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IL-15 Regulates Homeostasis and Terminal Maturation of NKT Cells

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Semi-invariant NKT cells are thymus-derived innate-like lymphocytes that modulate microbial and tumor immunity as well as autoimmune diseases. These immunoregulatory properties of NKT cells are acquired during their development. Much has been learned regarding the molecular and cellular cues that promote NKT cell development, yet how these cells are maintained in the thymus and the periphery and how they acquire functional competence are incompletely understood. We found that IL-15 induced several Bcl-2 family survival factors in thymic and splenic NKT cells in vitro. Yet, IL-15–mediated thymic and peripheral NKT cell survival critically depended on Bcl-xL, and effector differentiation, which is consistent with a role of T-bet in regulating terminal maturation.

During differentiation, NKT cells acquire the ability to produce IL-4 (ST1), IL-4 and IFN-γ (ST1 and ST2), or IL-4 and IFN-γ (ST3) in response to TCR stimulation (27, 41), and secretion of these cytokines is regulated by CSF-2 (31). At ST2 and ST3, NKT cells also acquire memory T cell markers (CD44^hi, CD62L^lo) as well as NK cell markers (NK2G2, Ly49, and NK1.1) and cytolytic function (granzyme B and perforin) (1, 42). Although a substantial fraction of ST2 cells remains in the thymus and matures to ST3, a small fraction egresses from the thymus and immigrates into the spleen and liver, whereupon further maturation occurs (41, 43, 44). Migration to these peripheral sites depends on CXCR6 (45), whereas continued development and maintenance in the liver require LFA-1 (46) and Id2 (40). Even though mature thymic and splenic NKT cells are phenotypically similar, they behave as distinct functional populations at these sites because the developmental cues and perhaps the agonistic ligands are distinct at different sites (44, 47, 48). Thus, unlike conventional T lymphocytes, which undergo complete ontogenetic maturation in the thymus but commit to effector differentiation only upon Ag recognition in the periphery, NKT cells complete both these processes within and outside the thymus. The instructive signals that differentially regulate the maturation and acquisition of effector functions of NKT cells remain poorly understood.

IL-15 is a pleiotropic cytokine that regulates the development and maintenance of several subsets of innate-like lymphocytes, including γδ T, CD8αα T, NK, and NKT cells (49). Furthermore, IL-15 signals the ontogeny, effector differentiation, and Mcl-1–dependent survival of NK cells (50). Moreover, thymic and peripheral NKT cells develop poorly in IL-15–deficient (IL-15^−/−) mice (35, 36). In the periphery, this is due to impaired NKT cell homeostatic proliferation (36). In the thymus, NKT cell proliferation appears intact, yet IL-15^−/− mice develop a severe blockade in NKT cell ontogeny at ST2, with leaky progression to ST3 (36). What role IL-15 signals play during thymic NKT cell ontogeny and whether IL-15 is essential for NK cell functional maturation in the periphery remain unknown.

In this study, we provide evidence that thymic and peripheral NKT cell survival critically depends on IL-15. One important downstream target of IL-15 appears to be Bcl-xL. Furthermore, we describe a new function for IL-15 by which it regulates ST2 to ST3 lineage progression and terminal NKT cell maturation, which is consistent with a role of T-bet in regulating ST2 to ST3 transition and terminal differentiation.

Materials and Methods

Mice

B6–IL-15^−/−, B6.129–IL-7α^−/−, B6.Jax^−/−, and B6.129–CD1d^TDT mice (34, 35, 51–53) were generous gifts from J. Peschon (Immunex), M. Taniguchi (RIKEN), and A. Bendelac (University of Chicago), respectively. B6–IL-15^−/− and B6.129-IL-7α^−/− mice were bred with B6-Eμ–Tα-Bcl-xL^−/− (55) mice. B6-C–IL-15^−/− Bcl-xL^−/− mice were backcrossed to B6–IL-15^−/− for six generations. All mouse crosses and experiments were performed under approval by Institutional Animal Care and Use Committee protocols.

Abs and reagents

All Abs used for the identification of NKT cells were from BD Pharmingen as described elsewhere. Anti-Brdu–Alexa 478 (PRB-1; Invitrogen); anti-human Bcl-xL–PE and IgG3 isotype control–PE (7B25 and B10; Southern Biotechnology Associates); anti-mouse Bcl-2–PE (3F11) and Armenian hamster IgG1K isotype control–PER (A19-3), anti-human Bcl-2–PE (6C8) and Armenian hamster IgG2A isotype control–PE (Ha4/8), anti–Mcl-1 rabbit polyclonal (Rockland); chicken anti–rabbit–Alexa 647 (Invitrogen); anti–T-bet–Alexa 478 (eBio4B10) and mouse IgG1 isotype control–Alexa 647 (P3; eBioscience); anti–Gata–3–Alexa 647 (L50-823) and mouse IgG1K isotype control–Alexa 647 (MOPC-21; Santa Cruz Biotechnology); and mouse IgG1 isotype control and anti-mouse IgG-FITC (eBioscience) Abs were purchased. α-galactosylceramide (αGalCer; KRN7000) was generously provided by Kirin Brewery Company (Gunma, Japan) or purchased from Funakoshi (Tokyo, Japan). Mouse αGalCer-loaded CD1d monomers were obtained from the National Institutes of Health Tetramer Facility (Emory University). Preparation of αGalCer-loaded CD1d tetramers from monomer and their use are described elsewhere (56).

