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The Thymic Microenvironment Differentially Regulates Development and Trafficking of Invariant NKT Cell Sublineages

Michael B. Drennan,* Srinath Govindarajan,† Katelijne De Wilde,* Susan M. Schlenner,‡ Sergei Nedospasov,§ Hans-Reimer Rodewald,‖ and Dirk Elewaut*  

The regulatory role of the thymic microenvironment during trafficking and differentiation of the invariant NKT (iNKT) cell lineage remains poorly understood. In this study, we show that fractalkine receptor expression marks emigrating subpopulations of the NKT1, NKT2, and NKT17 sublineages in the thymus and peripheral organs of naïve mice. Moreover, NKT1 sublineage cells can be subdivided into two subsets, namely NKT1a and NKT1b, which exhibit distinct developmental and tissue-specific distribution profiles. More specifically, development and trafficking of the NKT1a subset are selectively dependent upon lymphotoxin (LT)α2β1-LTβ receptor–dependent differentiation of thymic stroma, whereas the NKT1b, NKT2, and NKT17 sublineages are not. Furthermore, we identify a potential cellular source for LTαβ2 during thymic organogenesis, marked by expression of IL-7Rα, which promotes differentiation of the NKT1a subset in a non-cell-autonomous manner. Collectively, we propose a mechanism by which thymic differentiation and retention of the NKT1 sublineage are developmentally coupled to LTαβ2-LTβ receptor–dependent thymic organogenesis.  * The Journal of Immunology, 2014, 193: 5960–5972.

Invariant NKT (iNKT) cells constitute an innate-like T cell sublineage that is selected in the thymus by cortical thymocytes expressing the MHC class I–like molecule CD1d (1). In mice, iNKT cells express canonical Vα14-14Ia18 TCRα-chains associated with Vβ8, Vβ7, or Vβ2 capable of recognizing several endogenous as well as microbial agonist lipid Ags (2). Positive agonist selection of iNKT cell precursors in the thymus is associated with elevated levels of TCR signaling when compared with conventional VαT T lymphocytes (3, 4), and is characterized by enhanced expression of the transcription factor Egr2 (5). Increased TCR signaling in iNKT precursors results in induction of the promyelocytic leukemia zinc finger (PLZF) transcription factor (5), which in turn promotes effector iNKT cell differentiation. Initially, phenotypic and functional characterization of iNKT cell differentiation described the presence of four developmental stages in which effector differentiation was associated with up-regulation of the surface Ags CD44 and NK1.1, production of IFN-γ and IL-4, and stable expression of the Th1-lineage transcription factor T-bet (2). More recent studies have, however, shown that terminally differentiated iNKT thymocytes are composed of alternatively polarized Th1, Th2, and Th17 sublineages characterized by expression of the transcription factors T-bet, GATA-3, and RORγt, respectively (6). Whether iNKT polarization is a developmental consequence of lineage priming by transcription factors such as PLZF is currently an active field of research (7).

In this context, the physiological significance of thymic iNKT cell trafficking during development is a comparatively unexplored area in the field. What has been examined are proposed roles for sphingosine-1-phosphate receptor 1 (8) and neuropilin-1 (9) during thymic egress, and a requirement for CXCR3 during thymic iNKT retention (10). In contrast, a large number of studies have highlighted the importance of lymphocyte trafficking during conventional thymocyte development. For example, guided entry of T-progenitors into the thymus, positive selection in the thymic cortex, migration of thymocytes into the thymic medulla, and seeding of the peripheral compartment all require G protein–coupled transmembrane receptors (11). Thus, thymocytes receive distinct guidance cues from the thymic microenvironment, which in turn serves to promote their development and maturation (12). Similarly, a role for the thymic microenvironment in regulating iNKT cell trafficking has been previously reported (13). More specifically, thymic iNKT cell emigration was found to be impaired in animals deficient in either lymphotixin (LT)αβ or lymphotixin β receptor (LTβR), suggesting LTαβ-LTβR–dependent signaling in mouse thymus-regulated thymic iNKT cell trafficking during development.
LTβ and LTβR are members of the TNF superfamily (14) and are broadly implicated in primary and secondary lymphoid organ formation in mice (15). LTβ is predominantly expressed within the hematopoietic lineage as a membrane-bound heterotrimetric complex LTβ1β2, and engagement of LTβR by LTβ1β2 results in the upregulation of cell adhesion molecules and chemokines required for the maturation and organization of stromal organizer cells during lymphoid organ formation. In thymus, LTβ1β2-LTβR-dependent signaling has been shown to promote differentiation of the thymic epithelial compartment (17) and to facilitate positioning of mature T lymphocytes in the thymic medulla. Based on such findings, it was postulated (13) that LTβ signaling in thymus regulated the differentiation of the stromal microenvironment in a manner that specifically promoted the trafficking and emigration of iNKT thymocytes.

