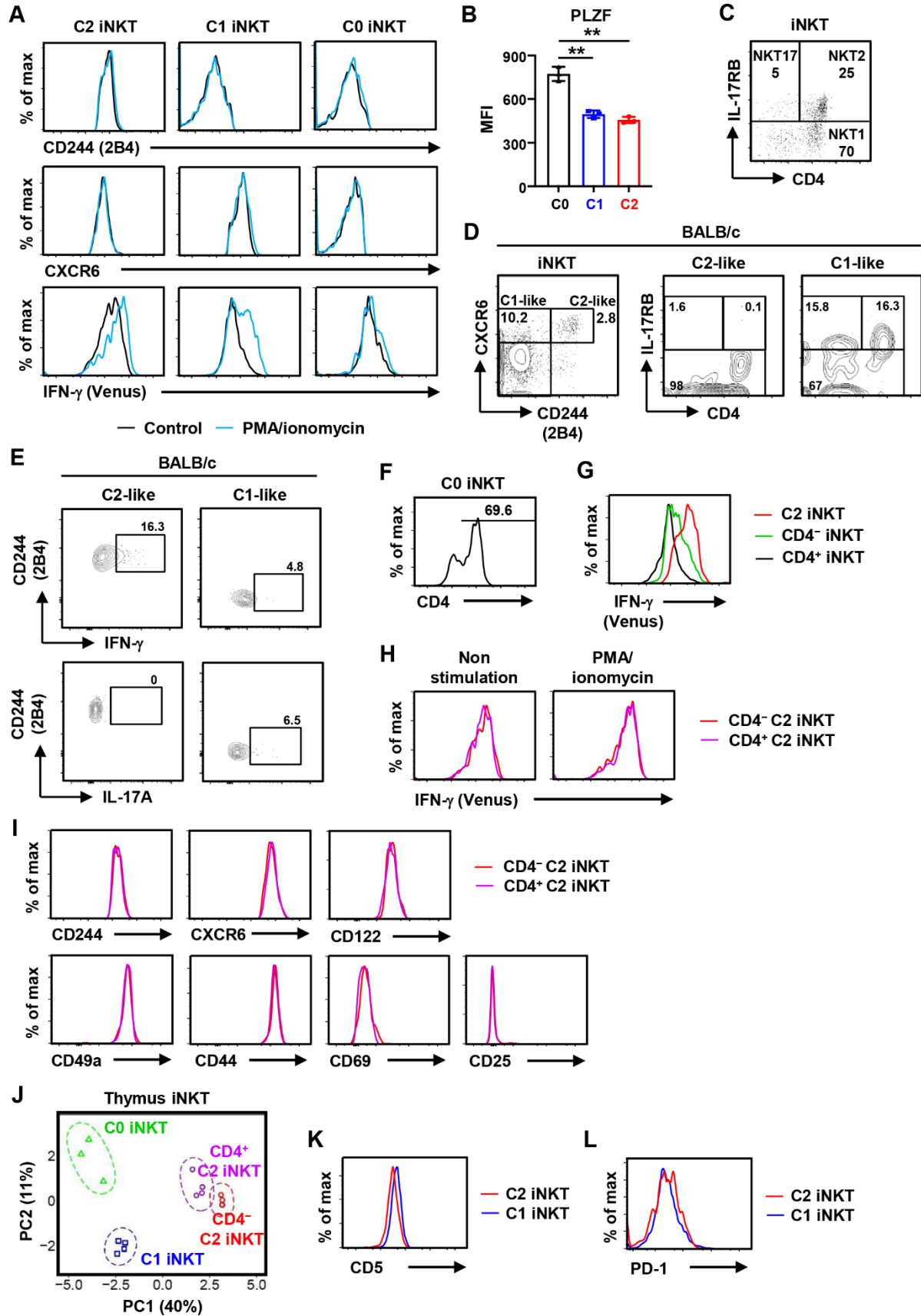
5 Tie2-Cre IL-15 cKO host mice were irradiated with two separate doses of 4.5 Gy with a three-
6 hour interval and injected intravenously with 5×10^5 bone marrow cells. After the
7 transplantation, the mice were maintained on water containing antibiotics for about 2 weeks to
8 prevent infection, and thymocytes were analyzed 5 weeks later.

9

10 **Tumor inhibition assay**

11 In vivo-pulsed effector cells (C2 iNKT, C1 iNKT, and NK cells) were isolated at day 4 from the
12 lungs of wild-type mice inoculated with 1×10^5 B16-F10 tumor cells by intravenous injection
13 and stimulated with 1 μ g/mouse α -galactosylceramide (α -Gal) at day 0 and day 2 by
14 intraperitoneal injection. For the target cell inhibition experiments, effector cells and 200 B16-
15 F10 tumor target cells/well were seeded into 96-well round-bottom plates at the indicated
16 effector to target ratios. Control wells were seeded with only 200 B16-F10 tumor target
17 cells/well. After 10 hours incubation with 100 ng/ml IL-15 at 37°C in 5% CO₂, each well was
18 gently washed with PBS, and the remaining target cells were stained with trypan blue. The
19 number of live target cells was counted, and the viability was calculated based on the number of
20 live cells and normalized to that in control wells.

21



1 **fig S1. Characterization of mouse C2 iNKT cells.**

2 (A) Flow cytometric analysis of CD244, CXCR6, and IFN- γ expression in the indicated thymic
3 iNKT cell subsets after stimulation with 25 ng/mL PMA and 1 μ g/mL ionomycin for 4 hours.

4 (B) The expression of PLZF in thymic iNKT cells presented as mean fluorescence intensity
5 (MFI) (n = 3). (C) Gating strategy for NKT1, NKT2, and NKT17 cells in the thymus of

6 C57BL/6 mice. (D) Flow cytometric analysis of C2- and C1-like iNKT cells in BABL/c mice.

7 (E) Flow cytometric analysis of IFN- γ and IL-17A expression in C2- and C1-like iNKT cells
8 from BALB/c mice after stimulation with PMA and ionomycin for 4 hours. (F) Flow cytometric

9 analysis of CD4 expression in thymic C0 iNKT cells. (G) Flow cytometric analysis of IFN- γ

10 expression in C2 iNKT cells, CD4⁺ and CD4⁻ iNKT cells. (H) Flow cytometric analysis of IFN- γ

11 expression in CD4⁺ and CD4⁻ C2 iNKT cells with or without PMA and ionomycin stimulation

12 for 4 hours. (I) Flow cytometric analysis of developmental and functional molecules in CD4⁺

13 and CD4⁻ C2 iNKT cells. (J) Principal component analysis (PCA) of CD4⁺ and CD4⁻ C2 iNKT

