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In vivo availability of the cytokine IL-7 constrains the survival and homeostasis of peripheral *i*NKT cells

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SUMMARY

Understanding the homeostatic mechanism of invariant natural killer T (*i*NKT) cells is a critical issue in *i*NKT cell biology. Because interleukin (IL)-15 is required for the thymic generation of *i*NKT cells, IL-15 has also been considered necessary for the homeostasis of peripheral *i*NKT cells. Here, we delineated the *in vivo* cytokine requirement for *i*NKT cells, and we came to the surprising conclusion that IL-7, not IL-15, is the homeostatic cytokine for *i*NKT cells. Employing a series of experimental mouse models where the availability of IL-7 or IL-15 was manipulated in peripheral tissues, either by genetic tools or by adult thymectomy and cytokine pump installation, we demonstrate that the abundance of IL-7, and not IL-15, limits the size of the peripheral *i*NKT cell pool. These results redefine the cytokine requirement for *i*NKT cells and indicate competition for IL-7 between *i*NKT and conventional αβ T cells.

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AUTHOR CONTRIBUTIONS

J.Y.P. and J.H.P. conceptualized and designed the research. J.Y.P., H.Y.W., D.T.D., C.L., K.H.K., T.H.K., N.S., and C.H. performed experiments and analyzed data. N.S., N.A., and R.E.G. provided reagents and analytic tools. J.Y.P. and J.H.P. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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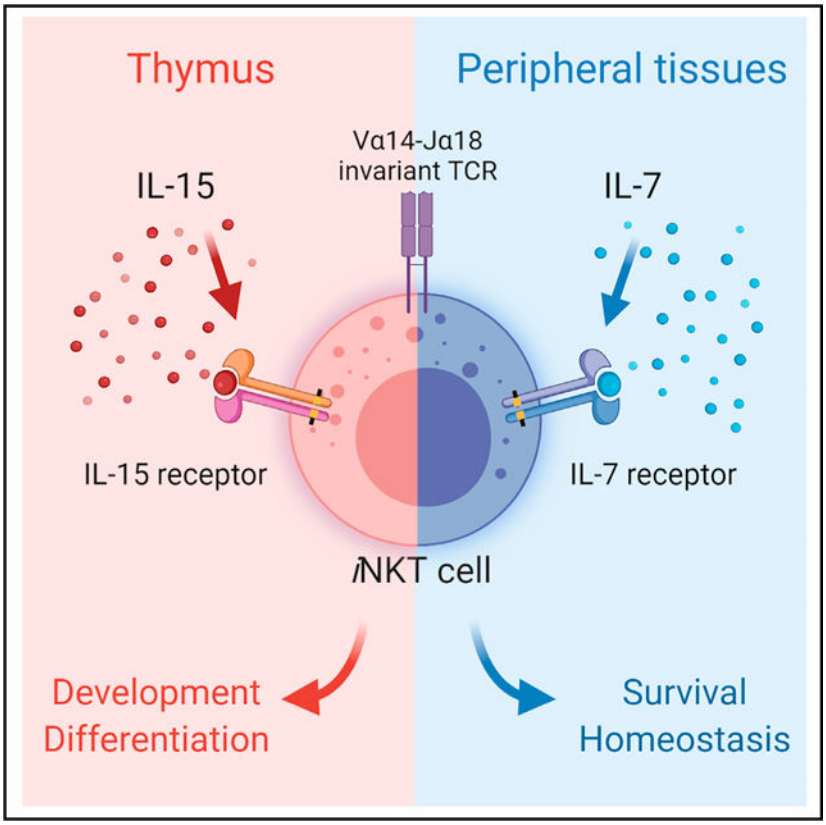
In brief

Park et al. assess the cytokine requirement for *i*NKT cells. While IL-15 is required for the thymic generation of *i*NKT cells, the survival and homeostasis of peripheral *i*NKT cells critically depend on the availability of IL-7 and not IL-15, revealing a dichotomy in the cytokine usage of *i*NKT cells.

Graphical abstract



INTRODUCTION

Natural killer (NK) T cells expressing the invariant Vα14-Jα18 T cell receptor (TCR) (*i*NKT cells) are CD1d-restricted immune regulatory cells that are uniquely positioned at the interface of the innate and adaptive immune systems (Godfrey and Kronenberg, 2004). As such, *i*NKT cells display functional characteristics and phenotypic markers of both innate NK cells and adaptive T lymphocytes (Godfrey and Kronenberg, 2004). Like T cells, *i*NKT cells express a somatically rearranged TCR that depends on RAG expression, and, like NK cells, *i*NKT cells express the NK lineage marker NK1.1 and are innate producers of pro-inflammatory cytokines (Bendelac, 1995a, b; Lantz and Bendelac, 1994). *i*NKT cells can recognize specific antigens and mount rapid and vigorous cytokine responses to play critical roles during microbial infection, inflammation, and in tumors (Bendelac et al., 2007; Godfrey et al., 2010).

Reflecting the shared characteristics of NK cells and T cells, the generation of *i*NKT cells also features a combined requirement of developmental signals that are specific to either NK cells or to T cells. Similar to conventional αβ T cells, *i*NKT cells are generated in the thymus from immature $CD4^+CD8^+$ double-positive (DP) thymocytes and require TCR-mediated positive selection (Bendelac, 1995b). This is in contrast to NK cells, which develop in the bone marrow and do not require a thymus (Haller et al., 1977). *i*NKT cells are similar to NK cells because their generation depends on interleukin (IL)-15 that is trans-presented by thymic medullary epithelial cells (Gordy et al., 2011; White et al., 2014). Such cytokine requirement is distinct from conventional T cells, which require IL-7, and not IL-15, for their development in the thymus. Thus, the generation of *i*NKT cells depends on a combination of cues that are characteristic for either conventional αβ T cells or NK cells.

In contrast to the generation of *i*NKT cells, however, the requirements for the survival and homeostasis of *i*NKT cells remain uncertain (Vahl et al., 2013). In particular, the role of cytokines for *i*NKT cell survival is incompletely understood. While conventional αβ T cells depend on IL-7, *i*NKT cells are proposed to mostly depend on IL-15 for their survival and homeostasis (Kennedy et al., 2000; Matsuda et al., 2002; Ranson et al., 2003a). As such, an IL-15 requirement had been documented by the paucity of liver *i*NKT cells of IL-15-deficient (*Il15*$^{-/-}$) mice (Matsuda et al., 2002), and the homeostatic proliferation of adoptively transferred *i*NKT cells, which depends on IL-15, and not IL-7 (Matsuda et al., 2002; Ranson et al., 2003a). The induction of the transcription factor T-bet, which promotes *i*NKT cell maturation (Townsend et al., 2004), also depends on IL-15 (Gordy et al., 2011; Townsend et al., 2004). Altogether, IL-15, and not IL-7, is currently considered as the homeostatic cytokine for mature *i*NKT cells.

However, IL-15 is also required for the generation of *i*NKT cells in the thymus (Kennedy et al., 2000). Thus, it was confusing whether *Il15*$^{-/-}$ mice lack peripheral *i*NKT cells because of the failure to generate them in the thymus or because of the failure to maintain them in the periphery. Additionally, the redundancy of IL-15 with other γc family cytokines has hampered further analysis of the homeostatic requirements for *i*NKT cells. In particular, a role for IL-7, which is a pro-survival factor for conventional αβ T cells (Schluns et al., 2000; Tan et al., 2001), remains unclear for *i*NKT cell homeostasis.

In the current study, we addressed these questions using a series of *in vivo* mouse models. While IL-15 was clearly a critical factor for the thymic development of *i*NKT cells, we were surprised to find that IL-15 was dispensable for *i*NKT cell survival in peripheral tissues. Instead, IL-7 and not IL-15 was essential to maintain the survival and homeostasis of *i*NKT cells. In fact, the *in vivo* availability of IL-7 constrained the size of the peripheral *i*NKT cell pool so that transgenic overexpression or administration of recombinant IL-7 proteins dramatically increased the numbers of *i*NKT cells. Conversely, in a mouse model where IL-7 is abundantly expressed in the thymus but not in peripheral tissues, *i*NKT cells were robustly produced in the thymus but succumbed in peripheral tissues. Collectively, this study unveils a non-redundant IL-7 requirement for *i*NKT cell homeostasis that has broad implications for understanding *i*NKT cell biology and effector function.

RESULTS

A requirement for γc cytokine receptors in thymic *i*NKT cell development

The thymus generates two distinct populations of mature ($CD24^{lo}TCR\beta^{hi}$) αβ T cells; namely, conventional αβ T cells and *i*NKT cells (Bendelac et al., 2007) (Figure 1A). *i*NKT cells differ from conventional αβ T cells in that they are selected by glycolipid-loaded CD1d molecules instead of self-peptide/MHC complexes (Benlagha et al., 2000). In C57BL/6 (B6) mice, approximately 8% of $CD24^{lo}$ mature thymocytes bind to glycolipid (PBS57)-loaded CD1d tetramers (CD1d Tet^{+}) and thus correspond to *i*NKT cells (Figure 1B). However, thymic *i*NKT cell frequencies can differ depending on the mouse strain, genetic background, age, or alterations in transcription factor expression (Constantinides and Bendelac, 2013; Esteban et al., 2003; Lee et al., 2013). Thus, *i*NKT cell development is controlled by a complex regulatory network, and its circuitry still needs to be deciphered. Notably, cytokines of the γc family also affect the generation of *i*NKT cells (McCaughtry et al., 2012). Along these lines, an *in vivo* requirement for γc cytokines is illustrated in the dramatic loss of mature ($CD24^{lo}TCR\beta^{hi}$) *i*NKT cells in γc-deficient (*Il2rg*$^{-/-}$) thymocytes (Figure 1A bottom and 1B). In contrast, the generation of conventional αβ T cells remained mostly unaffected in *Il2rg*$^{-/-}$ mice (Figure 1C). Thus, γc cytokines play a selective and non-redundant role in *i*NKT cell development in the thymus.

IL-15 is required for the generation, but not for the survival, of *i*NKT cells

The γc cytokine family consists of six members, and IL-15 is a γc cytokine that is considered to be essential for *i*NKT cell development (Kennedy et al., 2000; Ranson et al., 2003a). We confirmed an IL-15 requirement by assessing the *i*NKT cell population in thymocytes of IL-15-deficient (*Il15*$^{-/-}$) mice. Both the frequencies and numbers of *Il15*$^{-/-}$ thymic *i*NKT cells were dramatically reduced compared with those of wild-type (WT) *i*NKT cells, affirming IL-15 as a γc cytokine required for the thymic generation of *i*NKT cells (Figure 1D top and 1E left; Figure S1A).

IL-15 has also been considered necessary for *i*NKT cell survival and homeostasis in peripheral tissues (Gordy et al., 2011; Kennedy et al., 2000; Lodolce et al., 1998; Matsuda et al., 2002; Ranson et al., 2003a). Thus, we expected that the *i*NKT cells in peripheral tissues of *Il15*$^{-/-}$ mice would be substantially reduced. Surprisingly, however, the *i*NKT cell frequencies in the lymph nodes (LNs) and spleens of *Il15*$^{-/-}$ mice did not decrease (Figures 1D and S1A). Moreover, the total *i*NKT cell numbers pooled from multiple peripheral organs of *Il15*$^{-/-}$ mice (i.e., spleen, LN, liver, lung, bone marrow and fat) did not show significant differences to those of WT mice (Figures 1E and S1B). We confirmed such selective loss in thymic but not peripheral *i*NKT cells also in *Il15*$^{-/-}$ and littermate control mice (Figure S2), effectively excluding any potential differences in genetic background, age, or sex for these findings. Thus, contrary to the prevailing view (Kennedy et al., 2000; Ranson et al., 2003a), *i*NKT cell numbers in peripheral tissues were maintained independently of IL-15, and IL-15 appeared to be dispensable for the peripheral homeostasis of *i*NKT cells. Of note, we found that *i*NKT cells in the liver were an exception so that liver *i*NKT cell numbers were consistently diminished in *Il15*$^{-/-}$ mice (Figures 1F and S2C). However, such decrease in *i*NKT cell numbers was limited to the liver, and we did not find

reduced *i*NKT cell numbers in any other peripheral organs (Figures 1F, S1B, S2B, and S2C). It is currently not clear to us why liver *i*NKT cells are uniquely sensitive to the loss of IL-15, but we aim to address this in future studies. Collectively, these results document that IL-15 is required for the thymic development but not for the maintenance of *i*NKT cells, and that *i*NKT cells can survive by IL-15-independent homeostatic mechanisms in peripheral tissues.