Flow cytometry

Abs and staining procedures for the identification of NKT cells were as described elsewhere (56). NKT cells were identified as CD3ε^−/−tetramer^+ cells among B220^−/− spleenocytes and hepatic mononuclear cells or CD8^−/− thymocytes. Four-color flow cytometry was performed with a FACSCalibur instrument (BD Biosciences), whereas seven-color flow cytometry was performed with an LSR II instrument (BD Biosciences). Data were analyzed with FlowJo software (Tree Star). Absolute NKT cell numbers were calculated from the percentage of tetramer^+ cells and total number of cells recovered from each organ.

Ex vivo stimulation of thymocytes and splenocytes with IL-15

Thymocytes and splenocytes were stimulated in vitro with 100 ng/ml recombinant human (rh)IL-15 (Milenyi Biotec) for 5 d. Cells were then collected and stained with specific mAb for intracellular expression of Bcl-2, Bcl-xL, and Mcl-1.

Cytokine responses

Individual age-matched (6–10-wk-old) mice were injected i.p. with 5 μg of αGalCer or vehicle (0.1% Tween-20 in PBS) as a control. After 24 h, splenocytes, and hepatic mononuclear cells were prepared. Cells were stained with anti-CD3ε^+ mAb, tetramer, and anti–IFN-γ or –IL-4^+ and analyzed by flow cytometry. Sandwich ELISA was performed as described (56) to monitor serum cytokine response.

Cells were fixed and permeabilized with Cytofix/Cytoperm solution for intracellular staining or with Cytofix/Cytoperperm Plus (both from BD Pharmingen) for intracellular BrdU staining according to the manufacturer’s protocol. Staining for intracellular IFN-γ and IL-4 also used GolgiStop (BD Pharmingen). Staining for intracellular T-bet and Gata-3 used the Foxp3 staining buffer kit (eBioscience). Intracellular and intranuclear staining was performed according to the manufacturer’s protocol.

Thymocyte sorting

Thymic NKT cells were enriched by magnetic sorting using a Pan-T cell isolation kit in combination with CD8 beads (Milenyi Biotec) as instructed by the manufacturer. Enriched NKT cells from 5–10 mice were pooled and further purified by flow sorting CD3ε^−/−tetramer^+ cells (FACSARia; BD Biosciences). CD4 and CD8 DP thymocytes were flow sorted as above. Freshly purified NKT cells and DP thymocytes were >98% pure as judged by CD3ε^−/−tetramer^+ and CD4/CD8-specific staining (data not shown).

RNA preparation, microarray hybridization, and analyses

Freshly purified NKT cells were washed and lysed by passage through a Qiasheeder column (Qiagen). Total RNA was extracted per the manufacturer’s instructions (RNeasy; Qiagen). RNA yield was quantified spectrophotometrically (Nanodrop ND-1000; Nanodrop) and aliquots were used to determine the purity and quality (Agilent 2100 Bioanalyzer; Agilent Technologies). Microarray hybridizations were performed using one-color hybridization on a SurePrint G3 Mouse GE 60K Microarray (Agilent Technologies) by the Functional Genomics and Shared Resources Core (Vanderbilt University). Scanning was performed using a GenePix 4000B Microarray Scanner (Molecular Devices). The resulting images were processed with Agilent Feature Extraction software (Agilent Technologies) to generate raw probe intensity data, which was subsequently normalized by the quantile method (57). Differential expression analysis was carried out with an empirical Bayes approach on a linear model using the limma package (58, 59). In this study, we defined differentially expressed genes as those for which the log2 fold change values were >±1.5 or <±1.5 and for which normal p values were <0.001 (60). Next, we performed hierarchical clustering of the differentially expressed genes using the R AMAP Package (http://cran.r-project.org/web/packages/amap/). The microarray data are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dxgpxkoosiokmpm&acc=) under accession number GSE32568.
Real-time quantitative PCR

RNA was reverse transcribed with MuLV RT Reverse Transcriptase (Applied Biosystems) according to the manufacturer’s protocol. Quantitative PCR (qPCR) was performed in the presence of IQ RealTime Sybr Green PCR Supermix and target gene-specific primers (Supplemental Table I) in an iQ5 thermocycler (Bio-Rad). Results were analyzed using iQ5 software (Bio-Rad). Data were first normalized against β-actin control. As the threshold cycle (CT) values are obtained as log 2 values, the normalized ΔCT values of wild-type (wt) samples were subtracted from normalized ΔCT values of IL-15<sup>0</sup> samples. The resulting log<sub>2</sub> ΔΔCT values were transformed yielding relative expression, which enabled comparison between NKT cells isolated from the two strains. For this, the IL-15<sup>0</sup> sample, called the Calibrator, was set to 1 and relative expression calculated as fold change in gene expression in wt NKT cells relative to the Calibrator.