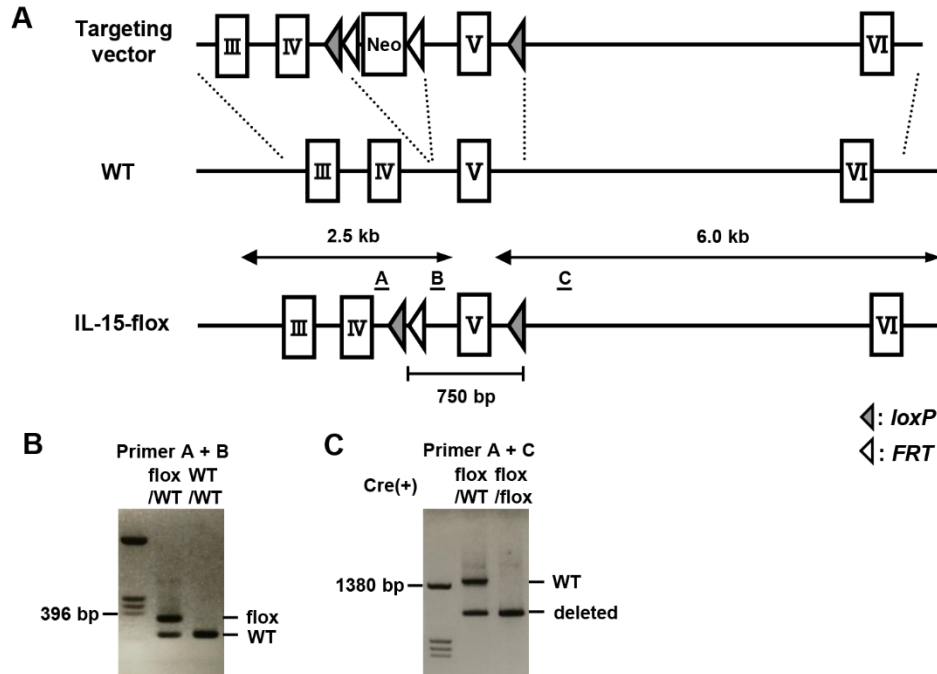
14 cells. Expression data were measured by dRNA-seq from 3–4 biological replicates. (K and L)

15 Flow cytometric analysis of CD5 (K) and PD-1 (L) in C2 and C1 iNKT cells. Data represent at

16 least two independent experiments with similar results (A, C-I, K, and L). Data are mean \pm SD

17 pooled from two independent experiments (B); with Student's *t*-test. **, *p* < 0.01.

18

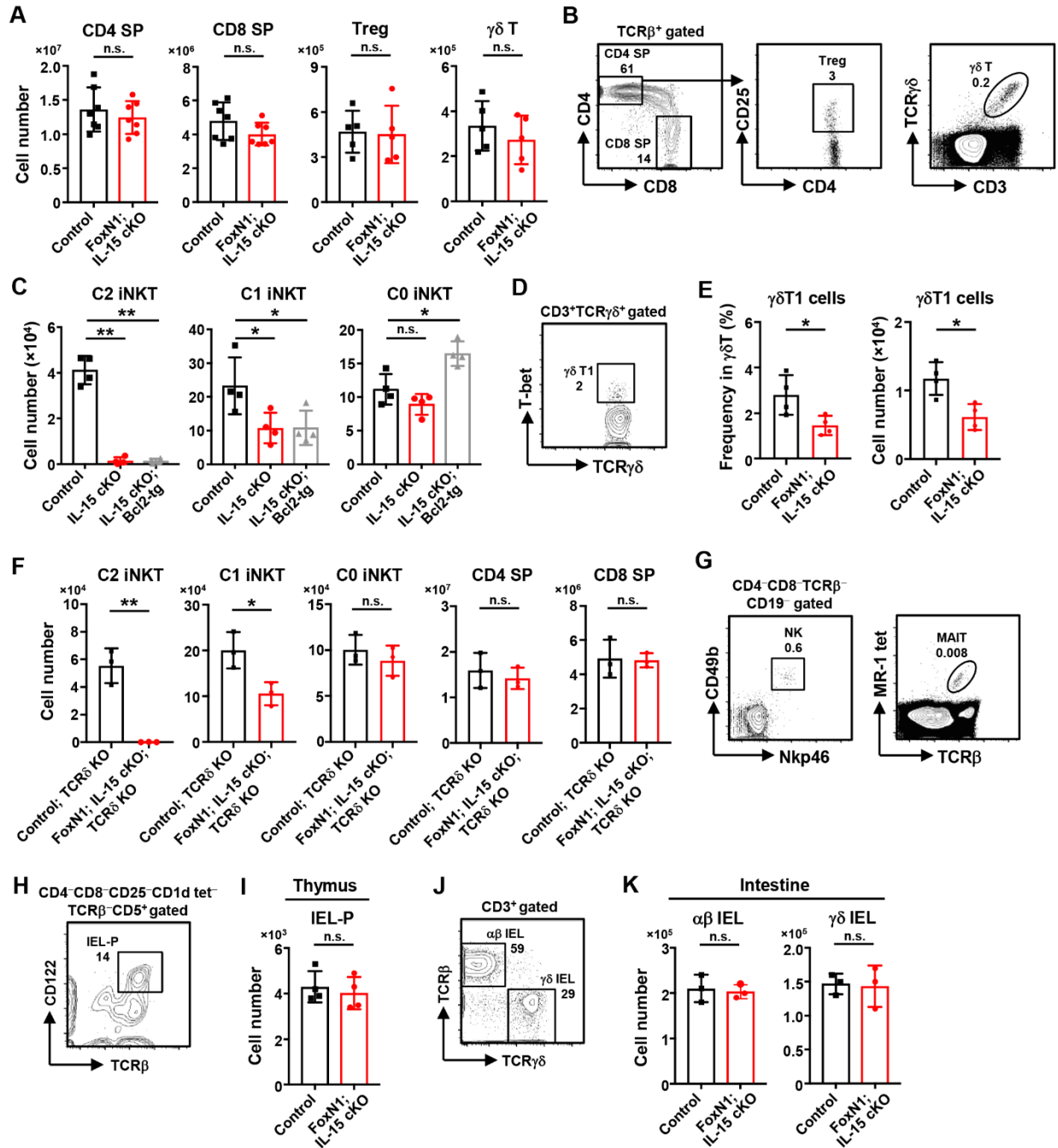


1

2 **fig. S2. Generation of IL-15-floxed mice.**

3 (A) Targeting strategy for the IL-15-floxed allele. Boxes indicate exons of the IL-15 locus. Gray
 4 and white triangles indicate loxP and FRT sequences, respectively. Horizontal bars indicate the
 5 primers for genotyping. The neomycin resistance gene cassette was excised by Flpe
 6 recombination. (B) Genotyping PCR to detect the IL-15-flox allele by the primers A and B
 7 shown in (A). (C) PCR analysis confirmed cKO in hematopoietic cells from Tie2-Cre; IL-15-
 8 flox/flox mice using the primers A and C shown in (A). Data represent at least four independent
 9 experiments with similar results (B and C).