To determine whether LTβR-dependent iNKT cell emigration was associated with the expression of a specific chemotactic mRNA profile, we isolated mRNA from emigrating iNKT and was associated with the expression of a specific chemotactic

780 (H57-597), T-bet-PE (eBio4B10), Gata-3-AlexaFluor 488 (TWAJ),Committee.

procedures were approved by the Institutional Animal Care and Ethics

according to the guidelines of the Ghent University vivarium. All animal

have been previously described (17). All mice were housed and bred

(CD79a-cre) (19), Tg(Itgax-cre)1-1Reiz (CD11c-cre)

(Lyz2-cre), and B6.Cg-Tg(Cdh5-cre)7Mlia/J (Cdh5-cre) mice were

Cy7 (PK136), Gr-1-PerCP-Cy5.5/allophycocyanin-eFluor 780 (RB6-

AlexaFluor 647 (eBio440c), CD19-PerCP-Cy5.5 (eBio1D3), NK1.1-PE-

CD11c-PE-Cy7 (N418), CD122 PerCP-eFluor 710 (TM-b1), Siglec-H-

allophycocyanin-eFluor 780 (RA3-6B2), CD11b-PE-Cy7 (M1/70),

phycocyanin (53-6.7), CD45-PerCP-Cy5.5 (30-F11), CD45R/B220-

FITC/allophycocyanin/PE-Cy7 (MEL-14),

CD44-FITC/allophycocyanin/allophycocyanin-eFluor 780 (IM7), CD62L-

medulla. Based on such findings, it was postulated (13) that LT

b microenvironment in a manner that specifically promoted the

results in the upregulation of cell adhesion molecules and che-

complex LT

a b 2 (16), and engagement of LT

b 1 b 2 expression within IL-7R

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To determine whether LTβR-dependent iNKT cell emigration was associated with the expression of a specific chemotactic mRNA profile, we isolated mRNA from emigrating iNKT and conventional αβ T cells and analyzed differentially expressed mRNA for genes previously associated with cellular chemotaxis. In this study, we show that emigrating iNKT cells differentially express fractalkine receptor and that mRNA for this receptor can be detected within the NK1T, NK2T, and NK1T1 sublineages in steady-state mouse thymus. In addition, fractalkine ligand was found to be expressed by endothelial cells within the thymus, predominantly at the corticomedullary junction (CMI). In the context of iNKT cell development, we show that expression of the fractalkine receptor is developmentally regulated within NK1T thymocytes in a LTβ1β2-LTβR-dependent manner and that seeding of the peripheral compartment by NK1T cells requires LTβ1β2-LTβR signaling. Furthermore, we show that the requirement for LTβ1β2 during NK1T development is not cell intrinsic but depends on LTβ1β2 expression within IL-7Rα-positive lymphoid precursors.

Materials and Methods

Mice

B6.129-P-Cxcr1tm1J(B6.129-P-Ly5tm1/cy5.5J) (Ly2-cre), and B6.Cg-Tg(Cdh5-cre)7Mlia/J (Cd79atm1(cre)Reth (CD79a-cre) (19), Tg(Itgax-cre)1-1Reiz (CD11c-cre)

(Lyz2-cre), and B6.Cg-Tg(Cdh5-cre)7Mlia/J (Cd79atm1(cre)Reth (CD79a-cre) (19), Tg(Itgax-cre)1-1Reiz (CD11c-cre)

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b 1 b 2 expression within IL-7Rα-positive lymphoid precursors.