TCRα rearrangement

cDNA was prepared from total RNA isolated from DP thymocytes. qPCR for Vα<sub>14</sub> to Jα<sub>18</sub> rearrangement was performed as described above using the following forward (F) and reverse (R) primers: Vα<sub>14</sub>F (5'-AGGTCTTGTGTCCTGTCAGC-3'), Jα<sub>18</sub>R (5'-CAGGTATGCAACTCGTGATCT-3'), Vα<sub>8</sub>F (5'-TCACAGCAACAGAGGACC-3'), and Jα<sub>5</sub>R (5'-AGTGAGCTGCCCCACAACCT-3'). PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistics

Comparisons of normally distributed continuous data were performed by one-way ANOVA with Tukey’s posttest to determine significance. The significance of gene expression analyzed by qPCR was determined by unpaired t test. Statistical significance of Vα<sub>14</sub>-Jα<sub>18</sub> rearrangement by FIGURE 1.

Defective NKT cell development and maintenance in IL-15<sup>0</sup> mice. A, Thymic, splenic, and hepatic NKT cells from B6 (n = 13) and B6–IL-15<sup>0</sup> (n = 9) mice were identified as CD3ε<sup>+</sup>tetramer<sup>+</sup> cells within electronically gated CD8<sup>lo</sup> thymic, B220<sup>lo</sup> splenic, or liver mononuclear cells. Numbers are percent NKT cells among total leukocytes within each organ. Data are representative of nine independent experiments. B, B6 and B6–IL-15<sup>0</sup> mice were injected i.p. with 2 mg BrdU daily for 3 consecutive d and sacrificed 1 d later. BrdU incorporation was determined by flow cytometry after extracellular lineage-specific staining and intracellular labeling with anti-BrdU Alexa 647 mAb. Overlaid histograms are of NKT cells identified as in A from vehicle (shaded) or BrdU injected (open) mice. The histograms show the expression levels of BrdU within NK1.1<sup>−</sup> (left panels) and NK1.1<sup>+</sup> (right panels) NKT cells within the thymus and spleen. Data are representative of three independent experiments. n = 6. C and D, Thymic and splenic mononuclear cells from B6 and B6–IL-15<sup>0</sup> mice were assessed for intracellular expression of Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1 detected with specific mAb within electronically gated B220<sup>lo</sup>CD3ε<sup>+</sup>tetramer<sup>+</sup> cells. Numbers refer to the difference in mean fluorescence intensity (ΔMFI) between isotype control (shaded) and specific mAb (open) staining. Data are representative of three independent experiments. n = 5. The flow data were acquired on two different instruments (FACS-Calibur and LSR-II; BD Biosciences) in different experiments. Even though the actual MFI varied, the represented trend remained the same nonetheless. Therefore, we have chosen to represent the ratio of expression between wt and IL-15<sup>0</sup> NKT cells. The ratio of Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1 expression by IL-15<sup>0</sup> (KO) and wt NKT cells is shown. A ratio of ~1 indicates no difference in expression; a ratio <1 indicates lower expression in IL-15<sup>0</sup> NKT cells.
qPCR was calculated by two-tailed, unpaired t test. Comparisons were performed using Prism software (GraphPad).

**Results**

*IL-15 induces Bcl-2 family survival factors within thymic but not peripheral NKT cells*

Previous reports demonstrated that NKT cell frequency is significantly lower in mice lacking IL-15 compared with wt mice due to altered NKT cell homeostasis within the thymus and spleen (35, 36). Although IL-15 regulates proliferation of splenic NKT cells, its role in homeostasis of the thymic subset has remained unclear (36). A re-evaluation confirmed that NKT cell frequency was altered in the absence of IL-15, and, hence, their numbers were reduced in the thymus, spleen, and liver (Fig. 1A). Likewise, in vivo BrdU incorporation experiments revealed that splenic but not thymic NKT cell proliferation was impaired in IL-15<sup>−/−</sup> mice (Fig. 1B). These data indicate that, in addition to its role in cell proliferation, IL-15 plays another role(s) during thymic NKT cell ontogeny.

Previous studies have suggested that IL-15 induces antiapoptotic Bcl-2 family members, including Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1, to prevent apoptosis and regulate homeostasis in vivo (49). For example, IL-15 promotes the survival of NK cells by inducing the survival factor Mcl-1 (50). An examination of the expression of the Bcl-2 family of survival factors by flow cytometry revealed that intracellular Bcl-2 expression was dramatically reduced in IL-15<sup>−/−</sup> thymic and splenic NKT cells (Fig. 1C). Although not as dramatic, intracellular Bcl-x<sub>L</sub> and Mcl-1 expression levels were also reduced either in thymic (Bcl-x<sub>L</sub>) or in both thymic and splenic (Mcl-1) IL-15<sup>−/−</sup> NKT cells when compared with wt NKT cells (Fig. 1C, 1D).