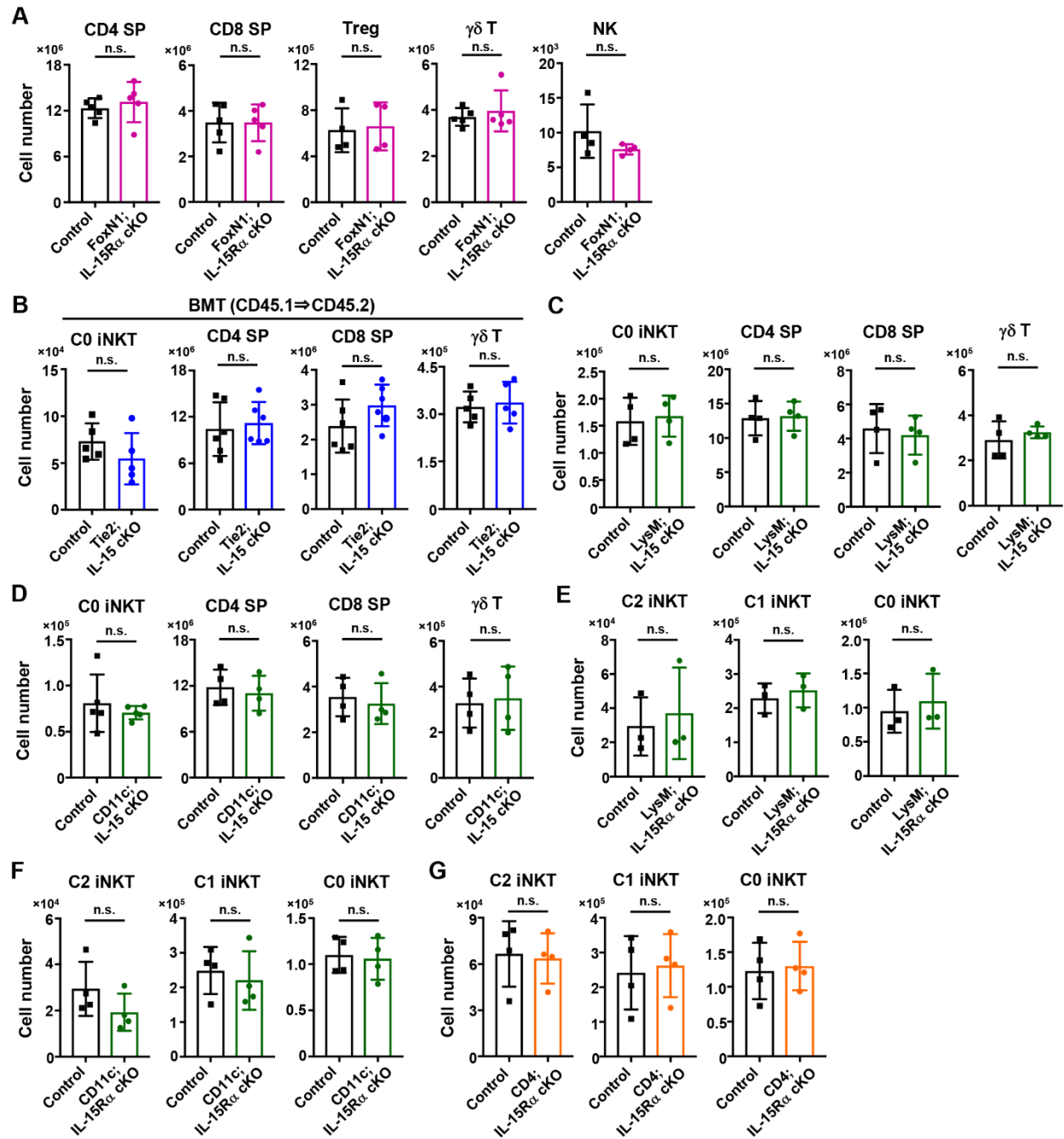
10



1
2 **fig. S3. Effects of TEC-derived IL-15 on thymocytes.**
3 (A) Number of indicated thymocytes in FoxN1-Cre IL-15 cKO (FoxN1; IL-15 cKO) mice and
4 littermate control (Control) (n = 5–7). (B) Gating strategy for SP, Treg, and γδ T cells from the
5 thymus. (C) Number of thymic C2, C1, and C0 iNKT cells in Bcl-2 transgenic (Bcl2-tg) FoxN1-

1 Cre IL-15 cKO mice (IL-15 cKO; Bcl2-tg), FoxN1-Cre IL-15 cKO mice (IL-15 cKO) and
2 littermate control mice (n = 4). **(D)** Gating strategy for $\gamma\delta$ T1 cells from the thymus. **(E)**
3 Frequency of $\gamma\delta$ T1 cells in $\gamma\delta$ T cells and number of $\gamma\delta$ T1 cells in the thymus of FoxN1-Cre IL-
4 15 cKO mice and littermate control (n = 4). **(F)** Number of indicated thymocytes in FoxN1-Cre
5 IL-15 cKO \times TCR δ KO mice (FoxN1; IL-15 cKO; TCR δ KO) and littermate control TCR δ KO
6 mice (n = 3). **(G)** Gating strategy for NK and MAIT cells from the thymus. **(H and I)** Gating
7 strategy (H) and number (I) of intraepithelial lymphocyte precursors (IEL-P) in the thymus of
8 FoxN1-Cre IL-15 cKO mice and littermate control (n = 4). **(J and K)** Gating strategy (J) and
9 number (K) of $\alpha\beta$ and $\gamma\delta$ intraepithelial lymphocytes (IELs) in the small intestine of FoxN1-Cre
10 IL-15 cKO mice and littermate control (n = 3). Data are means \pm SD and pooled from 2-4
11 independent experiments; Student's *t*-test or *t*-test with Welch's correction (A, E, F, I, and K)
12 and with one-way ANOVA (C). **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

13



1

2 **fig S4. IL-15 and IL-15R α trans-presentation in thymocyte development.**

3 (A) Number of indicated thymocytes in FoxN1-Cre IL-15R α cKO mice (FoxN1; IL-15R α cKO)
 4 and littermate control (Control) (n = 4–5). (B) Lethally irradiated hosts (Tie2-Cre IL-15 cKO
 5 mice and littermate control, CD45.2) were reconstituted with 1×10^6 bone marrow cells from
 6 wild-type mice (CD45.1). Number of indicated thymocytes in Tie2-Cre IL-15 cKO (Tie2; IL-15

1 cKO) or littermate control hosts 8 weeks after bone marrow transplantation (BMT) (n = 5–6). **(C**
2 **and D)** Number of indicated thymocytes in LysM-Cre IL-15 cKO mice (LysM; IL-15 cKO) (C)
3 (n = 4), CD11c-Cre IL-15 cKO mice (CD11c; IL-15 cKO) (D), and littermate control mice (n =
4 4–5). **(E and F)** Number of thymic C2, C1, and C0 iNKT cells in LysM-Cre IL-15R α cKO mice
5 (LysM; IL-15 R α cKO) (E) (n = 3), CD11c-Cre IL-15R α cKO mice (CD11c; IL-15 R α cKO)
6 (F) (n = 4), and littermate control mice. **(G)** Number of thymic C2, C1, and C0 iNKT cells in
7 CD4-Cre IL-15R α cKO mice (CD4; IL-15 R α cKO) and littermate control mice (n = 4). Data
8 are means \pm SD and pooled from 2-4 independent experiments; Student's *t*-test. n.s., not
9 significant.