*i*NKT cells are not homogeneous; rather, they comprise different subsets that are developmentally distinct and functionally diverse (Coquet et al., 2008; Lee et al., 2013; Michel et al., 2007; Pellicci et al., 2002; Terashima et al., 2008). Thus, we considered it important to examine if IL-15 is dispensable for the homeostasis of all *i*NKT cells or only a select subset of *i*NKT cells. Conventionally, mature *i*NKT cells have been divided into three distinct developmental stages based on their expression of CD44 and NK1.1 (Benlagha et al., 2002, 2005; Pellicci et al., 2002). Stage 1 *i*NKT cells are $CD44^{-}NK1.1^{-}$, followed by stage 2 *i*NKT cells, which are $CD44^{+}NK1.1^{-}$, and the most differentiated stage 3 *i*NKT cells are $CD44^{+}NK1.1^{+}$. The differentiation of stage 2 into stage 3 *i*NKT cells depends on IL-15 (Gordy et al., 2011), which in turn is necessary to upregulate expression of T-bet, a key transcription factor for terminal *i*NKT cell maturation (Townsend et al., 2004). In the thymus of C57BL/6 mice, the stage 3 subset comprises the vast majority (~90%) of *i*NKT cells (Benlagha et al., 2002). Consequently, the dramatic loss of thymic *i*NKT cells in *Il15*$^{-/-}$ mice is primarily due to decreases in the frequency and number of stage 3 *i*NKT cells (Figure 1G). In contrast, the number of stage 3 *i*NKT cells in peripheral lymphoid organs did not differ between *Il15*$^{-/-}$ and WT mice (Figure 1G right), indicating that peripheral stage 3 *i*NKT cells can either accumulate or arise independently of IL-15. Along these lines, stage 1 and stage 2 *i*NKT cell numbers in the periphery also did not differ between *Il15*$^{-/-}$ and WT mice (Figure S3A), indicating that IL-15 is dispensable for all stages of *i*NKT cells in peripheral tissues.

Recently, an alternative classification of *i*NKT cells has been put forward that places *i*NKT cells into three distinct effector subsets, namely, NKT1, NKT2, and NKT17 (Lee et al., 2013). NKT1 cells are characterized by high-level expression of the transcription factor T-bet, and they largely overlap with *i*NKT cells that are conventionally defined as stage 3 cells. In accordance with a role for IL-15 in T-bet expression (Gordy et al., 2011), NKT1 cells were dramatically reduced in the thymus of *Il15*$^{-/-}$ mice (Figures 1H and 1I). Importantly, however, NKT1 cell numbers in peripheral lymphoid organs remained comparable with those of WT mice (Figure 1I), demonstrating that IL-15 is required for the thymic generation but not for the peripheral maintenance of NKT1 cells. IL-15 was also dispensable for the maintenance of other *i*NKT subsets, as we did not find differences in NKT2 and NKT17 cell numbers of *Il15*$^{-/-}$ and WT mice (Figure S3B).

Because the IL-15-independent maintenance of *i*NKT cells was such a striking observation, we next wished to corroborate the validity of these results by confirming the lack of IL-15 activity in peripheral tissues of *Il15*$^{-/-}$ mice. NK cells are highly dependent on IL-15 (Ranson et al., 2003b). Consistent with their IL-15 requirement, we found a complete lack of NK cells in *Il15*$^{-/-}$ mice, confirming the IL-15 deficiency in these mice and documenting that *i*NKT cells are indeed maintained without IL-15 (Figure S4). We further wished to exclude potential contributions of the microbiota and/or facility-specific effects on *i*NKT

cell homeostasis, so we set up co-housing experiments of WT and *Il15*$^{-/-}$ mice. Here, we placed C57BL/6 WT mice with age- and sex-matched *Il15*$^{-/-}$ mice into the same cage for 5 weeks, and then analyzed the frequencies and numbers of *i*NKT cells in the thymus, LN, and spleen in co-housed mice (Figures S5 and S6). Notably, the thymic *i*NKT cell numbers remained dramatically diminished in *Il15*$^{-/-}$ mice, while the frequencies, numbers, and phenotypic features of LN and spleen *i*NKT cells were still comparable between WT and *Il15*$^{-/-}$ mice (Figures S5 and S6). Thus, we consider it unlikely that the microbiota or other environmental conditions would contribute to the IL-15-independent maintenance of peripheral *i*NKT cells. Lastly, to pinpoint that *i*NKT cell homeostasis is independent not only of IL-15 but of IL-15 signaling in general, we assessed the generation and maintenance of *i*NKT cells in IL-15Ra-deficient (*Il15ra*$^{-/-}$) mice. IL-15 signaling requires the transpresentation of the cytokine by the IL-15Ra receptor (Lodolce et al., 1998). Consequently, *Il15ra*$^{-/-}$ mice are impaired in IL-15 signaling, and we expected to find the same deficit in thymic *i*NKT cell generation but intact homeostasis of peripheral *i*NKT cells as in *Il15*$^{-/-}$ mice. This was indeed the case, as we observed a profound decrease in thymic *i*NKT cell numbers but no significant differences in the frequency, number, and phenotype of peripheral *i*NKT cells that included the LN, spleen, bone marrow, liver, and lung (Figures 1J, S7, and S8). Collectively, these data demonstrate an *in vivo* requirement for a γc cytokine in peripheral *i*NKT cell homeostasis that, surprisingly, is not IL-15, as has been previously thought.

IL-7 receptor is highly abundant on *i*NKT cells

To further examine which γc cytokine is required for *i*NKT cell maintenance, we assessed the expression of γc family cytokine receptors on *i*NKT cells. Consistent with their requirement for IL-15 signaling (Carson et al., 1994), the IL-2/IL-15 receptor β chain (IL-2/15Rβ) was abundantly expressed on *i*NKT cells, which differed from CD4 single-positive (SP) thymocytes or immature DP cells where IL-2/15Rβ was mostly absent (Figure 2A) (Gordy et al., 2011; Kennedy et al., 2000; Matsuda et al., 2002). *i*NKT cells additionally expressed other γc family cytokine receptors, specifically the IL-4Ra, the IL-7Rα, and the IL-21R (Figure 2A). Thus, *i*NKT cells are equipped to respond to various γc cytokines, and potentially any of these cytokines could act redundantly to IL-15 to provide survival.

Among γc cytokines, we found IL-7 of particular interest because the proprietary receptor for IL-7 (i.e., IL-7Rα), was highly expressed on *i*NKT cells in both the thymus and the spleen (Figures 2B and 2C). In fact, *i*NKT cells showed the largest abundance of IL-7Rα among all thymocyte subsets and peripheral αβ T cells (Figures 2B and 2C). Thus, we sought to examine a role of IL-7 for *i*NKT cells. IL-7 is essential for T cell development in the thymus so thymopoiesis is severely compromised in IL-7-deficient (*Il7*$^{-/-}$) mice (von Freeden-Jeffry et al., 1995). Both total thymocyte and *i*NKT cell numbers were dramatically reduced in *Il7*$^{-/-}$ mice, but the frequency of thymic *i*NKT cells did not decrease in the absence of IL-7 (Figure 2D), suggesting that there is no selective requirement for IL-7 for *i*NKT cell generation. In fact, there was a significant increase in thymic *i*NKT cell frequencies in *Il7*$^{-/-}$ mice (Figure 2E), indicating that *i*NKT cells are refractory to the lack of IL-7, presumably because they utilize IL-15 in the thymus. In peripheral tissues, however, *i*NKT cells were highly susceptible to the lack of IL-7, as reflected by the reductions in both

the frequencies and numbers of *i*NKT cells in lymphoid and non-lymphoid organs of *Il7*$^{-/-}$ mice (Figures 2D, 2E, and S9A). In fact, the cell numbers of all three *i*NKT subsets, i.e., NKT1, NKT2, and NKT17, were reduced in *Il7*$^{-/-}$ mice, indicating a common requirement for IL-7 in the homeostasis of all *i*NKT subsets (Figure S9B). Such an IL-7 requirement was specific to *i*NKT cells because the numbers of NK cells, which rely on IL-15, were mostly unaffected in *Il7*$^{-/-}$ mice (Figure S9C). These results document an IL-7 requirement for *i*NKT cell survival in peripheral tissues, and they further suggest that IL-7 is the γc cytokine required for *i*NKT cell homeostasis.

An *in vivo* IL-7 requirement for the maintenance of peripheral *i*NKT cells

To further clarify the significance of IL-7 for peripheral *i*NKT cells, we employed an animal model where IL-7 deficiency is limited to peripheral tissues. K7 mice are genetically engineered to express a thymocyte-specific IL-7 transgene on an *Il7*$^{-/-}$ background (Kim et al., 2012). Consequently, K7 mice express *Il7* mRNA in the thymus but not in peripheral tissues (Figure 3A). In agreement with robust IL-7 expression in the thymus, *i*NKT cell generation remained intact, and the frequency and number of thymic *i*NKT cells in K7 mice did not significantly differ from those of WT mice (Figures 3B and S10A). In contrast, the lack of IL-7 in peripheral tissues severely reduced the size of the *i*NKT cell pool, and *i*NKT cell numbers were dramatically reduced in both lymphoid and non-lymphoid tissues, including the liver (Figures 3B and S10A). These results indicated that IL-7 is critical for the homeostatic maintenance of *i*NKT cells in the periphery.

An IL-7 requirement for *i*NKT cell maintenance was further documented when we assessed the numbers of splenic *i*NKT cells relative to those of thymic *i*NKT cells. Compared with WT mice, the ratio of peripheral to thymic *i*NKT cells was dramatically reduced in K7 mice (Figure 3C) but did not alter their composition as assessed for each *i*NKT stage (Figure 3D). These results affirm a non-redundant role for IL-7 in maintaining all stages of *i*NKT cells in the periphery. Because IL-7 induces expression of the anti-apoptotic factor Bcl-2 (Rathmell et al.,2001), and because K7 *i*NKT cells contained significantly lower amounts of Bcl-2 than WT *i*NKT cells (Figure S10B), the loss of *i*NKT cells in K7 mice is most likely due to increased apoptosis. Thus, IL-7 is a critical survival factor for all peripheral *i*NKT cells. Finally, because the thymic output of *i*NKT cells affects the size of the peripheral *i*NKT cell pool (den Braber et al., 2012), we analyzed *i*NKT cell numbers in aged K7 mice (>6 months), where the thymic *i*NKT cell output was significantly reduced (Figure S10C). In aged K7 mice, peripheral *i*NKT cell numbers were still dramatically decreased (Figure S10C), demonstrating that IL-7 availability governs the homeostasis of peripheral *i*NKT cells independently of thymic output or aging.

Lastly, we considered it important to demonstrate a cell-intrinsic requirement for IL-7 signaling in *i*NKT cell survival. To this end, we utilized IL-7Rα floxed mice where IL-7 signaling can be conditionally terminated by IL-7Rα deletion using Cre recombinase expression (McCaughtry et al., 2012). For the selective IL-7Rα deletion in *i*NKT cells, we employed T-bet-Cre-expressing mice (Haddad et al., 2013), because we found T-bet being exclusively expressed in *i*NKT cells and not in other thymocytes (Figure 3E). Consequently, the T-bet-Cre-driven deletion of IL-7Rα in *Il7ra*$^{fl/fl}$T-bet^{Cre} mice was specific to *i*NKT cells,

permitting the testing of *i*NKT cell-intrinsic roles of IL-7 signaling (Figure 3F). In fact, over 90% of all thymic *i*NKT cells had deleted IL-7Rα in *Il7ra*$^{fl/fl}$T-bet^{Cre} mice, indicating that IL-7Rα expression is mostly dispensable for the generation of *i*NKT cells (Figures 3F and 3G). On the other hand, the frequency of IL-7Rα-negative *i*NKT cells dramatically decreased in peripheral tissues where IL-7 signaling is required for their survival (Figure 3G). Thus, IL-7R signaling is clearly a survival requirement for peripheral *i*NKT cells so that *i*NKT cells that have escaped IL-7Rα deletion prevailed and populated the peripheral *i*NKT compartment of *Il7ra*$^{wt/wt}$T-bet^{Cre} mice. Altogether, these results reaffirm a cell-intrinsic requirement for IL-7 in the homeostatic maintenance of *i*NKT cells.