Materials and Methods

Mice

B6.129-P-Cxcr1tm1J(B6.129-P-Ly5tm1/cy5.5J) (Ly2-cre), and B6.Cg-Tg(Cdh5-cre)7Mlia/J (Cd79atm1(cre)Reth (CD79a-cre) (19), Tg(Itgax-cre)1-1Reiz (CD11c-cre)

(Lyz2-cre), and B6.Cg-Tg(Cdh5-c cre)7Mlia/J (Cd79atm1(cre)Reth (CD79a-cre) (19), Tg(Itgax-cre)1-1Reiz (CD11c-cre)

(20), Tg(KRT1-cre)5132J(i-cre) (Krt-5-cre) (21), EpoRtm1GEPfehr/jk (Epo-iCre) (22), Tg(CAG-BgeoGFP/GFP)21LhC (ZEG) (23), ROtyr-cre (24), Tg(Foxi1-cre)1Kkh (25), and Ilyt2tm1/2/Cre (IL-7Rα+cre) (26) mouse strains were

provided by B. Lambrechts (Flanders Institute for Biotechnology, Ghent, Belgium), J. Haigh (Flanders Institute for Biotechnology), G. Eberl (Institute Pasteur, Paris, France), K. Kaestner (University of Pennsylvania, Philadelphia, PA), and H.-R. Rodewald (German Cancer Research Center, Heidelberg, Germany), respectively. Lyiht2tm1/2/Cre (LTβRFP) mice have been previously described (17). All mice were housed and bred according to the guidelines of the Ghent University vivarium. All animal procedures were approved by the Institutional Animal Care and Ethics Committee.

Flow cytometry and Abs

Cells were analyzed on FACSCantoII (BD Biosciences) and FlowJo software (TreeStar) and sorted on FACS Aria II (BD Biosciences). Abs were used: CD25-PE (PC61.5), CD72-allophycocyanin (LG7.P9), CD3e-V500 (500A2), CD4-PE/allophycocyanin-eFluor 780 (RM4-5), B6a-Ka allop hycocyanin (AF809), Tcrεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεεε
for NKT1<sup>a</sup> and NKT1<sup>b</sup> subsets revealed that both NKT1<sup>a</sup> and NKT1<sup>b</sup> cells express GATA-3, T-bet, and PLZF, but not RORγt (Fig. 2A), and that <10% of the NKT1<sup>b</sup> subset is positive for either T-bet or GATA-3 only (Fig. 2A). When stimulated in vitro with α-GalCer-loaded CD1d, hepatic NKT<sup>a</sup> and NKT<sup>b</sup> cells produced equivalent amounts of IL-6, IL-4, IFN-γ, and TNF-α, but no IL-2 (Fig. 2B). Furthermore, the NKT<sup>b</sup> subset comprises the majority of iNKT sublineage RTEs detected in the blood, spleen, and liver of C57BL/6 mice following intrathymic FITC injection (Fig. 2C), and upregulation of NK1.1 within iNKT RTEs occurs predominantly within the NKT1<sup>a</sup> and NKT1<sup>b</sup> subsets (Fig. 2C).

To assess whether CX3CR1 ligand was expressed in thymus, we performed an immunohistochemical analysis of thymus sections from C57BL/6 mice (Fig. 3A–G). In this work, CX3CL1-expressing cells could be detected within and at the edges of EpCAM-positive medullary thymic epithelium (Fig. 3A, 3B), in areas proximal to MECA-32–positive endothelium (Fig. 3C). Colocalization of CX3CL1-expressing cells with MECA-32–positive endothelium was subsequently confirmed by confocal microscopy (Fig. 3D–G), proving that CX3CL1 ligand is expressed by vasculature within the thymic medulla, as well as at the CMJ. Conversely, CX3CR1 deficiency did not significantly affect the development (Fig. 3H) or peripheral distribution of CX3CR1<sup>a</sup> iNKT sublineage cells (Fig. 3I) when compared with littermate controls. Thus, expression of CX3CR1 mRNA within the iNKT cell lineage can be correlated with a RTE phenotype in steady-state mice; however, the requirement for CX3CR1-dependent trafficking during iNKT cell export is functionally redundant.