10

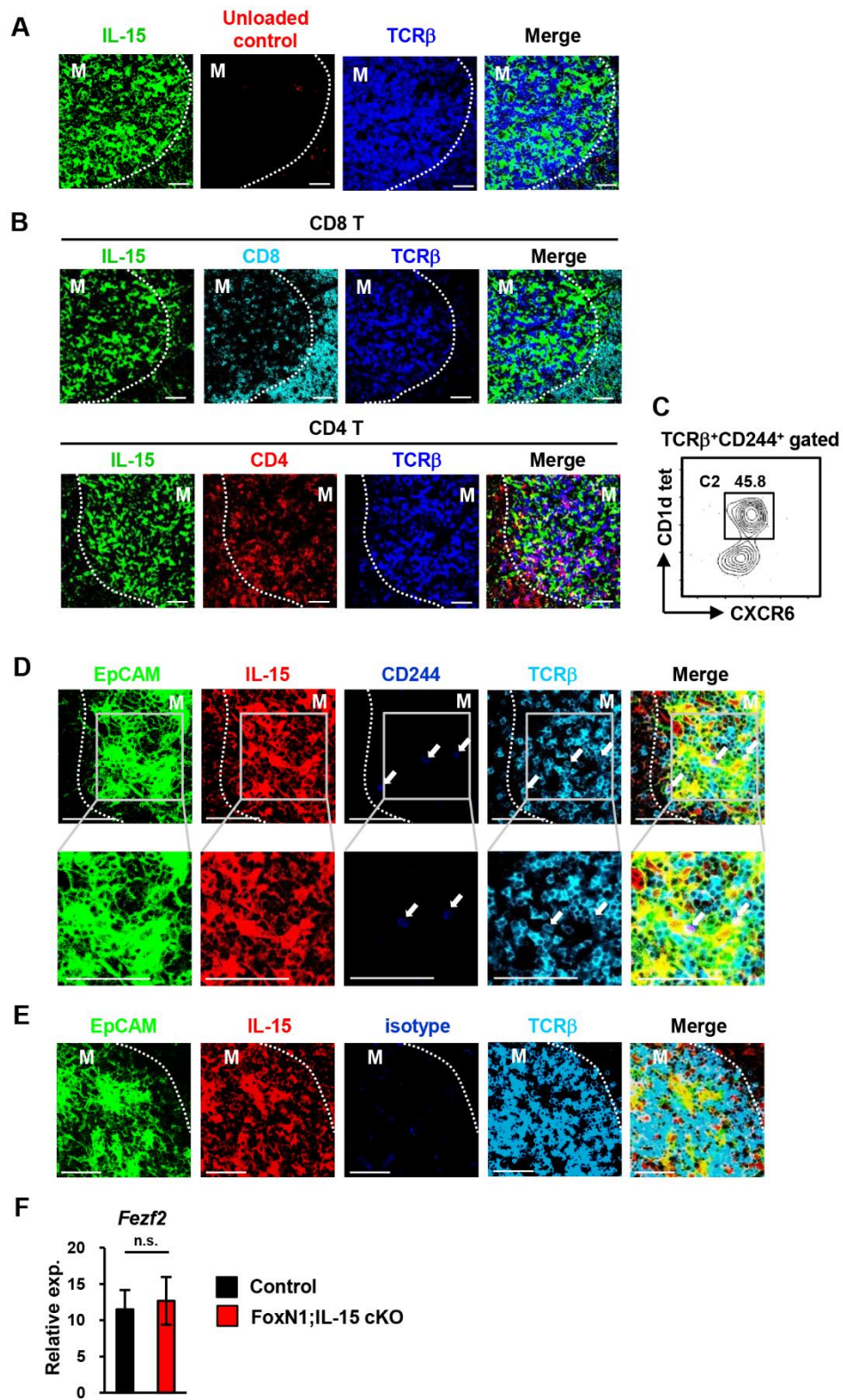
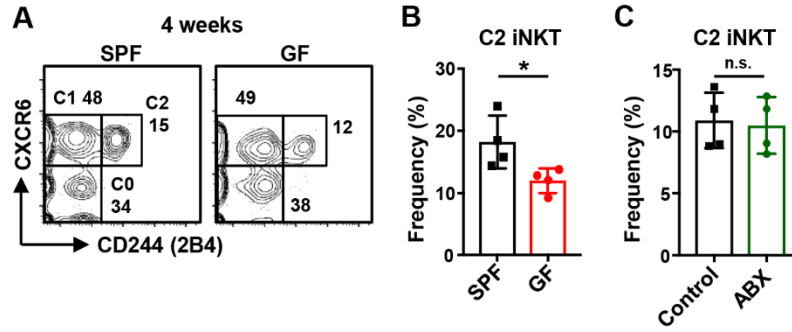


fig S5. Distribution of thymocytes and IL-15-producing mTECs in thymic medulla.

1 (A) Immunohistochemistry using PBS57-unloaded CD1d tetramer, the negative control for
2 PBS57-loaded CD1d tetramer in Fig. 3E (n = 2). M, thymic medulla. Scale bars, 50 μ m. (B)
3 Immunohistochemistry for CD8 SP and CD4 SP cells in the thymus of IL-15-CFP knock-in mice
4 (n = 3). M, thymic medulla. Scale bars, 50 μ m. (C) Frequency of C2 iNKT cells in
5 TCR β ⁺CD244⁺ thymocytes. (D) Immunohistochemistry for TCR β ⁺CD244⁺ cells in the thymus of
6 IL-15-CFP knock-in mice. Boxed areas in the upper panels are enlarged in the lower panels (n =
7 3). M, thymic medulla. Scale bars, 50 μ m. (E) Immunohistochemistry with isotype control for
8 anti-CD244 antibody in (D) (n = 2). M, thymic medulla. Scale bars, 50 μ m. (F) *Fezf2* mRNA
9 levels relative to *Hprt* in freshly isolated mTECs from FoxN1-Cre IL-15 cKO mice or littermate
10 control mice and measured by qPCR (n = 3). Data represent two (A and E) or three (B and D)
11 separate mice with similar results. Data represent three independent experiments with similar
12 results (C). Data are mean \pm SEM (F) and pooled from three separate mice with duplicate
13 measurement per mouse; Student's *t*-test. n.s., not significant.

14



1

2 **fig. S6. Effects of microbiome on C2 iNKT cells.**

3 (A) Flow cytometric analysis of thymic iNKT cells in SPF and germ-free (GF) mice. (B)

4 Frequency of thymic C2 iNKT cells in the mature NK1.1⁺ iNKT population shown in (A) (n = 4).

5 (C) Eight-week-old wild-type mice were fed antibiotics-containing water or control water for 4