STAT5 activation is required for *i*NKT cell survival

To understand the molecular basis of IL-7 requirement in *i*NKT cells, we next examined the effects of altering IL-7 receptor signaling in *i*NKT cells. IL-7 signaling triggers tyrosine phosphorylation at residue 449 (Y449) of the IL-7Rα cytoplasmic tail (Foxwell et al., 1995). Engineering Tyr449 to Phe449 (Y449F) disables IL-7-induced STAT5 recruitment and phosphorylation (Venkitaraman and Cowling, 1994) so that IL-7Rα Y449F knockin mice are impaired in IL-7-induced STAT5 phosphorylation (Osborne et al., 2007). While the lack of IL-7-induced STAT5 phosphorylation did not reduce the frequency or affect the differentiation of thymic *i*NKT cells (Figure 4A), both the frequencies and numbers of peripheral spleen *i*NKT cells were markedly diminished (Figure 4B). These results indicate that IL-7-induced STAT5 phosphorylation is dispensable for the generation of thymic *i*NKT cells but critical for the homeostasis of peripheral *i*NKT cells. Notably, the loss of peripheral *i*NKT cells was not due to impaired cytokine receptor expression because IL-2/15Rβ and γc expression levels were identical, and IL-7Rα expression was even higher on Y449F *i*NKT cells than on WT *i*NKT cells (Figure 4C).

To directly show that IL-7-induced pSTAT5 is required for *i*NKT cell survival, we next examined if peripheral Y449F *i*NKT cells are apoptotic. Caspase-3 activity is a prominent marker for programmed cell death (Lakhani et al., 2006). Spleen Y449F *i*NKT cells were indeed highly active for caspase-3, indicating that *i*NKT cells undergo massive apoptosis in the absence of pSTAT5 (Figure 4D). Lastly, to directly demonstrate that it is the lack of IL-7-induced pSTAT5 that led to *i*NKT cell death, we introduced a constitutively active STAT5b transgene (CA5^{Tg}) into Y449F mice (Burchill et al., 2003). As expected, constitutively active STAT5 was sufficient to restore *i*NKT cell survival (Figure 4E) and increase the number of peripheral *i*NKT cells, as documented in the spleen and liver of Y449F-CA5^{Tg} mice (Figure 4F). Altogether, these data demonstrate that *i*NKT cells require IL-7 receptor signaling to induce pSTAT5, which is necessary to prevent apoptosis.

Increased availability of IL-7 expands the homeostatic space for peripheral *i*NKT cells

To further corroborate that IL-7 controls the homeostasis of *i*NKT cells, we asked if increased IL-7 availability would increase the number of peripheral *i*NKT cells. To this end, we employed genetically engineered mice that express MHC-II promoter-driven mouse *Il7* cDNA transgenes (IL-7^{Tg}). The MHC-II promoter is mostly active in peripheral tissue so that the IL-7 abundance is profoundly increased in the periphery but not in the thymus (Mertsching et al., 1995). Consequently, any increase in peripheral *i*NKT or T cell numbers

is due to increased survival and not increased thymic output. As previously reported (Mertsching et al., 1995), peripheral T cell numbers were dramatically increased in IL-7^{Tg} mice, as illustrated by the highly elevated numbers of splenic $CD8^+$ T cells (Figure 5A). However, the numbers of NK cells, which depend on IL-15 and not on IL-7, remained mostly unaltered (Figure S11A) (Cooper et al., 2002; Prlic et al., 2003). On the other hand, IL-7 overexpression induced a dramatic increase in the frequency and number of peripheral *i*NKT cells (Figures 5B and 5C), concomitant with an increase in conventional αβ T cell numbers (Figure S11B). Importantly, the *i*NKT cell numbers in IL-7^{Tg} mice were increased without inducing cell proliferation, as documented in bromodeoxyuridine (BrdU) incorporation assays where BrdU was administered to WT and IL-7^{Tg} mice in their drinking water for 6 days (Figure 5D). These results support a role for IL-7 in the survival rather than proliferation of *i*NKT cells. Along these lines, the *i*NKT stage distribution in IL-7^{Tg} mice remained comparable with that of WT mice (Figures 5E and S11B), indicating that IL-7 is of equal importance to *i*NKT cells of all stages. Lastly, because thymic *i*NKT cell numbers are not increased in IL-7^{Tg} mice, these results permitted us to effectively exclude a scenario where increased thymic *i*NKT cell output would be responsible for increased *i*NKT cell numbers in the periphery. Therefore, these data fully align with our notion that IL-7 availability controls and constrains the size of the peripheral *i*NKT cell pool.

Increased IL-15 availability does not increase peripheral *i*NKT cell numbers

IL-15 is a homeostatic cytokine that partly overlaps with IL-7 in providing cell survival for αβ T cells (Ku et al., 2000; Park et al., 2019b; Tan et al., 2002). Accordingly, systemic overexpression of IL-15, such as in IL-15^{Tg} mice, resulted in dramatically increased numbers of peripheral CD8 T cells (Figure 6A), and in their acquisition of a memory phenotype as previously reported (Marks-Konczalik et al., 2000) and also reaffirmed in this study (Figure S12). Surprisingly, however, the increased availability of IL-15 did not increase peripheral *i*NKT cell numbers (Figures 6B and 6C). The increased level of IL-15 also did not alter the stages of the peripheral *i*NKT cell pool (Figure 6D). These results indicated that peripheral *i*NKT cells are refractory to increased IL-15 availability *in vivo*.

The IL-15 transgene is driven by the human EF-1α promoter and is consequently ubiquitously expressed in all tissues, including the thymus (Marks-Konczalik et al., 2000). In marked contrast to the periphery, however, the increase in IL-15 expression dramatically boosted both the frequency and number of thymic *i*NKT cells (Figure 6E). These results affirm the importance of IL-15 for *i*NKT cell development in the thymus and show that IL-15 availability controls the thymic *i*NKT cell output. Collectively, these data document disparate roles of IL-15 in *i*NKT cell biology where IL-15 drives the thymic development of *i*NKT cells but is dispensable for the maintenance of the peripheral *i*NKT cell pool.

The availability of IL-7, not IL-15, constrains the size of the peripheral *i*NKT cell pool

A major feature of the homeostatic mechanism is its capacity to expand the size of the existing T cell pool. A *bona fide* homeostatic cytokine would increase the number of *i*NKT cells in the absence of an influx of new *i*NKT cells from the thymus, and we aimed to demonstrate this for IL-7. First, we hypothesized that the size of the peripheral *i*NKT cell pool would rapidly shrink without the thymic contribution of new *i*NKT cells. However,

this has not been tested, and it has been unclear to what extent *i*NKT cell numbers can be maintained by purely peripheral homeostatic mechanisms. To establish these parameters, we utilized WT B6 mice that were adult thymectomized at 4 weeks of age, and we monitored the number of peripheral *i*NKT cells with time. Compared with sham-operated mice, *i*NKT cell numbers in thymectomized animals rapidly declined to approximately half the numbers in the spleen and less than a third in the LN between 2 and 3 months (Figure 7A). Curiously, liver *i*NKT cells in thymectomized mice did not show any statistically significant loss in their numbers compared with sham-operated animals (Figure S13A).

Thus, the liver *i*NKT cell pool is maintained independently of the thymic output, presumably because they comprise liver-resident non-circulating *i*NKT cells. For *i*NKT cells in other organs, however, there was a swift (12 days) loss of *i*NKT cells upon thymectomy, and *i*NKT cell numbers then stabilized at approximately 7 weeks (52 days post thymectomy; Figures 7A and S13B). These stabilized *i*NKT numbers were maintained at relatively constant levels for a prolonged time, up to 180 days post thymectomy (Figure 7A). Altogether, these data document that homeostatic mechanisms control the size of the peripheral *i*NKT cell pool.

Without new influx of thymus-derived *i*NKT cells, the number of peripheral *i*NKT cells stabilized at approximately 7 weeks after thymectomy. If the availability of the homeostatic cytokine is the constraining factor of *i*NKT cell numbers, we hypothesized that increasing the availability of the homeostatic cytokine would expand the size of the peripheral *i*NKT cell pool. To this end, we implanted cytokine-releasing osmotic pumps into thymectomized mice at day 52 post thymectomy and assessed the numbers of *i*NKT cells after 2 weeks. The osmotic pumps were designed to release either recombinant IL-15 or recombinant IL-7 at a constant rate over the 2-week period. The bioactivity of IL-15 and IL-7 was confirmed by the dramatically increased numbers of CD8 T cells in the spleen and LN of thymectomized mice that were implanted with the corresponding pumps (Figure 7B). Thus, conventional CD8 T cells are responsive to both IL-7 and IL-15, and these findings agree with previous reports that the survival and homeostasis of CD8 T cells are controlled by IL-7 and IL-15 (Schluns and Lefrancois, 2003; Tan et al., 2002). In marked contrast, *i*NKT cell numbers only increased in IL-7 and not in IL-15 pump-implanted mice (Figures 7C, S13C, and S13D). Thus, peripheral *i*NKT cells are refractory to the increased availability of IL-15, strongly opposing a role for IL-15 in constraining the homeostasis of *i*NKT cells. Instead, these results support a model where the peripheral *i*NKT cell pool is maintained by a homeostatic cytokine that is not IL-15, and the results from the IL-7 pump installation (Figure 7C) argue for a critical role of IL-7 in this process.

DISCUSSION

Unlike conventional αβ T cells that require IL-7 (Rochman et al., 2009), *i*NKT cells are considered to require IL-15 for their survival and homeostasis. Under this scenario, *i*NKT cells do not compete with other αβ T cells for IL-7, which is scarcely expressed *in vivo* and is only available in limited amounts (Hara et al., 2012; Kim et al., 2012; Park et al., 2004). Thus, the homeostasis of *i*NKT cells would be controlled independently of conventional αβ T cells. In marked contrast to this notion, here, we show that IL-7 is a non-redundant

requirement for peripheral *i*NKT cells and that the availability of IL-7, not IL-15, limits the homeostasis of *i*NKT cells. In fact, we uncovered a dual cytokine requirement for *i*NKT cells, where IL-15 is necessary for the thymic development and IL-7 is necessary for the survival of *i*NKT cells. These data demonstrate that *i*NKT cells and conventional αβ T cells are subject to the same cytokine constraint for peripheral homeostasis, indicating a common mechanism of cytokine utilization for the survival of *i*NKT and conventional αβ T cells.

While IL-7 is a critical factor for T cells, IL-7 is not produced by T cells or by other lymphocytes. Instead, IL-7 is expressed by non-lymphoid stromal cells and at low amounts, so that IL-7 availability is limited *in vivo*, and αβ T cells are forced to compete for IL-7 (Hara et al., 2012; Kim et al., 2012; Park et al., 2004). As a corollary, IL-7 availability limits the size of the αβ T cell pool in peripheral tissues (Park et al., 2004). Notably, *i*NKT cells were considered as exempt from this constraint because they supposedly require IL-15, not IL-7, for their survival. The current study challenges this idea because we made a string of experimental observations that were inconsistent with IL-15 being the homeostatic cytokine for *i*NKT cells. Instead, our data indicated that the *in vivo* availability of IL-7 controls the homeostasis and determines the size of the peripheral *i*NKT cell pool. As such, increased IL-7 availability in IL-7^{Tg} mice dramatically increased the peripheral *i*NKT cell numbers, but such an increase would not have been predicted if only IL-15 would control the size of the peripheral *i*NKT cell pool. Moreover, we found that a ubiquitously expressed IL-15 transgene increased *i*NKT cell numbers only in the thymus but not in the periphery, even though IL-15 was highly abundant in peripheral tissues. We know that the IL-15 transgene is expressed in the periphery because IL-15^{Tg} mice express elevated levels of serum IL-15 and contain dramatically increased numbers of CD8 T cells in the spleen and LNs (Marks-Konczalik et al., 2000). Thus, increased IL-15 expression expands the size of the CD8 T cell pool, but it fails to increase peripheral *i*NKT cell numbers. These results suggest that *i*NKT cell survival is either independent of IL-15 or that increased IL-15 expression alone is insufficient to promote *i*NKT survival. In the latter case, pro-survival factors such as tonic TCR signals could be potentially limiting and constrain the size of the *i*NKT cell pool. However, previous studies have suggested that *i*NKT cell survival is likely independent of TCR engagement (McNab et al., 2005). TCR ablation in peripheral *i*NKT cells further demonstrated that neither the survival nor the effector function of *i*NKT cells depend on tonic TCR signaling (Vahl et al., 2013). In addition, the overexpression of IL-7 dramatically increased the numbers of *i*NKT cells without necessitating alteration in TCR signaling. Collectively, these results agree with our proposition that *i*NKT cell survival is independent of IL-15.