Thymic LT<sub>a</sub>β<sub>2</sub>-LTβR–dependent signaling regulates development and emigration of NKT1<sup>a</sup> thymocytes

To begin assessing the requirement for LT<sub>a</sub>β<sub>2</sub>-LTβR–dependent signaling during thymic iNKT cell trafficking, we generated a germline LTβ-deficient mouse line (Krt-5-cre;LT<sup>bΔβ</sup>) by intercrossing male floxed LTβ mice (LT<sup>bΔβ</sup>) with females expressing a bovine cytokeratin 5 (Krt-5) cre-recombinase transgene. Previously, F<sub>2</sub> generation litters have been described to exhibit constitutive recombination between homozygous loxP sites when the Krt-5-cre transgene is expressed by the mother (21). Subsequent macroscopic examination of F<sub>2</sub> generation animals revealed defective secondary lymphoid organ formation in Krt-5-cre;LTβ<sup>bΔβ</sup> mice when compared with littermate controls (Fig. 4A–I), as is seen in LTβ<sup>−/-</sup> mice (15). Furthermore, the percentage and absolute numbers of iNKT cells isolated from the thymi of Krt-5-cre; LTβ<sup>bΔβ</sup> mice were comparable to controls (Fig. 4J, 4L), whereas all peripheral organs showed a reduction in iNKT counts when compared with controls (Fig. 4K, 4L), suggesting a developmental block in thymic export. Reduced thymic iNKT emigration in Krt-5-cre;LTβ<sup>bΔβ</sup> mice was subsequently shown for both young and mature FITC-injected animals in all peripheral organs examined (Fig. 4M–O). These results confirm that iNKT cell development and export require LT<sub>a</sub>β<sub>2</sub>-LTβR–dependent signaling (13) and that the Krt-5-cre;LTβ<sup>bΔβ</sup> mouse line accurately models such a requirement.

To examine the role of LT<sub>a</sub>β<sub>2</sub>-LTβR–dependent signaling during trafficking of iNKT cells in vivo, we backcrossed Krt-5-cre;LTβ<sup>bΔβ</sup> mice onto a CX3CR1<sup>gfp/wt</sup> background and analyzed iNKT sublineage development as a function of CX3CR1 and CXCR3 expression. Analysis of the percentage and absolute numbers of iNKT sublineage cells isolated from the thymi of triple-transgenic germline LTβ-deficient CX3CR1<sup>gfp/wt</sup> mice (i.e., Krt-5-cre;LTβ<sup>bΔβ</sup>;CX3CR1<sup>gfp/wt</sup>) was different from iNKT
FIGURE 1. Fractalkine receptor is upregulated in iNKT RTEs and characterizes distinct subsets of iNKT sublineage cells in steady-state mice. (A) Heat map of unbiased hierarchically clustered expression data for genes characteristic of emigrating and resident iNKT and CD4+ T cells. Data are representative of two independent experiments (A and B) pooled. P4, Res, Resident. (B) Analysis of stage 1–3 iNKT subset distribution within CX3CR1+CXCR3− (I), CX3CR1+CXCR3+ (II), CX3CR1−CXCR3− (III), and CX3CR1−CXCR3+ (IV) iNKT thymocytes. Data are one representative of three independent experiments (n = 4). (C–E) Fractalkine receptor (CX3CR1) expression for the NKT2 (CD27+IL-2Rb−), NKT1a (CD27+IL-2Rb+), NKT17 (CD27+IL-2Rb−), and NKT1b (CD27−IL-2Rb+) subsets present in the thymus, spleen, and livers of 8-wk-old CX3CR1gfp/wt mice, respectively. Dot blots are gated on CD3ε+CD1d−αGC+ lymphocytes from each organ and represent one of two independent experiments (n = 6). (F) Absolute number of CX3CR1+ iNKT lymphocytes in the thymus, blood, spleen, and liver of CX3CR1gfp/wt mice (n = 6).
sublineage counts in littermate controls (Fig. 5A, 5E); however, expression of CX3CR1 and CXCR3 within the NKT1\(^a\) subset was found to be dependent on LT\(b\) (Fig. 5A, 5B). Furthermore, up-regulation of CXCR3 within LT\(b\)-deficient NKT1\(^a\) thymocytes resulted in an enhanced migration index toward the CXCR3 ligand CXCL10 when compared with control migration indices (Fig. 5C).