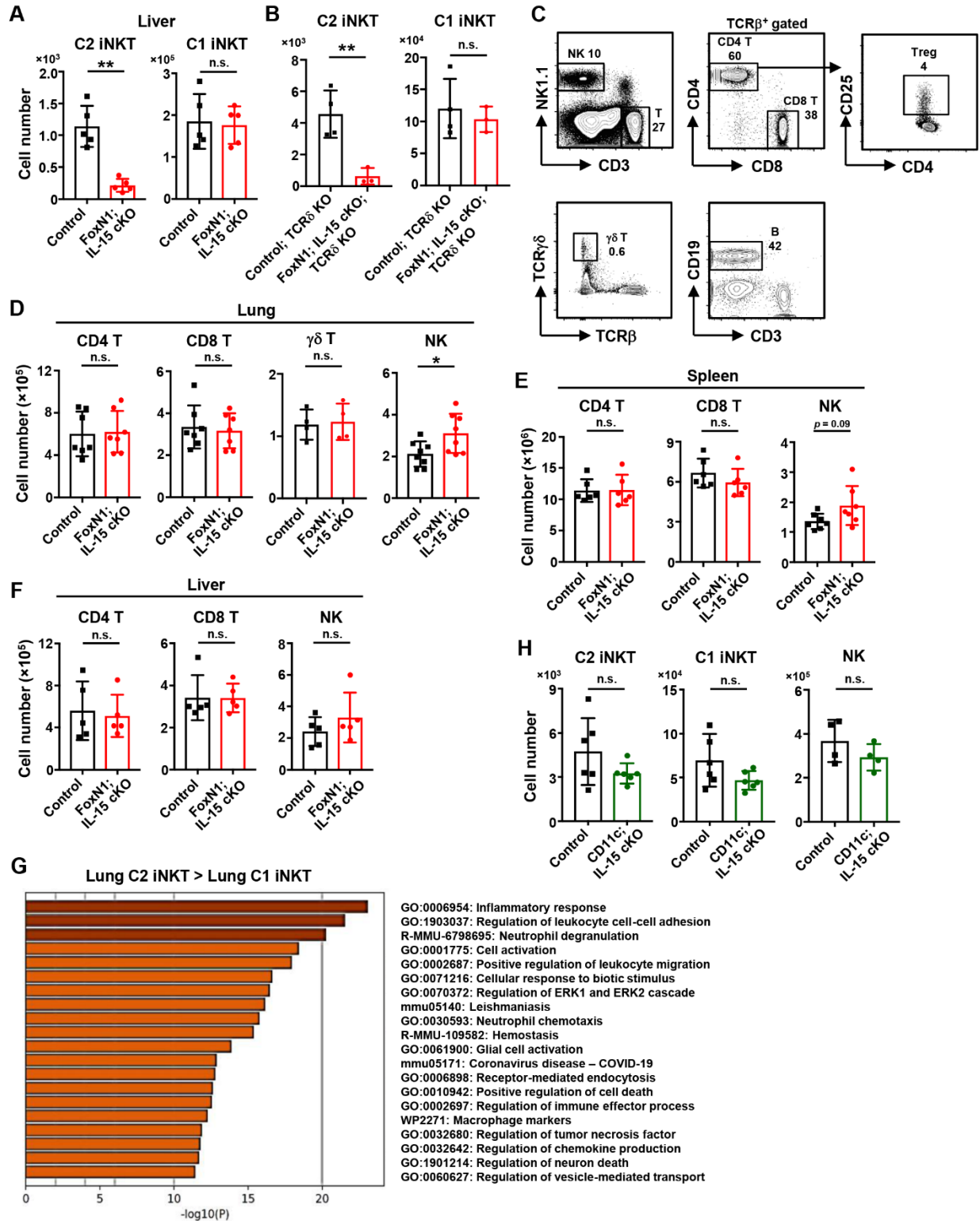
6 weeks. Frequency of thymic C2 iNKT cells in the mature iNKT population of antibiotic-treated

7 (ABX) and control 12-week-old mice (n = 4). Data represent two independent experiments with

8 similar results (A). Data are means \pm SD and pooled from two independent experiments (B and

9 C); Student's *t*-test. *, $p < 0.05$; n.s., not significant.

10



1

2

fig. S7. iNKT cells and relevant lymphocytes in peripheral tissues of IL-15 cKO mice.

1 (A) Number of liver iNKT cells in FoxN1-Cre IL-15 cKO mice (FoxN1; IL-15 cKO) and
2 littermate control mice (Control) (n = 5). (B) Number of iNKT cells in the lung of FoxN1-Cre
3 IL-15 cKO × TCRδ KO mice (FoxN1; IL-15 cKO; TCRδ KO) and control TCRδ KO mice (n =
4 3–4). (C) Gating strategy for the indicated lymphocytes from the lung. (D to F) Number of
5 indicated lymphocytes in the lung (D) (n = 4–8), spleen (E) (n = 6–7) and liver (F) (n = 5) of
6 FoxN1-Cre IL-15 cKO mice and littermate control mice. (G) Bar chart depicting the functional
7 analysis of differentially expressed genes between lung C2 and C1 iNKT cells. Normalized
8 enrichment scores were calculated using Metascape. (H) Number of indicated lung lymphocytes
9 in CD11c-Cre IL-15 cKO mice (CD11c; IL-15 cKO) (n = 4–6). Data are means ± SD and pooled
10 from 2-5 independent experiments (A, B, D, E, F, and H); Student's *t*-test. **, *p* < 0.01; *, *p* <
11 0.05; n.s., not significant.