In agreement with peripheral *i*NKT cell numbers being unaffected by the increased abundance of IL-15, we also did not find any significant changes in peripheral *i*NKT cell numbers by IL-15 deficiency. *i*NKT cell numbers remained unaltered in most peripheral tissues of *Il15*$^{-/-}$ mice, while the numbers of CD8 memory T cells and NK cells were dramatically reduced. These results indicated that most peripheral *i*NKT cells are refractory to the absence of IL-15, further strengthening our model where *i*NKT cell homeostasis operates independently of IL-15. Curiously, liver *i*NKT cells presented an exception to this concept because their frequency and number were substantially reduced in *Il15*$^{-/-}$ mice. It is currently unclear to us why liver *i*NKT cells would be uniquely sensitive to peripheral

IL-15. In fact, it was previously found that liver *i*NKT cells heavily rely on hepatic IL-7 for their survival (Liang et al., 2012). Thus, IL-15 and IL-7 appear to play non-redundant roles in liver *i*NKT cells homeostasis whereby we consider the possibility that IL-7 provides survival, but IL-15 could be critical for the recruitment or preservation of *i*NKT cells in hepatic tissues. Along these lines, liver *i*NKT cells are distinct from *i*NKT cells in other peripheral organs, as they depend on LFA-1-expressing liver-resident NK cells for their tissue retention (Emoto et al., 1999; Miyamoto et al., 2000). Thus, the number of *i*NKT cells in the liver is controlled not only by cell survival but also by cell adhesion and tissue residency. Because *Il15*$^{-/-}$ mice lack NK cells, such a scenario presents a plausible explanation for the selective paucity of *i*NKT cells in the liver, while total numbers of peripheral *i*NKT cells in *Il15*$^{-/-}$ mice can remain unchanged. Here, we also wish to point out that *i*NKT cells comprise a large fraction of lymphocytes in the liver but that the actual number of liver *i*NKT cells is diminutive (~200,000) compared with the number in the spleen (~800,000) or relative to the combined numbers of *i*NKT cells in all peripheral tissues (>1 million) (Godfrey et al., 2010; Gordy et al., 2011; Matsuda et al., 2002; Watarai et al., 2012). Consequently, interpreting the role of IL-15 in *i*NKT cells solely based on the frequency of *i*NKT cells and primarily relying on liver *i*NKT cells might result in obtaining a distorted and incomplete picture of peripheral *i*NKT cell homeostasis.

We acknowledge that our finding of IL-15-independent homeostasis of *i*NKT cells is in disagreement with studies by other groups that reported significantly reduced peripheral *i*NKT cell numbers of IL-15-deficient mice (Gordy et al., 2011; Matsuda et al., 2002). As a potential resolution, we wish to point out that our conclusion is based on the analysis of a large collection of peripheral organs that include LN, spleen, lung, bone marrow, and liver. These collected data points provided us with a more comprehensive picture of peripheral *i*NKT cell numbers than assessing *i*NKT cell numbers in a few selected organs. Also, we cannot exclude the possibility that environmental factors, including differences in the microbiota, could have contributed to the divergent findings of our study compared with previous reports. In fact, both the conventional gut microflora and an artificially restricted microflora can alter the generation and maintenance of *i*NKT cells (Wei et al., 2010), so differences in the housing conditions could account for dissimilar *i*NKT cell numbers between different studies.

Lastly, it remains unclear why IL-15 would not play a major role in *i*NKT cell maintenance but is important for *i*NKT cell generation in the thymus. In the same vein, it is unclear why IL-15 cannot be replaced by IL-7 to drive *i*NKT cell development in the thymus. IL-7 and IL-15 share the γc signaling pathway, and both cytokines induce phosphorylation of STAT5 and Akt to upregulate expression of anti-apoptotic Bcl-2 (Rochman et al., 2009). *i*NKT cells express high levels of IL-7Rα, and the intrathymic distributions of IL-7- and IL-15-producing thymic epithelial cells are similar and overlapping (Alves et al., 2009), so *i*NKT cells can bind to and encounter both IL-15 and IL-7 in the thymus (Colpitts et al., 2013; Cui et al., 2014; Sosinowski et al., 2013). However, it is IL-15 that is the major driver of thymic *i*NKT cell development. As a potential explanation, we postulated that only IL-15 would induce expression of *i*NKT cell lineage-specifying factors. IL-15 induces expression of the T-box transcription factor T-bet, which is required for *i*NKT cell maturation from stage 2 CD44^{hi}NK1.1^{-} cells into stage 3 CD44^{hi}NK1.1^{+} cells (Gordy et al., 2011). Thus,

IL-15 might play a non-redundant role in *i*NKT cell generation because it is required for upregulating the expression of T-bet.

We also acknowledge that an IL-7 requirement for *i*NKT cell survival is not entirely compatible with earlier reports, which proposed that IL-15, and not IL-7, is necessary for peripheral *i*NKT cells (Matsuda et al., 2002; Ranson et al., 2003a). How do we reconcile these differences? First, we used *in vivo* models of IL-7 deficiency or IL-7 overexpression that are limited to peripheral tissues to assess the IL-7 effect on peripheral *i*NKT cells, whereas other studies have relied on IL-7 germline deficiency or adoptive transfer models to address this question. Adoptive transfer experiments usually assess short term proliferation — and not survival—of adoptively transferred *i*NKT cells as an indicator for homeostasis. Importantly, homeostatic proliferation is distinct from steady-state homeostasis (Min et al., 2005; Surh and Sprent, 2008), and the effects of cytokine deficiencies on cell proliferation may not be applied to understand their effects on cell survival. Moreover, chronic lymphopenia induces profound changes in the commensal flora, which can lead to antigen-driven rapid proliferation of adoptively transferred T cells that is distinct from IL-7-driven slow homeostatic proliferation (Cho et al., 2007; Do et al., 2012; Goldrath and Bevan, 1999). Along these lines, it is interesting that adoptive transfers of Vα14^{+} *i*NKT cells into chronic lymphopenic host mice (*Rag2*$^{-/-}$*Il2rg*$^{-/-}$) resulted in massive proliferation of donor *i*NKT cells (>90% proliferation in 5 days) (Ranson et al., 2003a), whereas injection of *i*NKT cells into acute lymphopenic host mice (i.e., sublethally irradiated B6 mice) resulted in poor proliferation with only two or three cell divisions in 7 days (Matsuda et al., 2002). These data suggest that the host immune status can influence proliferation of adoptively transferred *i*NKT cells and that proliferation of adoptively transferred *i*NKT cells does not necessarily mirror survival and homeostasis of *i*NKT cells *in vivo*. In this regard, we think that steady-state *i*NKT cell numbers in peripheral IL-7-deficient K7 mice are a more reliable indicator for an IL-7 requirement because adoptive transfer and proliferation of donor cells are not required, thus directly demonstrating an IL-7 requirement for peripheral *i*NKT cell homeostasis.

Finally, an IL-7 requirement for *i*NKT cell survival is concordant with the study by Sprent and colleagues, who reported that a subset of IL-17-producing *i*NKT cells, i.e., NKT17 cells, depends exclusively on IL-7 for survival (Webster et al., 2014). IL-17-producing *i*NKT cells were identified and referred to as *i*NKT17 cells in the original study by Leite-de-Moraes and colleagues (Michel et al., 2007) and were then further characterized as mostly CD4^{-} NK1.1-negative *i*NKT cells by Godfrey and colleagues (Coquet et al., 2008). NKT17 cells are enriched in barrier tissues such as lung, skin, and peripheral LN (Doisne et al., 2009), but their ontogeny and survival requirements remain unmapped. Because we also found NKT17 cell numbers being limited by the availability of IL-7, our data align with the proposition that NKT17 cells are maintained by IL-7 (McNab et al., 2007; Webster et al., 2014). However, our results are disparate from the Sprent study, as we found IL-7 to be critical for not only NKT17 cells but also the survival of other NK T cell subsets. Thus, the current study confirms, but also expands, the role of IL-7 in *i*NKT cells, and it indicates a role for IL-7 in setting the size of the peripheral *i*NKT cell pool.

Limitations of the study

While we have demonstrated that the peripheral *i*NKT cell pool is controlled by IL-7, and not by IL-15, it is evident that the generation of *i*NKT cells in the thymus primarily depends on IL-15. The molecular basis of such a dichotomy in cytokine requirement between thymic and peripheral *i*NKT cells remains unclear. Therefore, our study is limited in its scope as it has not fully addressed these issues. Nonetheless, we consider the possibility that mature *i*NKT cells in the thymus could be tissue resident, and thus have different cytokine requirements compared with circulating peripheral *i*NKT cells. Tissue adaptation of peripheral *i*NKT cells could represent a potential mechanism, but such issues remain to be experimentally addressed and resolved.

STAR★METHODS

RESOURCE AVAILABILITY

Lead contact—Further information and requests for resources and reagents should be directed and will be fulfilled by the lead contact, Jung-Hyun Park (parkhy@mail.nih.gov).

Materials availability—All reagents generated in this study are available upon request with a completed material transfer agreement.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—C57BL/6NCrl (B6) and CD45.1 congenic B6 mice were obtained from the Frederick Cancer Research and Development Center, Frederick, MD. IL-7-transgenic (IL-7^{Tg}) mice, expressing a murine *Il7* cDNA under the control of the mouse *H2-Ea* promoter (Mertsching et al., 1995), were purchased from the Jackson Laboratories. Adult (4 weeks) thymectomized C57BL/6 male mice were purchased from the Jackson Laboratories. γc-deficient (*Il2rg*$^{-/-}$), IL-7-deficient (*Il7*$^{-/-}$), IL-15-deficient (*Il15*$^{-/-}$), IL-15Rα-deficient (*Il15ra*$^{-/-}$), IL-7Rα Y449 knock-in (Y449F) and IL-15 transgenic (IL-15^{Tg}) were previously described, (DiSanto et al., 1995; Kennedy et al., 2000; Lodolce et al., 1998; Marks-Konczalik et al., 2000; Osborne et al., 2007; von Freeden-Jeffry et al., 1995), and all strains were maintained on C57BL/6NCrl background. Peripheral IL-7 deficient mice (K7) were generated by introducing an *lck*-proximal promoter driven IL-7 transgene onto an *Il7*$^{-/-}$ background, as previously reported (Kim et al., 2012). Constitutively active STAT5b transgene (CA5^{Tg}) mice were previously described and kindly provided by Dr. Michael Farrar (Burchill et al., 2003). IL-7Rα floxed mice (*Il7ra*$^{fl/fl}$) (McCaughtry et al., 2012) was kindly provided by Dr. Al Singer (NCI), and T-bet-Cre (*Tbx21*^{Cre}) mice (Haddad et al., 2013) were obtained from the Jackson Laboratories. *Il7ra*$^{fl/fl}$ and *Tbx21*^{Cre} mice were intercrossed in-house to generate *Il7ra*$^{fl/fl}$ *Tbx21*^{Cre} mice. T-bet-ZsGreen reporter (TBGR^{Tg})

mice were a kind gift of Dr. Jinfang Zhu (NIAID) (Zhu et al., 2012). Animal experiments were performed with 6- to 12-week-old mice of both sexes and age-matched control mice, unless indicated otherwise. All animal experiments were approved by the NCI Animal Care and Use Committee, and all mice were cared for in accordance with NIH guidelines.

METHODS DETAILS

Flow cytometry—Cells were harvested and stained from the thymus and other organs. Data were acquired on LSR Fortessa or LSRII flow cytometers (BD Biosciences) and analyzed using software designed by the Division of Computer Research and Technology, NCI. Live cells were gated using forward scatter exclusion of dead cells stained with propidium iodide. The following antibodies were used for staining: TCRβ (H57–597), CD24 (30-F1), IL-7Rα (A7R34), NK1.1 (PK136), IL-2Rβ (TM-β1), IL-4Rα (M1), RORγt (Q31–378), T-bet (4B10), PLZF (9E12), and isotype control antibodies, all from eBioscience; CD44 (IM7), γc (4G3), CD4 (GK1.5 and RM4.5), and CD8α (53–6-7) from BD Biosciences; IL-21R (4A9) and CD45 (30-F11) from BioLegend. CD1d tetramers loaded with PBS-57 and unloaded controls were obtained from the NIH tetramer facility (Emory University, Atlanta, GA). Active caspase-3 was determined using the CaspGLOW™ fluorescein active caspase-3 staining kit (eBioscience).