**FIGURE 2.** Characterization of NKT1\(^a\) and NKT1\(^b\) subsets. (A) Analysis of GATA-3, T-bet, PLZF, and ROR\(\gamma\)t expression within the NKT1\(^a\) (CD27\(^+\)IL-2R\(\beta\)^b) and NKT1\(^b\) (CD27\(^+\)IL-2R\(\beta\)^b) subsets isolated from the liver of C57BL/6J mice. Isotype controls (shaded histograms). Numbers in parentheses represent mean fluorescence intensity for PLZF expression. Data represent one of two independent experiments (\(n = 5\)). (B) Sorted hepatic NKT1\(^a\) and NKT1\(^b\) subsets were stimulated in vitro with \(\alpha\)GalCer-loaded CD1d, and IL-2, TNF-\(\alpha\), IL-4, IL-6, and IFN-\(\gamma\) were measured in the supernatants at 72 h poststimulation. Data represent one of two independent experiments. (C) Analysis of the distribution of NKT1, NKT2, and NKT17 sublineages within CD44\(^{high}\)NK1.1\(^2\) and CD44\(^{high}\)NK1.1\(^+\) iNKT RTEs at 36 h postintrathymic FITC injection. Data represent one of two independent experiments of four pooled C57BL/6J mice.
FIGURE 3. Thymic localization of fractalkine expression and requirement for CX3CR1 signaling during NKT sublineage development. (A) C57BL/6J thymic sections show immunofluorescent staining for fractalkine (CX3CL1)-positive cells (red) and EpCAM-positive mTECs (blue). Dotted line denotes CMJ. (B) Immunofluorescent staining for control polyclonal IgG (red) and EpCAM-positive mTECs (blue) in C57BL/6J thymus sections. (C) Anti-Krt-5 (blue) and anti–MECA-32 (green) staining mark thymic mTEC and endothelial cell compartments, respectively. Arrow indicates MECA-32–positive endothelium at the CMJ. (D–G) Immunofluorescent staining shows colocalization of CX3CL1- and MECA-32–positive endothelium in thymus from 8-wk-old C57BL/6J mice. (H) iNKT sublineage distribution in CX3CR1-deficient (CX3CR1 gfp/gfp) mice compared with littermate controls (n = 8). (I) Absolute number of CX3CR1-positive iNKT cells in thymus, blood, spleen, and livers of CX3CR1 gfp/gfp mice compared with littermate controls (n = 5). Original magnification ×40 for (A)–(F) and ×63 for (G).
suggesting that LTα1b2-LTβR–dependent signaling regulates the CXCR3-dependent trafficking of NKT1α thymocytes (10). In addition, increased intrathymic trafficking of CXCR3-expressing NKT1α thymocytes is associated with the selective reduction of NKT1α lymphocytes in the blood, spleen, and livers of Krt-5-cre;LTβF/F mice relative to controls (Fig. 5D, 5E). Thus, the expression of CX3CR1 and CXCR3 within thymic NKT1α cells is regulated by thymic LTα1b2-LTβR–dependent signaling, which in turn regulates seeding of the peripheral compartment by NKT1α lymphocytes.

FIGURE 4. Generation and characterization of germline LTβ-deficient mice using a maternally transmitted bovine Krt-5 cre-recombinase transgene. Photographs show the presence or absence of axillary (A and F) and subiliac lymph nodes (B and G), Peyer’s patches (C, D, and H), and splenic MOMA-1+ marginal zone macrophages (yellow; E and I) in control (Krt-5-cre;LTβF/F) and germline LTβ-deficient (Krt-5-cre;LTβF/F) mice 60 min after injection of India ink, respectively. (A, B, F, and G) Injected India ink appears blue. Original magnification ×30 for (A), (B), (D), (F), and (G). Immunofluorescent images (E and I) represent splenic MOMA-1+ marginal zone macrophages (yellow) and collagen type IV–positive (green) glomerular basement membrane. Original magnification ×40. Results are representative of >25 mice examined macroscopically. (J and K) Percentage of iNKT cells isolated from the thymi and livers of Krt-5-cre;LTβF/WF (n = 11) and Krt-5-cre;LTβF/F (n = 12) mice, respectively. (L) Reduced absolute numbers of iNKT cells in peripheral organs of Krt-5-cre;LTβF/F mice compared with littermate controls (n = 11). Absolute numbers of FITC+ iNKT RTEs in the blood (M), spleen (N), and livers (O) of Krt-5-cre;LTβF/F and control mice at 3, 4, 5, 6, and 8 wk of age. Absolute iNKT cell counts represent two pooled mice per strain per time point and are representative data of two pooled experiments. *p < 0.05, **p < 0.005.
Non-cell-autonomous requirement for LTβ during NKT1α development