To test whether IL-15 induces survival factors in NKT cells, wt thymocytes and splenocytes were cultivated in the absence or presence of exogenous rhIL-15 for 5 d. Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1 expression in fresh or cultivated NKT cells (with or without rhIL-15) was evaluated using flow cytometry. Both fresh NKT cells and those cultivated without rhIL-15 were included as controls because expression of these survival factors changes during the 5-d culture. The data revealed that IL-15 upregulated the expression of Bcl-2 and Bcl-x<sub>L</sub> but not Mcl-1 in thymic NKT cells to levels above those expressed by freshly isolated NKT cells (Fig. 2A, 2B). Very few NKT cells cultivated without IL-15 expressed the three survival factors, and, hence, when compared with this control, IL-15 also appeared to induce Mcl-1 expression in thymic NKT cells (Fig. 2B). IL-15 also modestly induced Bcl-2 and Bcl-x<sub>L</sub> expression within splenic NKT cells but not to the levels found in freshly isolated NKT cells; their expression was above control NKT cells cultured in medium without rhIL-15 (Fig. 2A). These data indicate that IL-15 has the potential to induce or maintain the expression of survival factors of the Bcl-2 family in thymic or splenic NKT cells, respectively, and, hence, to support NKT cell survival during development.

*Bcl-x<sub>L</sub> overexpression in IL-15<sup>−/−</sup> mice supports NKT cell survival*

We took a genetic approach to further investigate the role of IL-15–induced genes in the survival of thymic and peripheral NKT cells.

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**FIGURE 2.** IL-15 upregulates expression of the survival factors Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1 within thymic and splenic NKT cells. Thymic and splenic mononuclear cells from B6 mice were cultivated in vitro in the absence or presence of 100 ng/ml of rhIL-15. After 5 d, intracellular expression of Bcl-2, Bcl-x<sub>L</sub> (A), and Mcl-1 (B) was detected with specific mAb within electronically gated B220<sup>−/−</sup>CD3ε<sup>−/−</sup>‘tetramer’ cells. Numbers refer to the ΔMFI between isotype control (shaded) and specific mAb (open). Data are representative of three independent experiments. n = 5. As for Fig. 1, the flow data were acquired on two different instruments in different experiments. Hence, the ratio of IL-15–induced Bcl-2, Bcl-x<sub>L</sub>, and Mcl-1 expression within NKT cells in comparison with unstimulated, freshly isolated NKT cells is shown. A ratio of <1 indicates no induction; a ratio >1 indicates IL-15–induced expression.
Bcl-xL overexpression restores NKT cell development in IL-15<sup>−/−</sup> mice. A, Thymic, splenic, and hepatic NKT cells from B6 (<i>n</i> = 8), B6–IL-15<sup>0</sup> (<i>n</i> = 10), B6–IL-15<sup>0</sup>;Bcl-2<sup>tg</sup> (<i>n</i> = 6), and B6–IL-15<sup>0</sup>;Bcl-x<sub>L</sub><sup>tg</sup> (<i>n</i> = 16) mice were identified as CD3<sup>ε</sup>°tetramer<sup>+</sup> cells within electronically gated CD8<sup>LO</sup> thymic, B220<sup>LO</sup> splenic, or liver mononuclear cells. Numbers are percent NKT cells among total leukocytes.

B, Absolute numbers of NKT cells in the thymus and spleen of B6, B6–IL-15<sup>0</sup>, B6–IL-15<sup>0</sup>;Bcl-2<sup>tg</sup>, and B6–IL-15<sup>0</sup>;Bcl-x<sub>L</sub><sup>tg</sup> mice were calculated from percent NKT cells in A and total cell count. Data are representative of six independent experiments showing mean ± SEM; <i>n</i>, as in A. The <i>p</i> value was calculated by one-way ANOVA with Tukey’s posttest.

C, Intracellular expression of the hBcl-x<sub>L</sub> and hBcl-2 transgene within double-negative, DP, CD4<sup>+</sup>CD8<sup>−</sup>, and CD8<sup>+</sup>CD4<sup>−</sup> thymocytes of B6.C-Bcl-x<sub>L</sub><sup>tg</sup> (right panels) and B6.Bcl-2<sup>tg</sup> (left panels) mice. Overlaid histograms represent isotype control (shaded) and specific mAb (open). Data are representative of three independent experiments. <i>n</i> = 5.

D, Thymic and splenic NKT cells from IL-7Rα<sup>+/+</sup> (<i>n</i> = 3), IL-7Rα<sup>−/−</sup> (<i>n</i> = 3), IL-7Rα<sup>−/−</sup>;Bcl-2<sup>tg</sup> (<i>n</i> = 3), and IL-7Rα<sup>−/−</sup>;Bcl-x<sub>L</sub><sup>tg</sup> (<i>n</i> = 2) mice were identified as CD3<sup>ε</sup>°tetramer<sup>+</sup> cells within electronically gated CD8<sup>LO</sup> thymocytes or B220<sup>LO</sup> splenocytes and liver mononuclear cells. Numbers are percent NKT cells among total leukocytes. Absolute NKT cell number (mean ± SEM) was calculated from percent NKT cells and total mononuclear cell number. Data are representative of two independent experiments. Note that because IL-7Rα<sup>−/−</sup> thymic size is small due to low cellularity, percent NKT cells appears artificially high despite extremely low NKT cell numbers. The <i>p</i> value was calculated by one-way ANOVA with Tukey’s posttest.