Lymphocyte isolation from non-lymphoid organs—Liver and lung mononuclear cells (MNCs) were prepared as previously described with minor modifications (Watarai et al., 2008; Zhang et al., 2005). In brief, liver tissues were pressed through a 70-μm cell strainer (BD Biosciences) and resuspended in PBS. Cell suspensions were centrifuged at 100g for 3 min, and supernatants were collected, spun down and washed again with cold PBS. Lungs were harvested after PBS perfusion, diced into pieces, and digested with collagenase IV (1 mg/ml in PBS, Life Technology) for 45 min at 37°C. Liver and lung samples underwent enrichment for lymphocytes by centrifugation in a two-step Percoll gradient (GE Life Sciences). Lymphocytes at the interphase were harvested, washed, and resuspended in cell culture media before further analysis. MNCs in fat tissues were isolated as previously described (Lynch et al., 2015). In brief, visceral fat was harvested and digested for 25 min at 37 °C with 20 ml of collagenase IV solution (1 mg/ml). After digestion, MNCs were isolated by filtration through a 40-μm nylon mesh (Fisher Scientific) and centrifugation for 5 min at 300g. All MNCs from non-lymphoid organs were identified by CD45 expression.

i*NKT subset staining*: In brief, *i*NKT cells were stained with PBS-57-loaded mouse CD1d tetramers followed by staining for other surface markers, as previously described (Park et al., 2019a). Specifically, for each analysis, 10 million thymocytes were stained with fluorochrome-conjugated CD1d tetramers in FACS buffer (0.5% BSA, 0.1% sodium azide in Ca^{2+}- and Mg^{2+}-free HBSS) for 20 min at 4°C. Without removing the tetramer reagents, antibodies against surface proteins were then added, and cells were incubated for an additional 30 min at 4°C. Excess reagents were then washed out with FACS buffer by centrifugation for 7 min at 1,500 rpm. Pelleted cells were then resuspended in 150 μl of 1:3 mixture of concentrate/diluent working solution of the Foxp3 Transcription Factor Staining Buffer kit (eBioscience Thermo Fisher) and 100 μl of FACS buffer and incubated at room

temperature for 20 minutes. Cells were then washed twice with 1x permeabilization buffer (eBioscience Thermo Fisher), before adding antibodies for transcription factors, such as PLZF, RORγt, and T-bet. Cells were incubated at room temperature for 1 hour, before washing out excess reagents with FACS buffer followed by flow cytometry analysis.

BrdU labeling—Mice were given intraperitoneal injections of 1 mg BrdU (5-bromo-2'-deoxyuridine) dissolved in PBS (10 mg/ml; Sigma-Aldrich). Injected mice were then kept for 6 days with 1 mg/ml BrdU in their drinking water before analysis. Cell staining was performed using a BrdU flow kit according to the manufacturer's instructions (BD Biosciences).

Quantitative real-time PCR—*i*NKT cells were electronically sorted from WT, *Il7*$^{-/-}$, and K7 spleen T cells. RNA was isolated using the RNEasy kit (Qiagen) and then reverse transcribed into cDNA with the QuantiTect Reverse Transcription kit (Qiagen). QuantiTect SYBR Green (Qiagen) and an ABI PRISM 7900HT Sequence Detection System was used for quantitative RT-PCR analysis. The following primers were used for detection: *Il7* (Forward: 5'-CTGATGATCAGCATCGATGAATTGG-3'; Reverse: 5'-GCAGCACGATTTAGAAAAGCAGCT T-3'), *Bcl2* (Forward: 5'-GGATAACGGAGGCTGGGATGCCT-3'; Reverse: 5'-CAGAGTGATGCAGGCCCCGAC-3'), *actinb* (Forward: 5'-GAGAGGG AAATCGTGCGTGA-3'; Reverse: 5'-ACATCTGCTGGAAGGTGG-3'), *Hprt* (Forward: 5'-TCATTATGCCGAGGATTTGGA-3'; Reverse: 5'-CAGAGGGCCACAATGTGATG-3'). Gene expression values were normalized to *actinb* or *Hprt* signals in the same sample.

Alzet osmotic pump installation—Recombinant mouse IL-7 or mouse IL-15 (R&D system) was administered into thymectomized mice using ALZET osmotic pumps (DURECT) following the manufacturer's instruction. The pumps were each set to release 5 μg/ml of cytokines per 24 hours.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are shown as the mean ± SEM. The two-tailed Mann-Whitney *U* test was used to calculate *P*-values, where *, $p<0.05$; **, $p< 0.01$; ***, $p<0.001$ were considered statistically significant. NS = not significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- IL-15 is required for the generation but not the survival of peripheral *i*NKT cells
- IL-7 is a homeostatic cytokine for *i*NKT cells in peripheral tissues
- IL-7 availability constrains the size of the peripheral *i*NKT cell pool

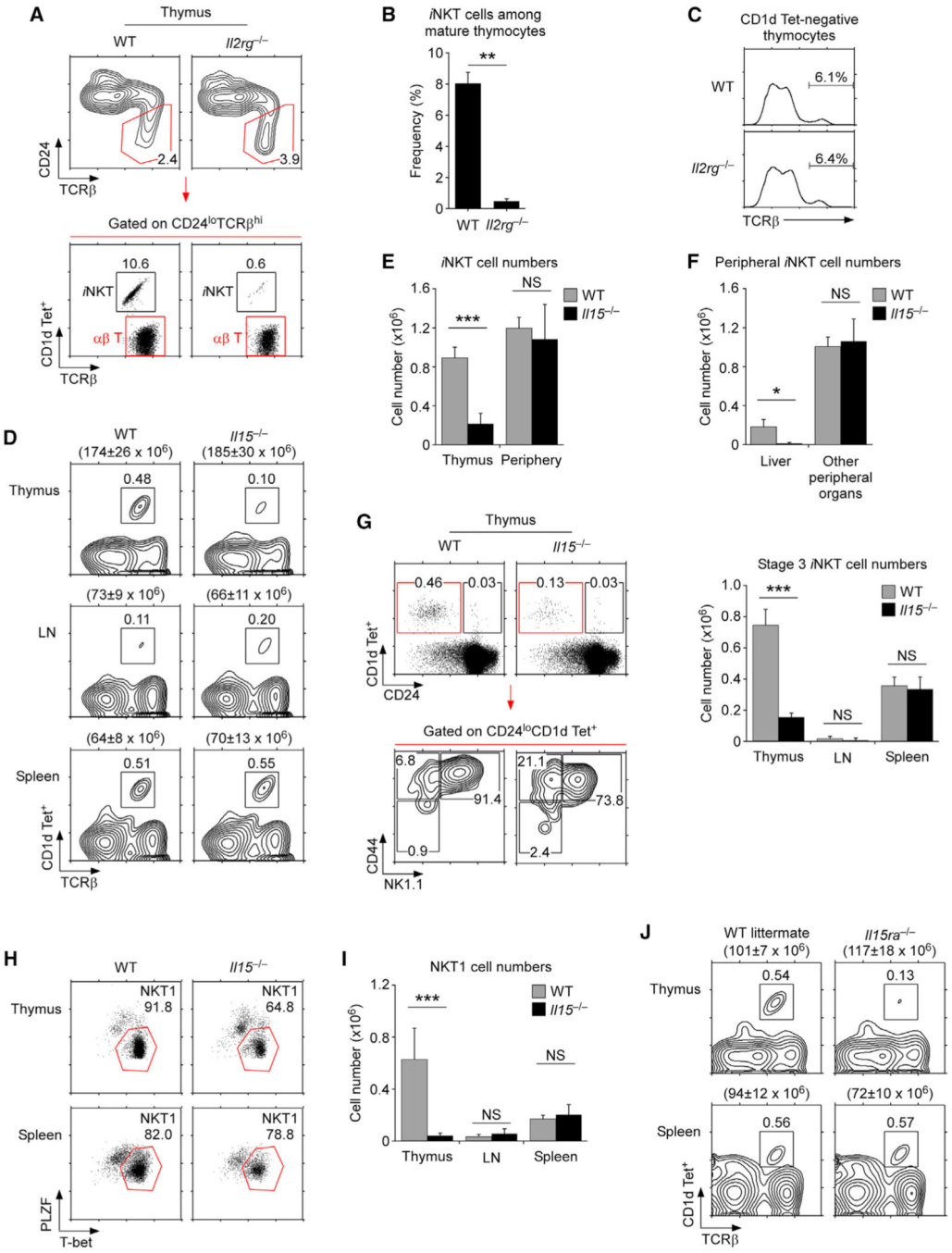

Figure 1. γc is required but IL-15 is dispensable for peripheral *i*NKT cells

(A) *i*NKT cell development in *Il2rg*$^{-/-}$ thymocytes. CD24^{lo}TCRβ^{hi} mature thymocytes from WT and *Il2rg*$^{-/-}$ mice were assessed for *i*NKT cells by staining with anti-TCRβ and CD1d tetramers loaded with the α-GalCer analog PBS-57 (CD1d Tet^{+}). Numbers in boxes indicate percentages of gated cells. Data are representative of five independent experiments.

(B) Frequency of thymic *i*NKT cells among CD24^{lo}TCRβ^{hi} mature thymocytes of WT and *Il2rg*$^{-/-}$ mice. Data show mean ± SEM of six WT and 13 *Il2rg*$^{-/-}$ mice.

(C) CD1d Tet-negative αβ TCR thymocytes in WT and *Il2rg*$^{-/-}$ mice. Numbers show percentages of TCRβ^{hi} mature T cells. Data are representative of three independent experiments.

(D) *i*NKT cell development and homeostasis in *Il15*$^{-/-}$ mice. *i*NKT cells in the indicated organs of WT and *Il15*$^{-/-}$ mice were identified by anti-TCRβ versus CD1d Tet staining. Total cell numbers are shown above each contour plot as mean ± SEM. Results are from three independent experiments with five WT and five *Il15*$^{-/-}$ mice.

(E) *i*NKT cell numbers in thymus and peripheral tissues of WT and *Il15*$^{-/-}$ mice. Periphery indicates the combined *i*NKT cells from spleen, LN, liver, lung, bone marrow, and fat tissues. Bar graph shows mean ± SEM from 15 WT and 18 *Il15*$^{-/-}$ mice.

(F) *i*NKT cell numbers were assessed among peripheral organs for either liver or other peripheral organs, which correspond to peripheral organs except for the liver. Bar graph shows mean ± SEM from 15 WT and 18 *Il15*$^{-/-}$ mice.

(G) *i*NKT cell differentiation in the thymus. Immature stage 0 and mature stage 1–3 *i*NKT cells in WT and *Il15*$^{-/-}$ thymocytes were identified by CD24 versus CD1d Tet staining (left, top). Stage 1–3 differentiation was determined on CD24^{lo}CD1d Tet^{+} thymocytes by CD44 versus NK1.1 analysis (left, bottom). Stage 3 *i*NKT cell numbers were determined in the indicated organs of WT and *Il15*$^{-/-}$ mice. Bar graphs show mean ± SEM from 10 WT and 13 *Il15*$^{-/-}$ mice from seven independent experiments (right).

(H) NKT1 cells were identified by intracellular promyelocytic leukemia zinc finger (PLZF) protein versus T-bet staining in the thymus and spleen of WT and *Il15*$^{-/-}$ mice. Dot plots are representative of four independent experiments with a total of five WT and five *Il15*$^{-/-}$ mice.

(I) NKT1 cell numbers in the indicated lymphoid organs of WT and *Il15*$^{-/-}$ mice. Bar graph shows the mean ± SEM from four WT and four *Il15*$^{-/-}$ mice from four independent experiments.

(J) *i*NKT cell development and homeostasis in *Il15ra*$^{-/-}$ mice. *i*NKT cells in thymus and spleen of *Il15ra*$^{-/-}$ and WT littermate control mice were identified by anti-TCRβ versus CD1d Tet staining. Total cell numbers are shown above each contour plot as the mean ± SEM. Results are from three independent experiments with five WT and six *Il15ra*$^{-/-}$ mice.