It has been previously demonstrated that thymocytes undergoing positive selection upregulate mRNA for LTα and LTβ (29, 30) and that positively selected thymocytes can promote differentiation of the thymic epithelial compartment (12, 30, 31). To investigate whether positively selected thymocytes served as a cellular source for LTβ during NKT1α development, we generated mice lacking LTβ within the CD4 T cell compartment (CD4-cre;LTβ<sup>F/F</sup>). Unexpectedly, the distribution of NKT1<sup>+</sup> lymphocytes in thymus and peripheral organs of CD4-cre;LTβ<sup>F/F</sup> mice was comparable to littermate controls (Fig. 6A, 6B), suggesting that LTβ functions during NKT1α subset development in a non-cell-autonomous manner. We subsequently generated mice lacking LTβ within the B lymphocyte (CD79a-cre;LTβ<sup>F/F</sup>), lymphoid tissue inducer (LTi, ROR<sup>γ</sup>t-cre;LTβ<sup>F/F</sup>), and dendritic cell (CD11c-cre;LTβ<sup>F/F</sup>) compartments, as previous studies have identified roles for these cellular lineages in secondary lymphoid organ formation.
and lymphocyte homeostasis (32–34). However, analysis of NKT1α development in CD79a-cre;LTβF/F (Fig. 6C, 6D), RORγt-cre;LTβF/F (Fig. 6E, 6F), or CD11c-cre;LTβF/F (Fig. 6G, 6H) mice showed no significant differences when compared with littermate controls, suggesting that LTβ expression within the B lymphocyte, LTi, or dendritic cell lineages is not involved in regulating intrathymic NKT1α development and trafficking. In addition, we found no significant differences in INKT cell development in mice lacking LTβ within the monocytic (Lyz2-cre;LTβF/F), epithelial (Krt-5Cre;LTβF/F), endothelial (Cdh5-cre;LTβF/F), mesenchymal (FoxL1-cre;LTβF/F), or erythroid precursor (Epo-icre;LTβF/F) lineages (data not shown), negating a role for LTβ expression within these lineages (35–37) in our model. Furthermore, we performed fate-mapping experiments to verify that all cre-recombinase strains tested in this work deleted efficiently within the cellular compartment being investigated (data not shown). Thus, we exclude a role for several distinct cellular lineages as a source for LTβ during NKT1 lineage development and propose that LTα1β2-LTβR-dependent patterning of thymic stroma regulates NKT1α subset development in a non-cell-autonomous manner.

Lymph node aplasia in germline LTβ-deficient mice does not indirectly regulate thymic INKT cell development and trafficking

Due to the absence of an INKT cell developmental defect in the cell-specific LTβ-deficient strains analyzed in this work (Fig. 6), we sought to exclude any indirect role lymph node aplasia may have on thymic INKT development and trafficking in germline LTβ-deficient mice (Figs. 4, 5). To address this, we injected pregnant C57BL/6 mice at gestational day 9 (E9) with a LTβR antagonist (LTβR-Ig) fusion protein, as this treatment regimen has been previously shown to disrupt lymph node development in mice (38). In this study, macroscopic analysis of adult mice treated in utero with LTβR-Ig revealed defective anterior and posterior lymph node organogenesis (Supplemental Fig. 1C–F), whereas total INKT cell counts in the thymus, blood, spleen, and livers of young and mature mice treated with LTβR-Ig showed no significant difference when compared with LFA3-Ig-treated littermate controls (Supplemental Fig. 1G–J). Based on these results, we conclude that perturbed peripheral lymphocyte homeostasis in the absence of lymph nodes has a negligible effect on INKT cell development and trafficking in the thymus and that the requirement for LTβ during NKT1α development reflects a role for LTβR-dependent signaling during development of the thymus itself.