E, Intracellular expression of the hBcl-x<sub>L</sub> and hBcl-2 transgenes within NKT and NK cells of B6.C-Bcl-<sup>−</sup> (Figure legend continues)
Thus, Bcl-2 and Bcl-xL transgenes were independently introgressed into IL-15<sup>−/−</sup> mice. Analysis of the resulting mice at age 6–8 wk, when thymic and splenic cellularity is similar between the different strains, revealed that enforced Bcl-xL but not Bcl-2 overexpression rescued the frequency and absolute numbers of thymic, splenic, and hepatic NKT cells in IL-15<sup>−/−</sup> mice (Fig. 3A, 3B).

Because the Bcl2 (Ig<sup>α</sup>) and Bclxl (pLck) transgenes are under the control of different promoters (54, 55), we assessed the expression patterns of both transgenes within different thymocyte subsets. The data revealed that both transgenes were expressed as early as the CD4<sup>+</sup>CD8<sup>−</sup> double-negative stage of development, through the DP stage, and within CD4<sup>+</sup> and CD8<sup>+</sup> single-positive thymocytes (Fig. 3C). Consistent with Bcl-2 expression during early thymocyte development, Bcl-2 transgene introgression into IL-7R<sup>α</sup> null mice, which poorly if at all develop conventional T and NKT cells (34, 61), rescued the development of both lineages (Fig. 3D). Therefore, the failure of Bcl-2 overexpression to restore NKT cell numbers in IL-15<sup>−/−</sup> mice was not due to the lack of proper transgene expression or functionality, but most likely due to the fact that Bcl-2 plays a minor role when compared with Bcl-xL in conveying IL-15 signals for NKT cell survival. Together, the above data indicate that IL-15 relays specific, Bcl-xL–dependent survival signals during NKT cell ontogeny.

A previous study reported that IL-15–induced Mcl-1 specifically supported NK cell survival in vivo and in vitro (50) and that overexpression of Bcl-2 in mice lacking IL-2Rβ, the common β-chain of IL-2R and IL-15R, or in IL-15<sup>−/−</sup> mice repopulates NK cells in vivo (62, 63). Because NKT and NK cells are closely related innate-like lymphocyte lineages and because both lineages use IL-15 for survival, we ascertained whether IL-15 induces survival of the two cell types by similar mechanism(s). This question was addressed because Bcl-2 and Bcl-xL transgenes were expressed in NK cells from B6–Bcl-2<sup>tg</sup> and C–Bcl-xL<sup>tg</sup> mice (Fig. 3E). We found that, as previously reported (62, 63), Bcl-2 partially rescued hepatic but not splenic NK cells. Nonetheless, Bcl-xL overexpression rescued NK but not hepatic or splenic NK cells in IL-15<sup>−/−</sup> mice (Fig. 3F), which is consistent with the reported model in which NK cells predominantly use Mcl-1 for IL-15–induced survival (50). Thus, IL-15 appears to regulate survival of distinct lineages in a unique, cell type-specific manner, with Bcl-xL serving as an IL-15–induced survival factor for NKT cells.

Because Bcl-xL is required for DP thymocyte survival and Va14 to Ja18 rearrangement (4), it is possible that IL-15<sup>−/−</sup> DP thymocytes poorly rearrange these distal gene segments, thereby resulting in low NKT cell numbers. To test this, Va14 to Ja18 rearrangement within DP thymocytes flow sorted from wt, IL-15<sup>−/−</sup>, Ja18<sup>−/−</sup>, and Cd1d<sup>−/−</sup> mice, which rearrange Va14 to Ja18 gene segments but are deficient in positive selection of NKT cells (52), was quantified. No defect in the canonical Va14 to Ja18 rearrangement was seen in IL-15<sup>−/−</sup> DP thymocytes (Fig. 4). Likewise, the more distal Va8 to Ja5 rearrangement was also unaffected in IL-15<sup>−/−</sup> DP thymocytes as gauged by nonquantitative PCR (Fig. 4B). Interestingly, however, a 1.5-fold higher Va14 to Ja18 rearrangement was observed in DP thymocytes of IL-15<sup>−/−</sup>Bcl-xL<sup>tg</sup> mice (Fig. 4A), which was statistically insignificant. This result suggests that any increase in the initial pool of NKT cell precursors in DP thymocytes of IL-15<sup>−/−</sup>Bcl-xL<sup>tg</sup> mice less likely led to increased numbers of NKT cells in these mice.