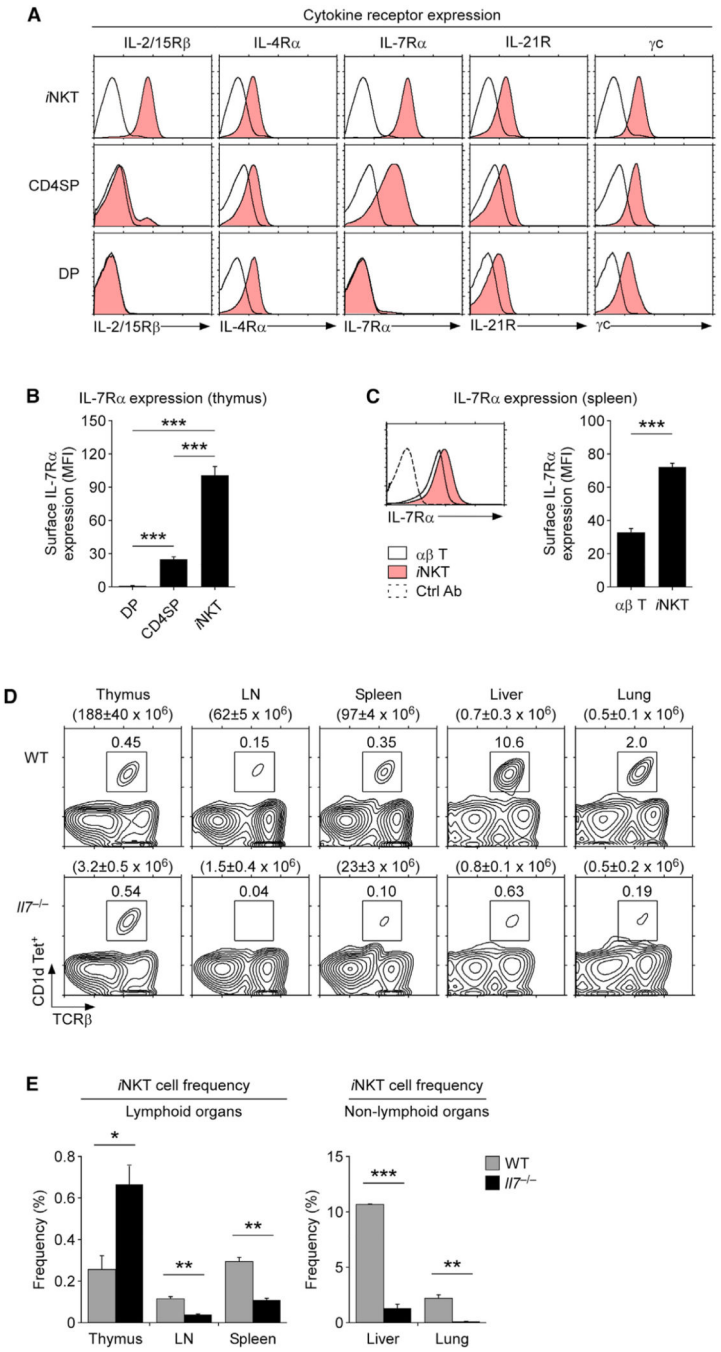

Figure 2. IL-7 is critical for the maintenance of *i*NKT cells in peripheral tissues

(A) Expression of γc family cytokine receptors (shaded histograms) on *i*NKT, CD4SP, and DP thymocytes of WT mice. Control antibody staining is shown in open histograms. Results are representative of four independent experiments.

(B) Surface IL-7Rα expression on *i*NKT, CD4SP, and DP thymocytes from WT mice. Mean fluorescence intensity (MFI) values are the summary of three independent experiments (mean ± SEM).

(C) Surface IL-7Rα levels on spleen αβ T cells and *i*NKT cells. MFI values are the summary of three independent experiments (mean ± SEM).
(D) *i*NKT cells in the indicated tissues and organs of WT and *Il7*$^{-/-}$ mice. Total cell numbers are shown above contour plots as the mean ± SEM. Data are representative of two independent experiments with a total of four WT and three *Il7*$^{-/-}$ mice.
(E) Frequencies of *i*NKT cells in lymphoid (left) and non-lymphoid tissues (right) of WT and *Il7*$^{-/-}$ mice. Bar graphs show the summary of two independent experiments with a total of four WT and three *Il7*$^{-/-}$ mice.

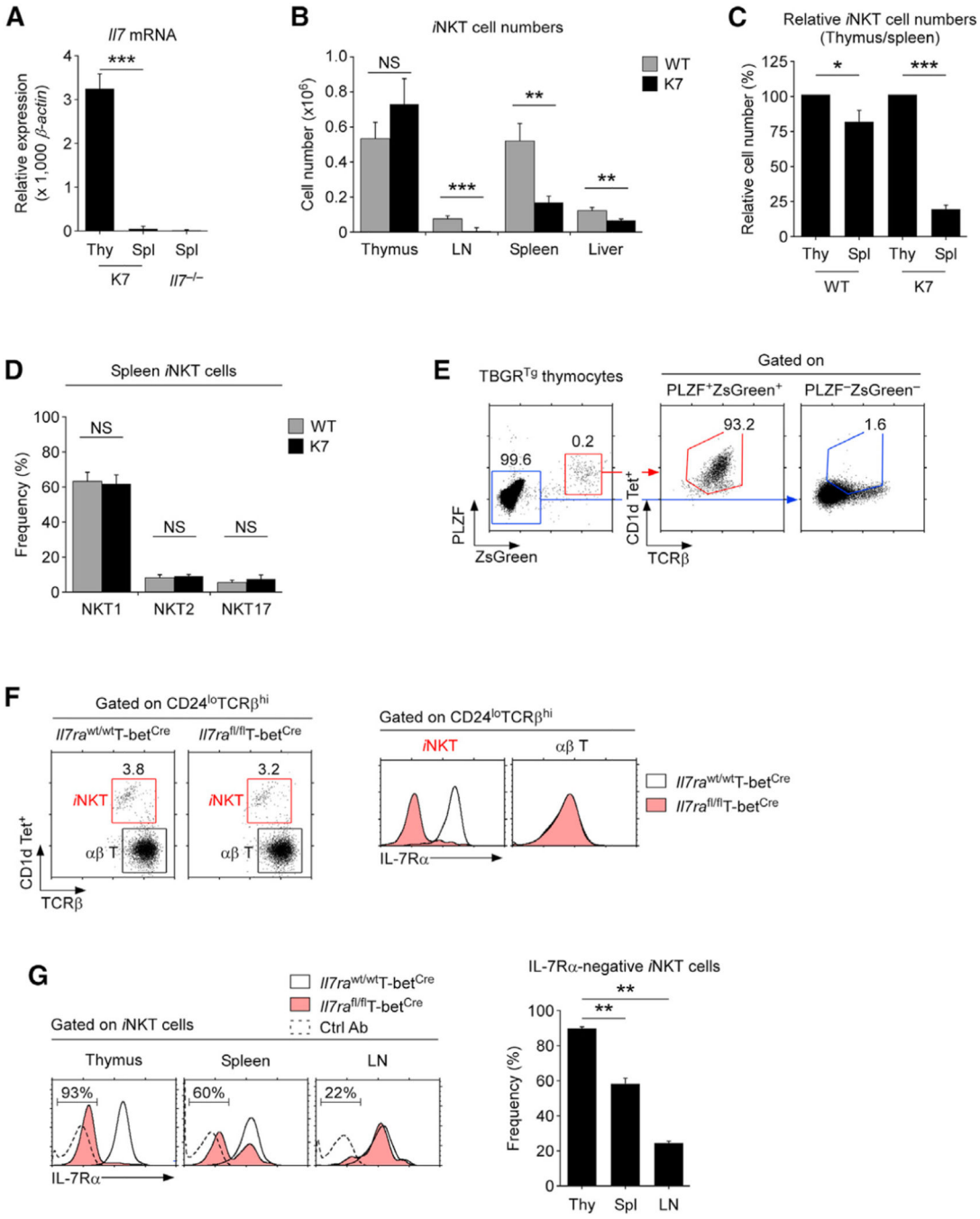

Figure 3. *i*NKT cell generation and survival in K7 mice

(A) Quantification of IL-7 mRNA in the thymus (Thy) and spleen (Spl) of K7 mice. *Il7* mRNA expression was assessed from thymus and spleen tissues of K7 mice by quantitative real-time PCR. Spleen mRNA from *Il7*$^{-/-}$ mice was used as a negative control. Data are the summary of three independent experiments.

(B) *i*NKT cell numbers in the indicated organs of WT and K7 mice. Bar graph shows *i*NKT cell numbers as the mean ± SEM from six WT and seven K7 mice from three independent experiments.

(C) Relative *i*NKT cell numbers in the thymus and spleen of WT and K7 mice where thymic *i*NKT cell numbers of each mouse strains were set to 100%. Data are the summary of 13 independent experiments with a total of 17 WT and 18 K7 mice.
(D) Subset composition of splenic *i*NKT cells in WT and K7 mice. The frequency of each subset was determined among spleen *i*NKT cells of WT and K7 mice by intracellular staining for PLZF, RORγt, and T-bet. Data show summary of six independent experiments with seven WT and eight K7 mice.
(E) Cellular identity of ZsGreen^{+} cells among thymocytes of TBGR^{Tg} mice. Total thymocytes of TBGR^{Tg} mice were stained for PLZF and plotted against ZsGreen reporter expression (left). The frequency of CD1dTet^{+} *i*NKT cells was then determined in PLZF^{+}ZsGreen^{+} versus PLZF^{-}ZsGreen^{-} thymocytes (right).
(F) IL-7Rα deletion in mature *i*NKT and conventional abT cells of *Il7ra*$^{fl/fl}$T-bet^{Cre} thymocytes. iNKT and conventional αβ T cells were identified among CD24^{lo}TCRβ^{hi} thymocytes of *Il7ra*$^{fl/fl}$T-bet^{Cre} and control *Il7ra*$^{wt/wt}$T-bet^{Cre} mice and assessed for surface IL-7Rα expression. Results are representative of four independent experiments.
(G) Surface IL-7Rα expression on *i*NKT cells of *Il7ra*$^{fl/fl}$T-bet^{Cre} mice. *i*NKT cells were identified in the thymus, spleen, and LN of *Il7ra*$^{fl/fl}$T-bet^{Cre} and littermate control *Il7ra*$^{wt/wt}$T-bet^{Cre} mice and assessed for IL-7Rα expression (left). Bar graph shows the frequency of IL-7Rα^{+} *i*NKT cells of the indicated mice (right). Results are from four independent experiments with a total of six *Il7ra*$^{wt/wt}$T-bet^{Cre} and nine *Il7ra*$^{fl/fl}$T-bet^{Cre} mice.

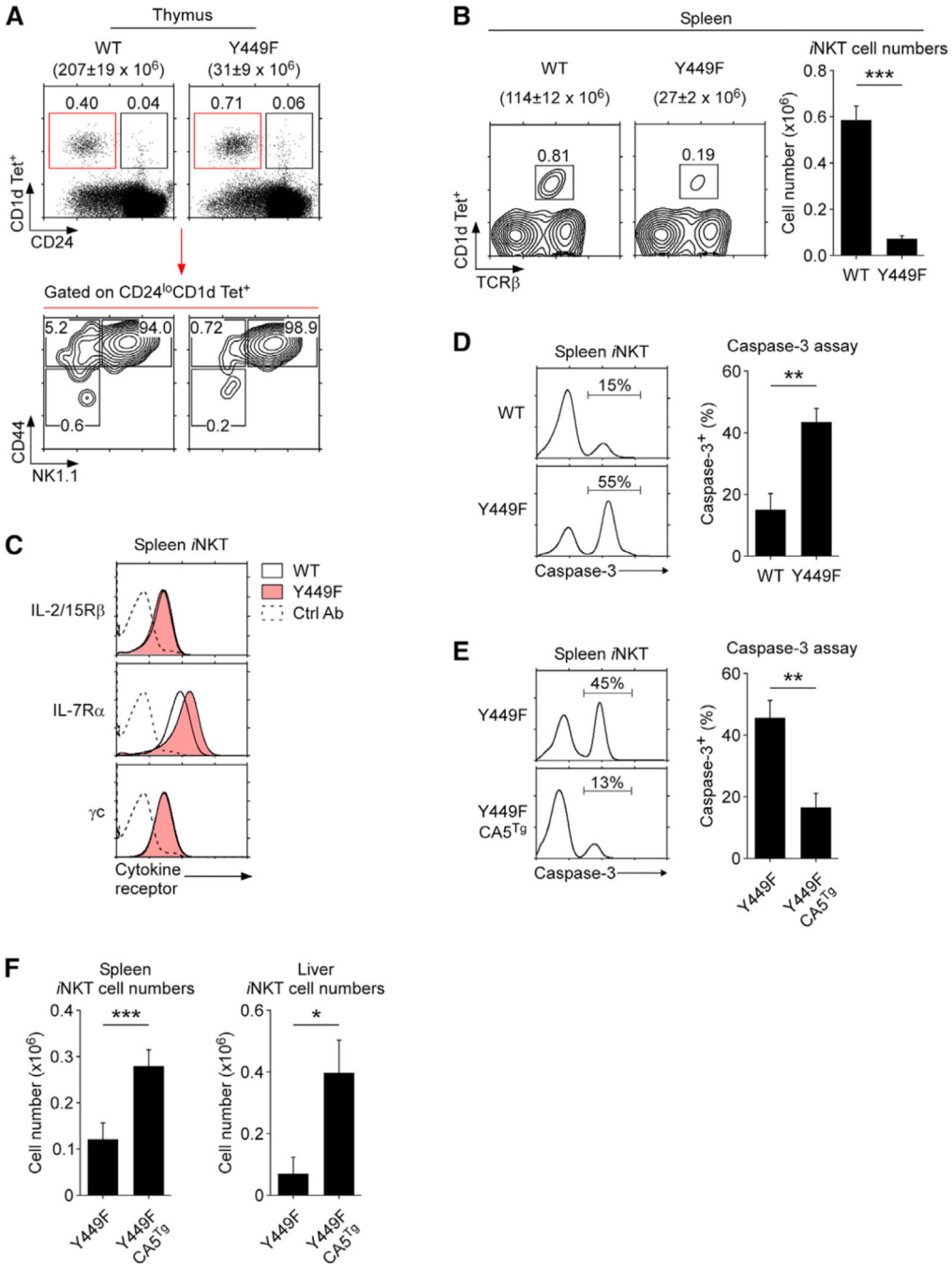

Figure 4. *i*NKT cell survival requires STAT5 activation downstream of IL-7 receptor signaling

(A) Thymic *i*NKT cell development in Y449F mice. Immature (stage 0; $CD24^{hi}$) and mature (stage 1–3; $CD24^{lo}$) *i*NKT cells were identified by CD24 versus CD1d tetramer staining in WT and Y449F thymocytes (top). Stage 1–3 *i*NKT cells were further determined on gated $CD24^{lo}$CD1d Tet^{+} thymocytes (bottom).