IL-7Rα-expressing hematopoietic cells regulate thymic NKT1α development in a LTα1β2-LTβR-dependent manner

Based on published results (13) as well as data presented in this study, the requirement for LTβ during differentiation of the NKT1α subset must fulfill a number of criteria, as follows. First, the cellular source for LTβ must be present within the thymic anlage during, or prior to, E15 (13). Second, the cellular lineage(s) expressing LTβ should potentially exclude those already investigated in this study. Three, potential cellular lineage(s)-expressing LTβ should presumably have a nonredundant role during thymic organogenesis. Based on these criteria, we postulated that hematopoietic and/or lymphoid progenitors to the T lymphocyte lineage may be viable candidates as they can be detected within the thymic anlage from E11.5 onward (39, 40) and are reported to promote differentiation of thymic stromal tissue (12, 41). Thus, to assess the role of hematopoietic and/or lymphoid progenitors as a cellular source for LTβ during NKT1α development, we selected a mouse line expressing cre-recombinase under the control of the IL-7Rα promoter (26). Importantly, IL-7Rα-expressing cells can be detected within the thymic anlage at E11.5 (39, 40). IL-7Rα expression marks promoter activity within common lymphoid organs...
progenitors and pro-T cells in adult animals (26), and stimulation of IL-7Rα induces expression of LTα1β2 in IL-7Rα+ c-Kit+ progenitors (42). In this study, the generation and macroscopic analysis of IL-7Rα-cre;LTβF/F mice found them to phenocopy Krt-5-cre;LTβF/F mice (Fig. 4A–I) in that they lacked anterior and posterior lymph node structures (Fig. 7A, 7B, 7F, 7G), Peyer’s patches (Fig. 7C, 7D, 7H), and MOMA-1+ marginal zone macrophages in the spleen (Fig. 7E, 7I). Analysis of iNKT sublineage development in IL-7Rα-cre;LTβF/F mice revealed a selective reduction in peripheral NKT1α lymphocytes (Fig. 8A, 8B), as was observed for Krt-5-cre;LTβF/F;CX3CR1gfp/wt mice (Fig. 5D, 5E), and thymic iNKT cell export to the blood, spleen, and liver was impaired when compared with littermate controls (Fig. 8C, 8D).

In addition, thymic NKT1α development was assessed by backcrossing IL-7Rα-cre;LTβF/F mice onto a CX3CR1 gfp/wt background, as was done for Krt-5-cre;LTβF/F;CX3CR1 gfp/wt mice (Fig. 5). Subsequent analysis of thymic NKT1α cell development in wild-type controls (IL-7Rα-cre;LTβF/F;CX3CR1 gfp/wt) revealed that the NKT1α subset can be subdivided into three subpopulations, namely CX3CR1+CXCR3+ (I), CX3CR1+CXCR3+ (II), and CX3CR1+CXCR3+ (III), respectively (Fig. 8E). Importantly, IL-7Rα-cre;LTβF/F;CX3CR1 gfp/wt mice exhibited a selective reduction in the frequency and absolute cell counts of subpopulations I and II relative to controls (Fig. 8E, 8F), whereas subpopulation III was selectively enriched for in IL-7Rα-cre;LTβF/F;CX3CR1 gfp/wt thymi (Fig. 8E, 8F). Furthermore, NKT1α thymocytes isolated from the thymi IL-7Rα-cre;LTβF/F;CX3CR1 gfp/wt mice expressed double the amount of cell surface CXCR3 when compared with littermate controls (Fig. 8G), which coincided with a selective reduction in NKT1α lymphocytes in all peripheral organs examined, including the liver (Fig. 8H, data not shown).

Based on these findings, we conclude that an IL-7Rα-expressing hematopoietic and/or lymphoid precursor regulates the differentiation of NKT1α thymocytes in a noncell-autonomous manner. In the absence of LTα1β2-LTβR-dependent signaling, NKT1α thymocytes fail to upregulate mRNA for CX3CR1 and are induced to overexpress CXCR3. We propose that this phenotypic change is associated with retention of stage III NKT1α thymocytes within the thymic microenvironment, with concomitant reduced export of the NKT1α subset.

Discussion

The current study shows that CX3CR1 expression can be used to define subpopulations of iNKT sublineages present in the thymus and peripheral organs of naive mice. We propose that upregulation of CX3CR1 within thymic iNKT sublineages can be correlated with cells destined for export; however, our results argue against a functional requirement for CX3CR1-dependent trafficking during active iNKT cell export. In addition, we characterize two new NKT1 sublineage subsets, termed NKT1α and NKT1β, which have distinct developmental as well as tissue distribution profiles. More specifically, the development and trafficking of the NKT1α subset are dependent upon noncanonical signaling regulated by LTβR, whereas the NKT1β, NKT2, or NKT17 sublineages are not. Furthermore, the data imply that CXCR3-dependent trafficking of
NKT1a thymocytes is regulated by thymic LTα1β2-LTβR-dependent organogenesis. More specifically, we propose that deleting membrane-bound LTα1β2 within hematopoietic precursors to the T lymphocyte compartment disrupts thymic organogenesis in a manner that directly perturbs intrathymic development, trafficking, and retention of NKT1a thymocytes.