FIGURE 4. Normal TCRα rearrangement in IL-15<sup>−/−</sup> mice. A, Va14-to-Ja18 rearrangement within DP thymocytes flow sorted from the indicated strains was assessed by real-time qPCR and represented as mean ± SD of two independent experiments. β-actin was used as the internal control for normalization. The p value calculated by two-tailed, paired t test indicated that Va14-to-Ja18 rearrangement in B6, B6–IL-15<sup>−/−</sup>, and B6–IL-15<sup>−/−</sup>; Bcl-xL<sup>tg</sup> DP thymocytes was significant only when compared with that in B6-Ja18<sup>−/−</sup> DP cells. The ~1.5-fold increased Va14-to-Ja18 rearrangement seen in B6–IL-15<sup>−/−</sup>Bcl-xL<sup>tg</sup>DP thymocytes compared with the others was statistically insignificant (p < 0.0097). B, RT-PCR assessment of Va8-to-Ja5 and Va14-to-Ja18 rearrangements. Data are representative of two experiments.

IL-15 regulates terminal maturation of NKT cells

Because IL-15 deficiency reduced the numbers of thymic CD44<sup>+</sup> NKT1.1<sup>−</sup>st2 and CD44<sup>+</sup>NK1.1<sup>−</sup>st3 NKT cells, and because forced Bcl-xL overexpression in IL-15<sup>−/−</sup> mice rescued total thymic and peripheral NKT cell numbers (Fig. 3), whether IL-15 has additional roles in NKT cell ontogeny was ascertained. Therefore, we determined whether IL-15 regulates terminal maturation of developing NKT cells as well, particularly at st3, in which NKT cells acquire most of their NK cell-like effector functions. Thus, the phenotype of IL-15<sup>−/−</sup>;Bcl-xL<sup>tg</sup> NKT cells was compared with wt and IL-15<sup>−/−</sup> NKT cells. Surprisingly, akin to IL-15<sup>−/−</sup> NKT cells, phenotypic st2 to st3 transition, as measured by acquisition of the NK1.1 surface marker, was still partially impaired in IL-15<sup>−/−</sup>Bcl-xL<sup>tg</sup> mice (Fig. 5A, 5B), despite near wt numbers of NKT cells in these mice. Additional phenotypic analyses revealed that expression patterns of few other mature NKT cell markers, such as NKG2D, Ly6C, CD69, and Ly49C/I, reflected those of thymic NKT cells that were blocked at ST2 (Fig. 5C). As these markers are predominantly expressed by NK1.1<sup>+</sup> NKT cells (Fig. 6A). Of those markers, the expression of Ly6C was also altered in hepatic but not splenic NKT cells in IL-15<sup>−/−</sup> and IL-15<sup>−/−</sup>Bcl-xL<sup>tg</sup> mice (Fig. 5C). Thus, IL-15 is required not only for lineage sur-

x<sub>1.1</sub> and B6-Bcl-2<sup>−/−</sup> mice. Overlaid histograms represent isotype control (shaded) and specific mAb (open) staining. Data are representative of two independent experiments; n, as in C. F. Splenic and hepatic NK and NKT cells from B6 (n = 6), B6–IL-15<sup>−/−</sup> (n = 7), B6–IL-15<sup>−/−</sup>Bcl-xL<sup>tg</sup> (n = 4), and B6–IL-15<sup>−/−</sup>Bcl-xL<sup>tg</sup> (n = 10) mice were identified as NK1.1<sup>−</sup> "tetramer<sup>SNO</sup>" and NK1.1<sup>−</sup> "tetramer" cells, respectively, within electronically gated B220<sup>−</sup> splenic or hepatic mononuclear cells. Data are representative of four independent experiments.
vival but also for terminal maturation of NKT cells, especially of the thymic subset. Furthermore, the differential requirement for IL-15 in terminal differentiation (e.g., acquisition of Ly6C, NK1.1) of some (thymic and/or hepatic) NKT cells and not all (splenic) NKT cells suggests that distinct signals instruct NKT cell maturation in distinct lymphoid organs.

**IL-15 regulates multiple gene expression changes during ST2 to ST3 NKT cell transition**

To define the mechanism by which IL-15 regulates terminal maturation of thymic NKT cells, microarray experiments were performed. For this, wt and IL-15<sup>−/−</sup> thymic NKT cells were flow sorted, and the derived RNA was subjected to hybridization to a mouse microarray chip containing 60,000 genes; hybridization was performed in duplicate (IL-15<sup>−/−</sup>) or triplicate (wt). Select genes differentially expressed (minimally log<sub>2</sub> fold change ≥1.5 or nominal p value < 0.001) in wt and IL-15<sup>−/−</sup> NKT cells (Fig. 6A) were then validated by real-time qPCR. We found that Tbx21 (T-bet) was upregulated in wt NKT cells compared with IL-15<sup>−/−</sup> NKT cells (Fig. 6A, 6B). This finding is consistent with the previously reported IL-15–induced Tbx21-encoded T-bet expression in NKT cells in vitro (20). Furthermore, multiple Tbx21-regulated genes, such as Ifng, Gzma (granzyme A), Gzmc, Hopx, and several NK cell receptor genes (KlrA [NKG2E], KlrBc [NK1.1], KlrC1 [NKG2A/B], and Krlk1 [NKG2D]) (20, 29), were upregulated in wt thymic NKT cells, which predominantly consist of st3 NKT cells (Fig. 6B). Consistent with the lack of intracellular Bcl-2 in IL-15<sup>−/−</sup> NKT cells (Fig. 1C), the Bcl2 gene was poorly expressed in these cells (Fig. 6B). Conversely, Zbtb16 (PLZF), Tcf7 (T-cell factor 1), and Rorc (retinoic acid-related orphan receptor γ) genes, all signatures of ST1 and ST2 NKT cells, were upregulated in IL-15<sup>−/−</sup> thymic NKT cells (Fig. 6C), consistent with developmental arrest at ST2. Because T-bet is a critical regulator of differentiation in other lineages with cytotoxic properties (CTL and NK cells), our results are consistent with a role of Tbx21 in regulating ST2 to ST3 transition.