(B) Splenic *i*NKT cells in WT and Y449F mice (left). Bar graph shows splenic *i*NKT cell numbers from six WT and nine Y449F mice (right).

(C) Surface cytokine receptor expression on WT and Y449F splenic *i*NKT cells. Data are representative of three independent experiments with one mouse each.
(D) Active caspase-3 detection in freshly isolated WT and Y449F splenic *i*NKT cells (left). Bar graph shows the summary of three independent experiments (right).
(E) Active caspase-3 was assessed in freshly isolated Y449F and Y449F.CA5^{Tg} splenic *i*NKT cells (left). Bar graphs show the summaries of three independent experiments (right).
(F) Numbers of *i*NKT cells in peripheral tissues of Y449F.CA5^{Tg} mice. *i*NKT cells were identified by TCRβ versus CD1d tetramer staining of whole splenocytes and CD45^{+} liver mononuclear cells. *i*NKT cell numbers are shown as the mean ± SEM from eight Y449F and four Y449F.CA5^{Tg} mice.

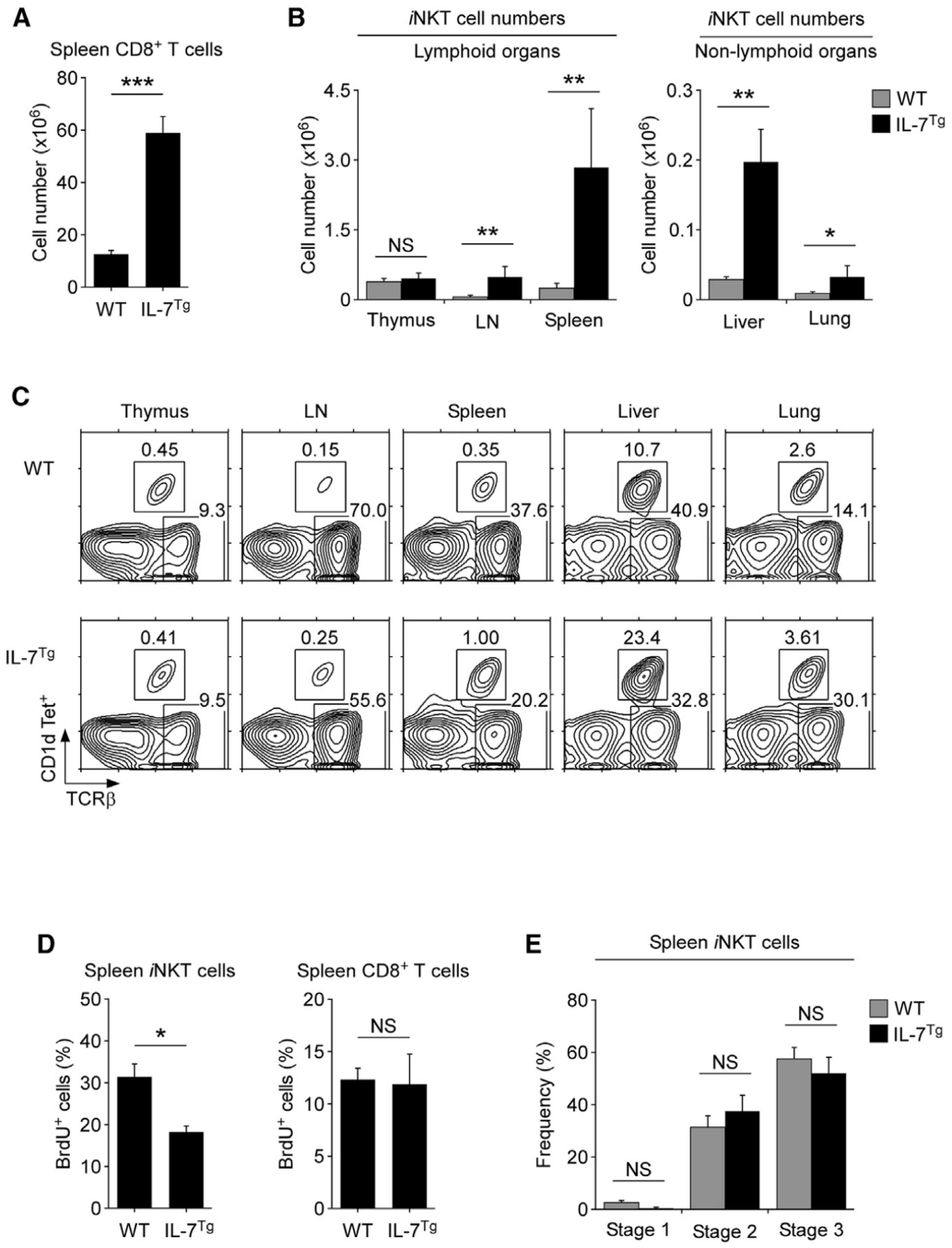

Figure 5. IL-7 overexpression expands size of the peripheral *i*NKT cell pool

(A) IL-7 overexpression results in increased CD8 T cell numbers in the spleen. Bar graph shows the summary of seven WT and five IL-7^{Tg} mice (mean ± SEM).

(B) *i*NKT cell numbers in lymphoid and non-lymphoid tissues of IL-7^{Tg} mice. Bar graphs show mean ± SEM from eight WT and six IL-7^{Tg} for lymphoid tissues and three WT and three IL-7^{Tg} mice for non-lymphoid tissues.

(C) *i*NKT cell frequencies in various tissues of IL-7^{Tg} mice. Data are representative of two independent experiments.

(D) BrdU labeling of *i*NKT cells and CD8 T cells in the spleen of WT and IL-7^{Tg} mice. BrdU incorporation was assessed after 6 days of BrdU treatment in drinking water. Bar graph shows the summary of three independent experiments with total six WT and four IL-7^{Tg} mice.

(E) *i*NKT cell stages in IL-7^{Tg} mice. Bar graph shows the frequency of each stages among spleen *i*NKT cells in IL-7^{Tg} and WT mice. Results show mean ± SEM from 12 WT and 10 IL-7^{Tg} mice.

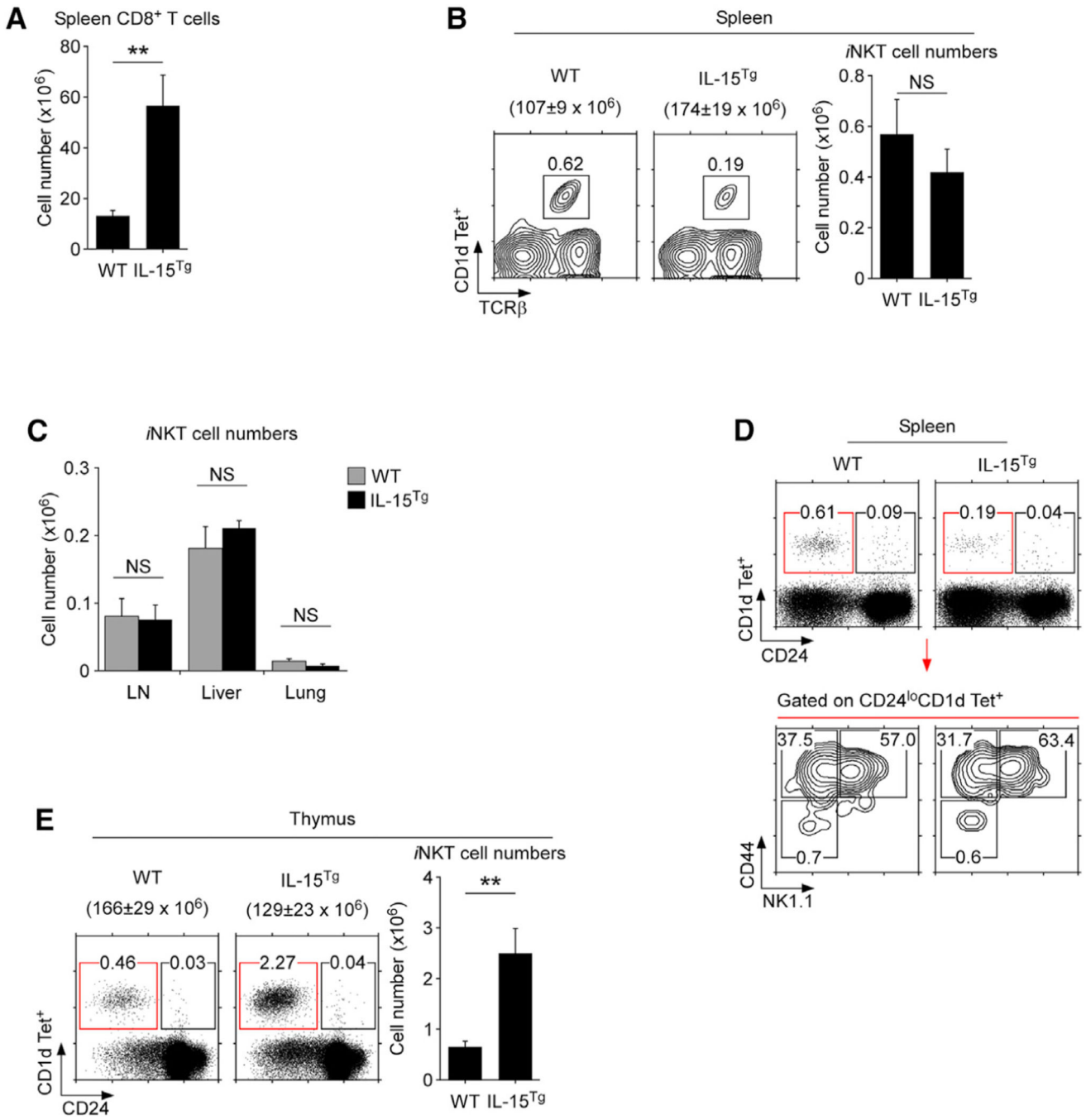

Figure 6. Peripheral *i*NKT cell numbers remain unaltered in IL-15^{Tg} mice

(A) IL-15 overexpression increases the number of CD8 T cells. Bar graph shows the summary of splenic CD8 T cell numbers from seven WT and five IL-15^{Tg} mice (mean ± SEM).

(B) *i*NKT cells in the spleens of WT and IL-15^{Tg} mice (left). Bar graph shows *i*NKT cell numbers (mean ± SEM) from eight WT and 10 IL-15^{Tg} mice from six independent experiments (right).

(C) *i*NKT cell numbers in the indicated tissues of IL-15^{Tg} mice. Bar graph shows *i*NKT cell numbers (mean ± SEM) from six WT and four IL-15^{Tg} mice.
(D) *i*NKT cell differentiation in the spleens of WT and IL-15^{Tg} mice. Immature and mature *i*NKT cells were identified by CD24 versus CD1d tetramer staining in splenocytes of WT and IL-15^{Tg} mice. *i*NKT stage 1–3 were determined on gated mature (CD24^{lo}CD1d Tet^{+}) splenocytes (bottom). Data are representative of five independent experiments.
(E) Thymic *i*NKT cell development in IL-15^{Tg} mice. Dot plots show CD24 versus CD1d tetramer staining of total thymocytes in WT and IL-15^{Tg} mice (left). Bar graph shows thymic *i*NKT cell numbers (mean ± SEM) from six independent experiments with a total of six WT and eight IL-15^{Tg} mice (right).