12

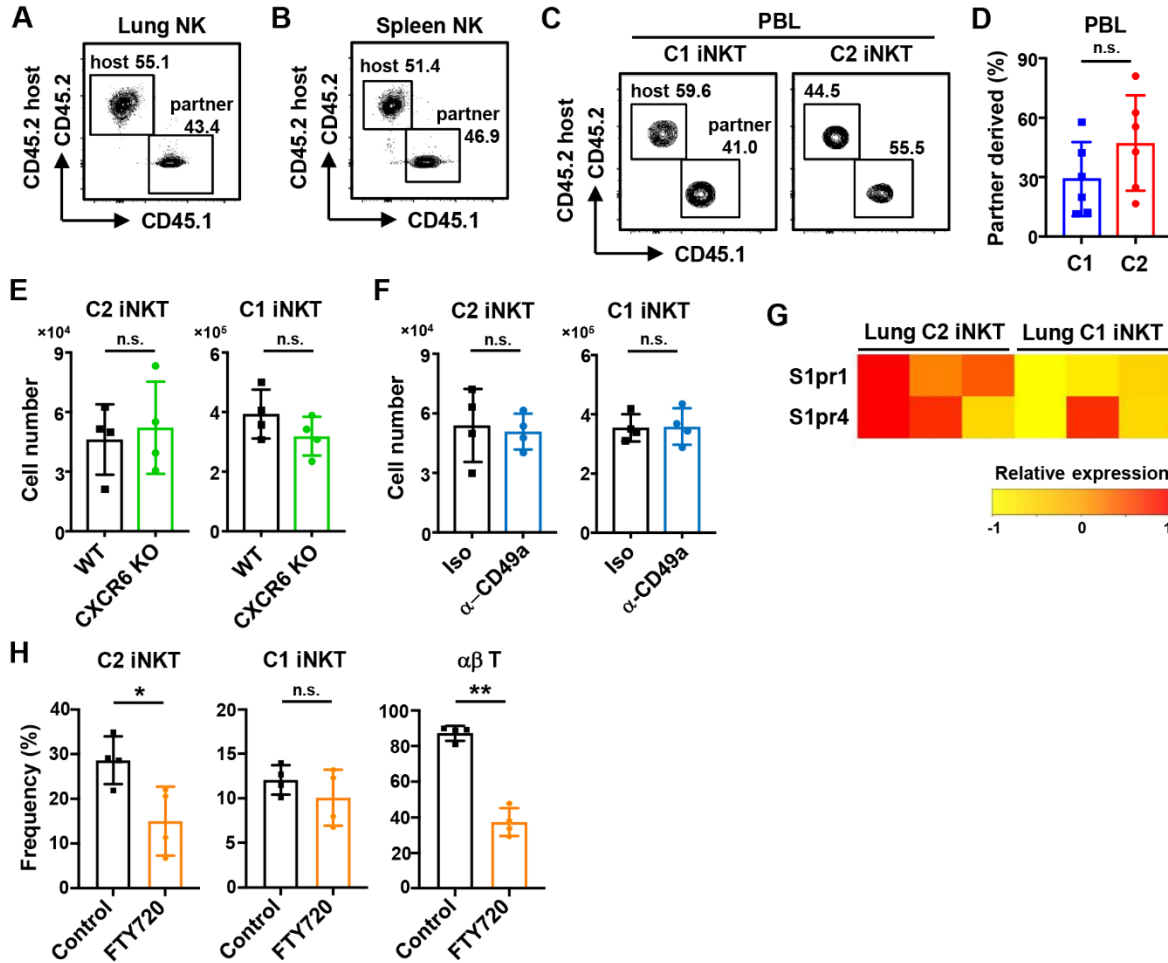
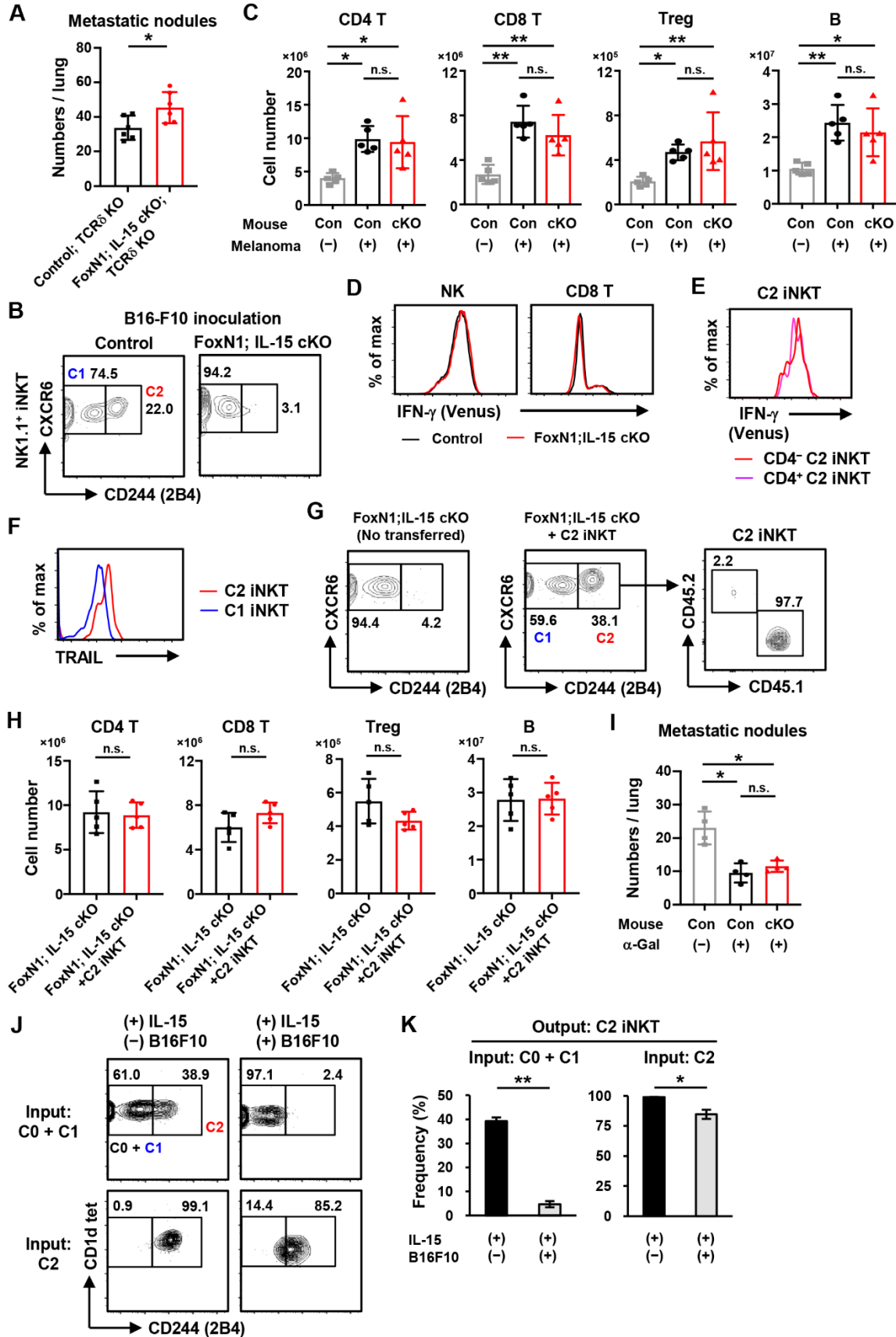


fig. S8. Circulation and retention of iNKT cells and NK cells.

(A and B) Genetically marked CD45.1 and CD45.2 mice of the same age underwent parabiosis surgery and were analyzed 60 days later. Flow cytometric analysis for partner-derived and host-derived NK cells in the lung (A) or spleen (B) (n = 6). (C and D) Flow cytometric analysis of partner-derived and host-derived C2 and C1 iNKT cells among peripheral blood lymphocytes (PBL) after parabiosis surgery (C). Frequency of partner-derived C2 and C1 iNKT cells of PBL (D) (n = 6). (E) Number of thymic C2 and C1 iNKT cells in CXCR6-GFP/GFP mice (CXCR6 KO) and wild-type (WT) mice (n = 4). (F) WT mice were administered anti-integrin $\alpha 1$ (α -CD49a) blocking antibody or isotype control (Iso) by intravenous injection every two days. Number of thymic C2 and C1 iNKT cells 6 days after the first antibody administration (n = 4).

1 (G) Expression of S1pr1 and S1pr4 in C2 and C1 iNKT cells measured by dRNA-seq from three
2 biological replicates. (H) WT mice were administered FTY720 by intraperitoneal injection for
3 three days. Frequency of CD45⁺ intravascular cells among the indicated lymphocytes by the
4 intravascular staining of CD45 (n = 4). Data represent three independent experiments with
5 similar results (A to C). Data are means \pm SD and pooled from 2-3 independent experiments (D-
6 F and H); Student's *t*-test. **, $p < 0.01$; *, $p < 0.05$; n.s., not significant.

7



1 **fig. S9. Alterations of relevant lymphocytes in a B16-F10 melanoma cell metastasis model.**

2 (A) Mice were intravenously injected with 4×10^4 B16-F10 melanoma cells, and the number of
3 metastatic nodules in the lung of FoxN1-Cre IL-15 cKO \times TCR δ KO mice (FoxN1; IL-15 cKO;
4 TCR δ KO) or littermate control TCR δ KO mice after 14 days of inoculation is shown (n = 6).

5 (B) Flow cytometric analysis of C1 and C2 iNKT cells in the lung 14 days after the inoculation.