**IL-15 regulates functional maturation of NKT cells**

Terminally mature NKT cells produce the highest amounts of effector cytokines. Because phenotypically this subset of NKT cells appears impaired in IL-15<sup>−/−</sup> and IL-15<sup>−/−</sup>Bcl-x<sub>L</sub>tg mice, we determined whether they were functionally competent. For this purpose, the potent NKT cell ligand αGalCer (64) was administered...
Compared to IL-15+/−2 mice, significantly fewer splenic and hepatic NKT cells from IL-15−/− and IL-15−/−;Bcl-xLtg mice expressed intracellular IL-4 and IFN-γ in response to in vivo stimulation (Fig. 7A, 7B). Furthermore, NKT cells that expressed IL-4 (splenic) and IFN-γ (splenic and hepatic) in IL-15−/− and IL-15−/−;Bcl-xL−/− mice expressed these cytokines at significantly lower levels compared with those in IL-15+/− mice (Fig. 7A, 7B).

Consistent with this expression pattern, the serum IFN-γ and IL-4 responses were also poor in IL-15−/− and IL-15−/−;Bcl-xL−/− mice compared with their IL-15+/− counterparts (Fig. 7C, 7D). Thus, IL-15 signaling is required for fully competent NKT cell responses in vivo.

IFN-γ and IL-4 responses by NKT cells require transcriptional regulation by T-bet and Gata-3 (17, 19). T-bet is also required for the terminal maturation of thymic NKT cells, as T-bet deficiency blocked NKT cell ontogeny at the st2 to st3 transition (20).

**FIGURE 6.** IL-15 induces Tbx21- and T-bet–regulated genes in developing NKT cells. A, Cluster analysis of all (left panel) or select genes (right panel) differentially expressed in B6 (n = 3) and IL-15−/− (n = 2) NKT cells from microarray experiments are shown. Differential expression was defined as those genes that showed log2 fold change ≥1.5 or ≤−1.5 with a nominal p value <0.001. B and C, Select differentially expressed genes identified in A were validated by qPCR using RNA isolated from flow-sorted B6 and B6–IL-15−/− thymic NKT cells. Genes upregulated in B6-derived thymic NKT cells (B) and genes upregulated in B6–IL-15−/−–derived NKT cells (C) are shown. β-actin was used to normalize expression levels. Data represent mean ± SD of two independent experiments; each qPCR was performed in duplicate for an n value of 4 to calculate the p value by unpaired t test.
similar to that seen with IL-15 deficiency (Fig. 5). Because Tbx21 expression is reduced in IL-150 NKT cells, and IL-15 induces Tbx21 expression in NK cells in vitro (20), we investigated whether impaired NKT cell function in IL-150 and IL-150;Bcl-xLtg mice was due to altered levels of T-bet and Gata-3 expression. For this, thymic and splenic NK1.1+ and NK1.1+ NKT cells from wt and IL-150 mice were probed for intracellular expression of T-bet and Gata-3. We found that the level of T-bet expression was reduced by 30% (splenic) to 50% (thymic) in IL-150 compared with wt NKT cells (Fig. 7E, 7G). Additionally, a slightly reduced percentage of thymic, but not splenic, NKT cells expressed T-bet in IL-150 mice. Similarly, intracellular Gata-3 expression was ∼15–20% lower in thymic and splenic NK1+ NKT cells but about similar in NK1+ NKT cells of IL-150 mice compared with their wt counterparts (Fig. 7F, 7G), consistent with somewhat more preserved IL-4 production in IL-150 NKT cells when compared with IFN-γ response. In wt NKT cells, Gata-3 expression level was constant, whereas T-bet levels increased with st3 differentiation (Fig. 7G). Hence, the reduced T-bet expression (Fig. 7E) in IL-150 mice is consistent with reduced st3 NKT cells in these mice. Thus, IL-15 signals are partially required for T-bet and Gata-3 expression in NKT cells and, possibly, for the robust IFN-γ and IL-4 cytokine responses seen in wt but not in IL-150 and IL-150; Bcl-xLtg mice.