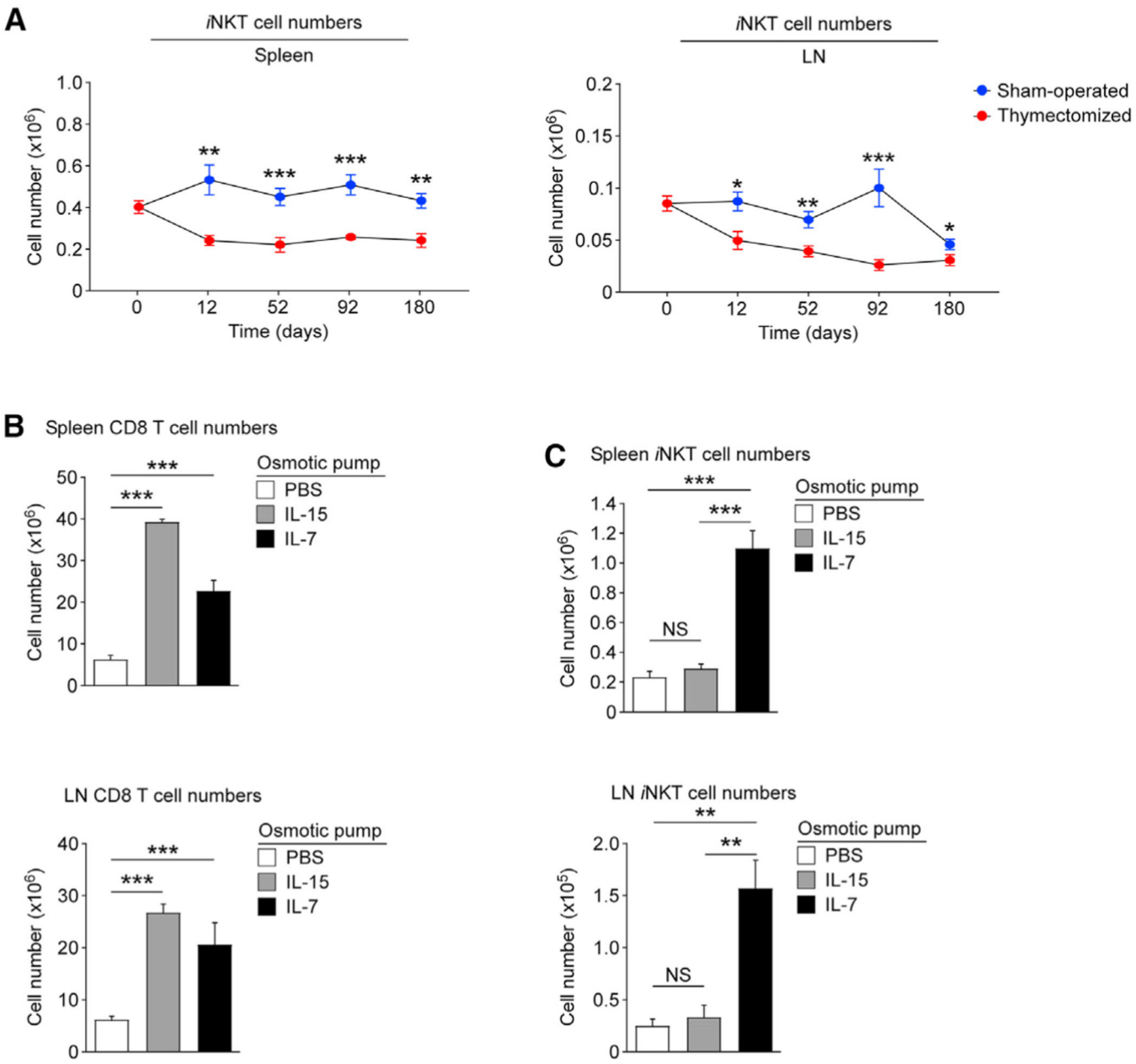

Figure 7. Increased IL-7 availability expands the size of the peripheral *i*NKT cell pool

(A) *i*NKT cell numbers were assessed in the spleens (left) and LNs (right) of adult thymectomized mice at the indicated days after surgery. Each data point shows the mean ± SEM of *i*NKT cell numbers for either sham-operated or thymectomized mice. For sham-operated mice: day 12, eight mice; day 52, 14 mice; day 92, eight mice; day 180, 10 mice. For thymectomized mice: day 0, 11 mice; day 12, eight mice; day 52, 16 mice; day 92, 12 mice; day 180, 10 mice. Mice were analyzed in three independent experiments.

(B) Splenic (top) and LN (bottom) CD8 T cell numbers in sham-operated or adult thymectomized mice where recombinant IL-15 or IL-7 was infused for 2 weeks using Alzet osmotic pumps. Bar graph shows the summary of four independent experiments with

seven IL-15 pump-installed mice, seven IL-7 pump-installed mice, and five PBS control pump-installed mice.

(C) Splenic (top) and LN (bottom) *i*NKT cell numbers in sham-operated or adult thymectomized mice where recombinant IL-15 or IL-7 was infused for 2 weeks using Alzet osmotic pumps. Bar graph shows the summary of four independent experiments with seven IL-15 pump-installed mice, seven IL-7 pump-installed mice, and five PBS control pump-installed mice.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC anti-TCRβ (H57–597)	eBioscience Thermo Fisher	Cat# 11–5961-82, RRID:AB_465322
PE-Cy7 anti-TCRβ (H57–597)	eBioscience Thermo Fisher	Cat# 25–5961-82, RRID:AB_2573506
PE-Cy7 anti-CD24 (30-F1)	eBioscience Thermo Fisher	Cat# 12–0242-82, RID:AB_10853806
PE anti-IL-7Rα (A7R34)	eBioscience Thermo Fisher	Cat# 12–1271-82, RRID:AB_465844
eFluor660 anti-IL-7Rα (A7R34)	eBioscience Thermo Fisher	Cat# 50–1271-82, RID:AB_11219081
PE anti-NK1.1 (PK136)	eBioscience Thermo Fisher	Cat# 12–5941-82, RRID:AB_466050
PE anti-IL-2Rα (CD25; PC61.5)	eBioscience Thermo Fisher	Cat# 12–0251-82, RRID:AB_465607
PE anti-IL-2Rβ (CD122; TM-b1)	eBioscience Thermo Fisher	Cat# 12–1222-82, RRID:AB_465836
eFluor 660 anti-T-bet (eBio4B10)	eBioscience Thermo Fisher	Cat# 50–5825-82, RID:AB_10596655
PE anti-IL-4Rα (CD124; mIL4R-M1)	BD Biosciences	Cat# 552509, RRID:AB_394407
APC anti-CD44 (IM7)	BD Biosciences	Cat# 559250, RRID:AB_398661
PE anti-γc (CD132; 4G3)	BD Biosciences	Cat# 554457, RRID:AB_395404
PE-Cy7 anti-CD4 (GK1.5)	BD Biosciences	Cat# 563933, RRID:AB_2738492
PE-Cy7 anti-CD4 (RM4.5)	BD Biosciences	Cat# 558107, RRID:AB_397030
Alexa Fluor 647 anti-CD8α (53–6-7)	BD Biosciences	Cat# 557682, RRID:AB_396792
BV786 anti-RORγt (Q31–378)	BD Biosciences	Cat# 564723, RRID:AB_2738916
PE anti-IL-21R (4A9)	BioLegend	Cat# 131906, RRID:AB_1279430
Pacific Blue anti-CD45 (30-F11)	BioLegend	Cat# 103126, RRID:AB_493535
PE anti-PLZF (9E12)	BioLegend	Cat# 145804, RRID:AB_2561973
CD1d tetramers (PBS-57 loaded)	NIH tetramer facility	Emory University, Atlanta, Georgia
Chemicals, peptides, and recombinant proteins		
BrdU (5-bromo-2’-deoxyuridine)	Sigma-Aldrich	Cat# B5002
QuantiTect SYBR® Green PCR Kits	Qiagen	Cat# 204145
RNeasy Plus Micro kit	Qiagen	Cat# 74034
QuantiTect reverse transcription kit	Qiagen	Cat# 205313
Collagenase IV	Thermo Fisher	Cat# 17104019

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Percoll	GE Life Sciences	Cat# 17089101
Recombinant mouse IL-7	R&D system	Cat# 407-ML-025/CF
Recombinant mouse IL-15	R&D system	Cat# 447-ML-010/CF
Critical commercial assays		
eBioscience Fixation/Perm diluents	eBioscience Thermo Fisher	Cat# 00–5223-56
eBioscience Fixation/Perm concentrate	eBioscience Thermo Fisher	Cat# 00–5213-43
Permeabilization Buffer 10x	eBioscience Thermo Fisher	Cat# 00–8333-56
CaspGLOW™ fluorescein active caspase-3 staining kit	eBioscience Thermo Fisher	Cat# 88–7004-42
Experimental models: Organisms/strains		
Mouse: C57BL/6 (C57BL/6NCrl)	Charles River Laboratories	Stock# 24107773, 24107757
Mouse: γc-deficient (*Il2rg*$^{-/-}$)	The Jackson Laboratory	(DiSanto et al., 1995) Stock #003174
Mouse: IL-15-deficient (*Il15*$^{-/-}$)	In house	(Kennedy et al., 2000)
Mouse: IL-15Rα-deficient (*Il15ra*$^{-/-}$)	The Jackson Laboratory	(Lodolce et al., 1998) Stock #003723
Mouse: IL-7-deficient (*Il7*$^{-/-}$)	In house	(von Freeden-Jeffry et al., 1995)
Mouse: K7	In house	(Kim et al., 2012)
Mouse: IL7Rα floxed (*Il7Ra*$^{fl/fl}$)	Provided by Dr. A. Singer	(McCaughtry et al., 2012)
Mouse: T-bet-ZsGreen reporter (TBGR^{Tg})	Provided by Dr. J. Zhu	(Zhu et al., 2012)
Mouse: T-bet^{cre}	The Jackson Laboratory	(Haddad et al., 2013) Stock #024507
Mouse: IL-7RαY449 knock-in (Y449F)	In house	(Osborne et al., 2007)
Mouse: Constitutively active STAT5b transgene (CA5^{Tg})	Provided by Dr. M. Farrar	(Burchill et al., 2003)
Mouse: IL-7-transgenic (IL-7^{Tg}) mice	The Jackson Laboratory	(Mertsching et al., 1995) Stock# 008334
Mouse: IL-15 transgenic (IL-15^{Tg})	In house	(Marks-Konczalik et al., 2000)
Mouse: Thymectomized adult mice 4 weeks (C57BL/6)	The Jackson Laboratory	Stock# 000664
Oligonucleotides		
qRT-PCR *Il7* Forward: 5'-CTGATG ATCAGCATCGATGAATTGG-3'	Integrated DNA Technologies	This paper

REAGENT or RESOURCE	SOURCE	IDENTIFIER
qRT-PCR *Il7* Reverse: 5'-GCAGC ACGATTTAGAAAAGCAGCT T-3'	Integrated DNA Technologies	This paper
qRT-PCR *Bcl2* Forward: 5'-GGATA ACGGAGGCTGGGATGCCT-3'	Integrated DNA Technologies	This paper
qRT-PCR *Bcl2* Reverse: 5'-CAGAG TGATGCAGGCCCCGAC-3'	Integrated DNA Technologies	This paper
qRT-PCR *actinb* Forward: 5'-GAGA GGGAAATCGTGCGTGA-3'	Integrated DNA Technologies	This paper
qRT-PCR *actinb* Reverse: 5'-ACATC TGCTGGAAGGTGG-3'	Integrated DNA Technologies	This paper
qRT-PCR *Hprt* Forward: 5'-TCATT ATGCCGAGGATTTGGA-3'	Integrated DNA Technologies	This paper
qRT-PCR *Hprt* Reverse: 5'-CAGAG GGCCACAATGTGATG-3'	Integrated DNA Technologies	This paper
Software and algorithms		
GraphPad Prism 7	GraphPad software	https://www.graphpad.com
FlowJo software version 10.2	FlowJo	https://www.flowjo.com
Flow cytometry analysis software. Active Control 4.2.0.7	Division of Computer Research and Technology, NIH	This paper
Other		
Canvas X	Canvas GFX	https://www.canvasgfx.com
BD FACS LSRII	BD Biosciences	https://www.bdbiosciences.com
BD FACS LSRFortessa	BD Biosciences	https://www.bdbiosciences.com
ALZET® Osmotic Pumps	DURECT	Cat# Model 2002