6 (C) FoxN1-Cre IL-15 cKO (cKO) mice or littermate control mice (Con) were administered (+)
7 or without (-) 4×10^4 B16-F10 melanoma cells 14 days before the analysis. Number of indicated

8 lymphocytes in the lung is shown (n = 5). (D and E) Flow cytometric analysis of IFN- γ

9 expression in NK cells and CD8 T cells (D) and C2 iNKT cells (E) in the lung of FoxN1-Cre IL-
10 15 cKO \times IFN- γ -Venus reporter mice and control IFN- γ -Venus reporter mice 14 days after the

11 inoculation of melanoma cells. (F) Flow cytometric analysis for TRAIL expression in iNKT cells

12 after culture with B16-F10 melanoma cells in the presence of IL-15. (G) CD45.1 C2 iNKT cells

13 were adoptively transferred into CD45.2 FoxN1-Cre IL-15 cKO mice. Flow cytometric analysis

14 of transferred CD45.2 C2 iNKT cells in the lung of recipients 4 days after the inoculation of

15 melanoma cells. (H) Number of indicated relevant lymphocytes 14 days after the inoculation of

16 melanoma cells in CD45.2 FoxN1-Cre IL-15 cKO recipients with or without the adoptive

17 transfer of CD45.1 C2 iNKT cells (n = 5). (I) FoxN1-Cre IL-15 cKO mice (cKO) and littermate

18 control mice (Con) were administered α -galactosylceramide (α -Gal) (+) or control PBS (-). The

19 number of metastatic nodules in the lung of the indicated mice 14 days after the inoculation of

20 melanoma cells (n = 4). (J) Flow cytometric analysis of C2 and C0+C1 thymic iNKT cells after

21 4 days of culture with or without B16-F10 melanoma cells in the presence of IL-15. (K)

22 Frequency of the C2 iNKT cells shown in (J) (n = 3). Data are means \pm SD and pooled from 2-3

23 independent experiments; Student's *t*-test (A, H and K) and one-way ANOVA (C and I). Data

1 represent at least three independent experiments with similar results (B, D-G, and J). **, $p <$

2 0.01; *, $p < 0.05$; n.s., not significant.

3

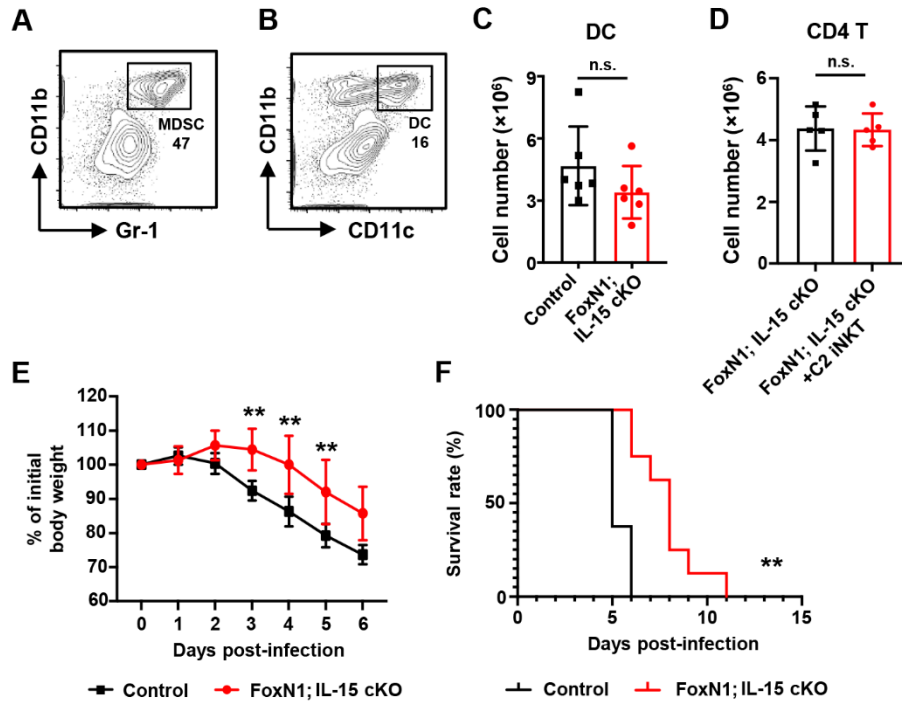
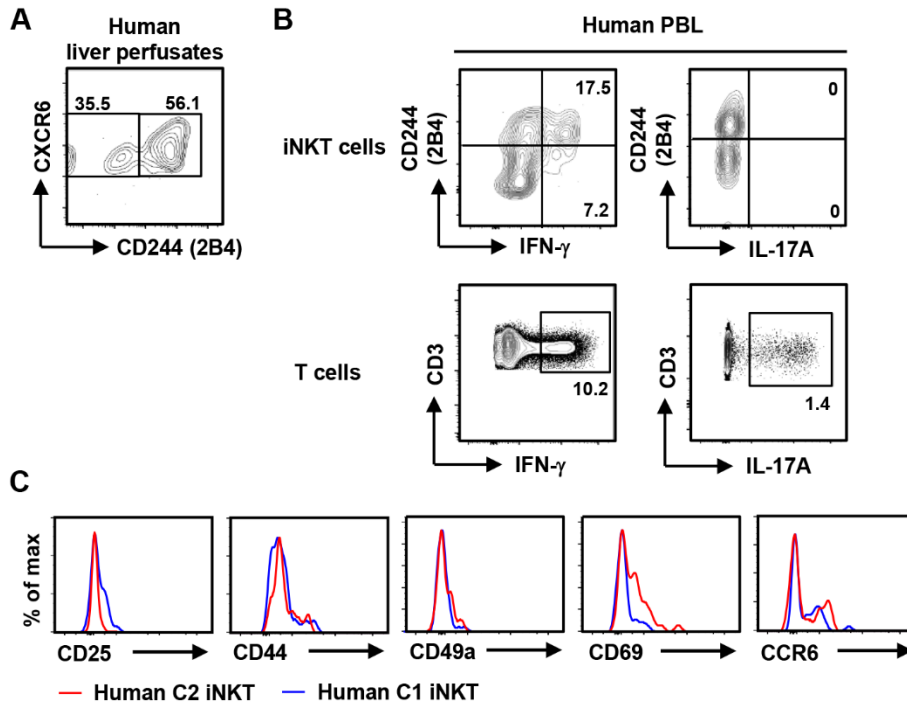


fig. S10. Alterations of relevant lymphocytes in an influenza virus infection model.

(A) Gating strategy for myeloid-derived suppressor cells (MDSCs) in the lung of mice on day 7 post infection by 0.5 LD₅₀ of influenza A virus (IAV). (B and C) Gating strategy (B) and number (C) of DCs in the lung of FoxN1-Cre IL-15 cKO and control mice on day 7 post infection (n = 6). (D) Number of CD4 T cells in the lung of FoxN1-Cre IL-15 cKO mice with or without the adoptive transfer of C2 iNKT cells on day 7 post infection (n = 5). (E) Body weight changes of age-matched FoxN1-Cre IL-15 cKO and control mice at the early phase post inoculation with a lethal dose (2 LD₅₀) of IAV. Each group consists of 8 female mice. (F) Survival rate of age-matched FoxN1-Cre IL-15 cKO and control mice following intranasal infection with IAV at the lethal dose (2 LD₅₀). Mice were monitored daily until death occurred or euthanized in case of greater than 20% weight loss. Each group consists of 8 female mice, and the difference was evaluated by the log rank test. Data are means \pm SD (C, D, and E) and pooled from 2-4 independent experiments; Student's t-test. **, $p < 0.01$; n.s., not significant.