Discussion

We describe three unique features of IL-15 that are essential for functional NKT cell development: 1) it induces survival of thymic and peripheral NKT cells by regulating Bcl-xL and Bcl-2 expression; 2) IL-15 regulates T-bet expression and signals st3 NKT cell induction and differentiation; and 3) it regulates effector differentiation that is consistent with the role of T-bet in this process. Thus, IL-15 plays a central role in NKT cell development and function.

It appears as though NKT cells narrowly escape death at each stage of their development: immediately after positive selection,
they turn on Nur77 expression in st0 NKT cells (65), perhaps owing to high-avidity interactions with their cognate ligand, namely, CD1d-self-lipid complexes. In T cells, the transcription factor Nur77, in addition to inducing an apoptotic program, when targeted to the mitochondrion binds to the otherwise antiapoptotic factor Bcl-2 and, by changing its conformation, converts it into a proapoptotic protein (66). Therefore, onward development of NKT cells perhaps requires survival signal(s) to protect the lineage from death. We have identified one such survival signal to be IL-15. It induces both Bcl-2 and Bcl-xL within thymic NKT cells in vitro. Yet, only genetic overexpression of Bcl-xL but not Bcl-2 confers survival potential to st1 and st2, but not st3, NKT cells. These data are consistent with a previous report showing that enforced Bcl-2 expression does not rescue NKT cell development in IL-15Rα–null mice (62). A recent report suggested that forced Bcl-2 overexpression or intromission of Bim deficiency modestly rescues NKT cell development in the IL-15–presenting IL-15Rα–null mice (38). The differences in survival mechanisms in these three animal models currently remain unknown.

The global gene expression analysis of wt and IL-15–deficient NKT cells, as well as our published data (14), indicate that st3 but not st0–2 NKT cells express high levels of Bcl-2. Hence, it is possible that Bcl-2 functions as an important survival factor only upon commitment and development into st3 NKT cells. Because the st3 NKT cells are absent in IL-15–, IL-15Rα–, and IL-15Rβ–null mice, another factor must be necessary to support NKT cell survival up to this stage. Our data suggest Bcl-xL provides that function. The failure of transgenic Bcl-2 to do so in this study perhaps reflects the need for an IL-15–induced, st3-specific accessory factor to execute its function.

Previous studies have demonstrated that thymic and peripheral NKT cells represent distinct functional subsets (44, 47, 48). The factor(s) that impacts differentiation of these two subsets remained incompletely defined. We found that most thymic NKT cells in IL-15–;Bcl-xL–mice were blocked at the st2-to-st3 ontogenic transition and lacked activation/memory marker expression, as were IL-15– NKT cells. In striking contrast, splenic and hepatic NKT cells in IL-15–;Bcl-xL–mice underwent almost complete phenotypic maturation, suggesting that developmental cues in the thymus and periphery are somewhat distinct. In contrast, gene expression analyses indicated reduced T-bet expression in both thymic and splenic NKT cells of IL-15– mice. T-bet was previously shown to regulate the st2-to-st3 NKT ontogenic transition (20, 29). Thus, reduced T-bet expression is consistent with the st2-to-st3 developmental block in IL-15– NKT cells. Furthermore, low levels of T-bet may in part explain the poor expression of NK cell receptors as well as the deficiency in st3–specific IFN-γ response in IL-15– mice. Taken together, we conclude that IL-15 differentially impacts central and peripheral NKT cell maturation in vivo.

It is noteworthy that T-bet belongs to a group of transcription factors whose functions are regulated not only by their presence or absence but also by absolute levels. An example of such regulation is seen in effector CD8 T cell fate determination via graded expression of T-bet (67). Differentiation into effector and memory CD8 T cell fates was dictated by the levels of T-bet, in which high T-bet expression induced an effector cell fate, whereas lower T-bet level directed cells into the memory pool. Such a model would in principle be consistent with a past report (38) and ours suggesting that partially reduced levels of T-bet expression in IL-15– mice could indeed significantly impair the NKT cell differentiation program. Finally, because T-bet regulates expression of IL-15/IL-2Rβ (CD122) on NKT cells (this report and 29) and therefore IL-15 responsiveness, the two factors (IL-15 and T-bet) generate a positive-feedback regulatory loop. The property of all such loops is that small changes in gene expression are amplified by self-sustained and self-amplified system oscillation. Therefore, we predict that the reduced but not the complete absence of T-bet observed in IL-15– NKT cells may be sufficient to prevent complete effector differentiation.

The importance of NKT cell function(s) is implicated in multiple infectious diseases. Because NKT cells acquire most of their NK cell-like properties at st3 of their differentiation, and because IL-15 seems to regulate acquisition of these properties, we speculate that IL-15–encoded NKT cell functions will be important in controlling in vivo responses to infections. This will be an exciting area for future investigations. In conclusion, IL-15 functions by supporting survival of developing NKT cells and perhaps by inducing Tbx21 expression as an st3-specific differentiation factor in NKT cells. NKT cell share this feature with NK cells and CD8 T cells and are expected to collaborate with these lineages in executing Tbx21–induced functions in vivo.

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Disclosures
The authors have no financial conflicts of interest.

References

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Supplementary Table 1. List of qPCR primer pairs used to validate gene expression data

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