1



2

3 **fig. S11. Characterization of human iNKT cells.**

4 (A) Flow cytometric analysis of human C1 (CD244⁻CXCR6⁺) and C2 (CD244⁺CXCR6⁺) iNKT
5 cells in liver perfusates. (B) Flow cytometric analysis of IFN- γ and IL-17A expression in human
6 T cells, C1, and C2 iNKT cells after stimulation with 25 ng/mL PMA and 1 μ g/mL ionomycin
7 for 4 hours. (C) Flow cytometric analysis of developmental and functional molecules in human
8 C1 and C2 iNKT cells. Data represent at least two independent experiments with similar results
9 (A-C).

10

1 **Table S1. List of antibodies used for flow cytometry**

Mouse

Antibody	Fluorescence	Clone	Company	Catalog number	RRID
TCR β	FITC	H57-597	BioLegend	109206	AB_313429
TCR β	PE	H57-597	BioLegend	109208	AB_313431
TCR β	APC	H57-597	BioLegend	109211	AB_313434
TCR β	APC/Cy7	H57-597	BioLegend	109219	AB_893626
TCR β	Brilliant Violet 421	H57-597	BioLegend	109230	AB_2562562
CD3 ϵ	FITC	145-2C11	BioLegend	100306	AB_312671
CD3 ϵ	PE/Cy7	145-2C11	Thermo Fisher Scientific	25-0031-82	AB_469572
CD4	APC-eFluor 780	RM4.5	Thermo Fisher Scientific	47-0042-82	AB_1272183
CD5	FITC	53-7.3	BioLegend	100605	AB_312734
CD8 α	eFluor 450	53-6.7	Thermo Fisher Scientific	48-0081-82	AB_1272198
CD8 α	PE/Cy7	53-6.7	TONBO	60-0081	AB_2621832
CD11b	APC/Cy7	M1/70	BioLegend	101225	AB_830641
CD11c	PE/Cy7	N418	BioLegend	117318	AB_493568
CD25	FITC	PC61	BioLegend	102005	AB_312854
CD44	PE/Cy7	IM7	Thermo Fisher Scientific	25-0441-82	AB_469623
CD49a	PE	Ha31/8	BD Bioscience	562115	AB_11153117
CD49a	Brilliant Violet 421	Ha31/8	BD Bioscience	740046	AB_2739815
CD49b	Pacific Blue	DX5	BioLegend	108918	AB_2265144
CD69	PE/Cy7	H1.2F3	Thermo Fisher Scientific	25-0691-82	AB_469637
CD45	APC/Cy7	30-F11	BioLegend	103115	AB_312980
CD45.1	Brilliant Violet 510	A20	BioLegend	110741	AB_2563378
CD45.2	FITC	104	Thermo Fisher Scientific	11-0454-82	AB_465061
CD122	Biotin	TM- β 1	BioLegend	123205	AB_940607
CD244.1	APC	C9.1	Thermo Fisher Scientific	17-2440-82	AB_2573188
CD244.2	Alexa Fluor 647	m2B4(B6)458.1	BioLegend	133509	AB_2072854
CD244.2	PE/Cy7	m2B4(B6)458.1	BioLegend	133511	AB_2564338
CD244.2	Biotin	m2B4(B6)458.1	BioLegend	133505	AB_1626229
CD244.2	Biotin	2B4	BD Bioscience	557452	AB_396714
NK1.1	PE	PK136	BioLegend	108708	AB_313395
NK1.1	APC	PK136	BioLegend	108709	AB_313396

TCR $\gamma\delta$	APC	GL3	BioLegend	118116	AB_1731813
TCR $\gamma\delta$	PE/Cy7	GL3	BioLegend	118124	AB_11204423
CXCR6	FITC	SA051D1	BioLegend	151107	AB_2572144
CXCR6	APC	SA051D1	BioLegend	151105	AB_2572142
TRAIL	PE	N2B2	BioLegend	109305	AB_2303575
KLRG1	APC/Cy7	2F1	BioLegend	138425	AB_2566553
IFN- γ	FITC	XMG1.2	Thermo Fisher Scientific	11-7311-82	AB_465412
IFN- γ	PE/Cy7	XMG1.2	BioLegend	505826	AB_2295770
IL-17A	PE/Cy7	eBio17B7	Thermo Fisher Scientific	25-7177-82	AB_10732356
IL-17RB	eFluor 660	MUNC33	Thermo Fisher Scientific	12-7361-82	AB_2572658
PD-1	APC	29F.1A12	BioLegend	135209	AB_2251944
ICOS	PE	C398.4A	Thermo Fisher Scientific	12-9949-81	AB_466277
P2X7R	PE	1F11	BioLegend	148703	AB_2650951
Gr-1	FITC	RB6-8C5	BioLegend	108406	AB_313371
F4/80	PE	BM8	BioLegend	123109	AB_893498
EpCAM	FITC	G8.8	BioLegend	118207	AB_1134106
Ly-51	Alexa Fluor 647	6C3	BioLegend	108311	AB_2099614
Bcl-2	PE	BCL/10C4	BioLegend	633507	AB_2043939
Bcl-X	PE	7B2.5	GeneTex	GTX46035	AB_774264
Ki-67	PE	SolA15	Thermo Fisher Scientific	12-5698-82	AB_11150954
PLZF	PE	9E12	BioLegend	145803	AB_2561966
T-bet	PE	4B10	Thermo Fisher Scientific	12-5825-82	AB_925761
Granzyme B	eFluor 660	NGZB	Thermo Fisher Scientific	50-8898-82	AB_11219679
Perforin	APC	S16009B	BioLegend	154403	AB_2721464

Human

Antibody	Fluorescence	Clone	Company	Catalog number	RRID
CD3	APC	UCHT1	BioLegend	300458	AB_2564151
CD4	APC/Cy7	RPA-T4	BioLegend	300518	AB_314086
CD25	PE	BC96	BioLegend	302606	AB_314276
CD44	PE	IM7	BioLegend	103008	AB_312959
CD49a	PE	TS2/7	BioLegend	328303	AB_1236441
CD69	PE	FN50	BioLegend	310905	AB_314840
CD94	PE	DX22	BioLegend	305506	AB_314536
CD161	PE	HP-3G10	BioLegend	339903	AB_1501086
CD244	PE/Cy7	C1.7	BioLegend	329520	AB_2572017
CXCR6	FITC	K041E5	BioLegend	356020	AB_2715815

CCR5	PE	2D7	BD Bioscience	555993	AB_396279
CCR6	PE	11A9	BD Bioscience	559562	AB_397273
ICOS	PE	C398.4A	Thermo Fisher Scientific	12-9949-81	AB_466277
IFN- γ	PE	4S.B3	BioLegend	502508	AB_315233
IL-17A	PE	BL168	BioLegend	512305	AB_961395

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