The requirement for LTβR-dependent signaling during thymic organogenesis has been investigated by a number of groups. For example, differentiation of medullary thymic epithelial cells (mTECs) has been shown to require LTα1β2 expression within positively selected thymocytes (30, 31), which constitutes a continuous process that can be disrupted in adult animals. Our results, however, argue against a role for the adult program of mTEC differentiation during iNKT development, as treatment of adult C57BL/6 mice with LTβR-Fc fusion protein (13) or deletion of LTβ in positively selected thymocytes, B cells, or dendritic cells failed to regulate trafficking and export of NKT sublineages (Fig. 6). Moreover, we have assessed, and can exclude, a functional requirement for LTβ expression within the RORγt+ LTi compartment during development of the NKT1a subset (Fig. 6D). Based on these findings, we conclude that the fetal role of LTi during mTEC differentiation (43) serves a limited role in our model. We do, however, provide data that support a role for LTβR expression within IL-7Rα–positive hematopoietic cells during NKT1a subset development (Fig. 8).

FIGURE 8. Defective NKT1 sublineage development in IL-7Rα-cre;LTβF/F mice. (A and B) iNKT sublineage frequency and absolute counts in the thymus, blood, spleen, and liver of control and IL-7Rα-cre;LTβF/F mice, respectively (n = 8; three independent experiments). Dot blots are gated on CD3ε+CD1dα3Gc+ lymphocytes. (C and D) Frequency and absolute counts of FITC+ iNKT RTEs in the blood, spleen, and liver of control and IL-7Rα-cre;LTβF/F mice at 5 wk of age. Percentage of iNKT RTEs constitutes two pooled mice per strain and is representative data of two independent experiments. (E) Analysis of CX3CR1 and CXCR3 expression within NKT1a thymocytes isolated from control and IL-7Rα-cre;LTβF,F,Cx3cr1gfp/wt and thymi. In this figure, NKT1a thymocytes are divided into CX3CR1+CXCR3+ (I), CX3CR1+CXCR3+ (II), and CX3CR1 CXCR3− (III) subsets. (F) Quantification of the absolute numbers of NKT1a subsets I, II, and III in control (LTβF/F) and IL-7Rα-cre;LTβF,F,Cx3cr1gfp/wt (LTβF/F) thymi. Data are representative of three pooled experiments (n = 9 and 6, respectively). (G) Histogram of median CXCR3 expression on NKT1a thymocytes in control (F/F) and IL-7Rα-cre;LTβF,F,Cx3cr1gfp/wt (F/F) thymi. Numbers in parentheses denote CXCR3 median expression (n = 8; three independent experiments). (H) Distribution of hepatic iNKT sublineages in control and IL-7Rα-cre;LTβF,F,Cx3cr1gfp/wt mice (n = 8; three independent experiments). *p < 0.05.

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cortical thymic epithelial cell differentiation can be regulated in a LTβR-dependent fashion (45). Furthermore, hematopoietic progenitors that seed the thymus comprise a relatively heterogeneous mixture of cells, including common lymphocyte progenitors and early thymic progenitors, which all express IL-7Rα (26, 40). This being said, we are unable to experimentally exclude a potential role for more mature lymphoid lineages in the thymus with a history of IL-7Rα expression, although the absence of a NKTR1α subset phenotype in CD4-crateLTβRα, CD79a-crateLTβRα, and CD11c-crateLTβRα mice (Fig. 5) would argue against such an interpretation.

In the context of thymocyte trafficking during T cell development and selection, the developmentally regulated expression of CX3CR1 and CXCR3 within thymic NKT sublineages clearly distinguishes them from chemokine receptor expression profiles used to define trafficking of conventional T cells within the thymus (11). To our knowledge, CX3CR1 expression within developing iNKT thymocytes has not been previously reported and, apart from one study that defines neuropilin-1 as a marker for emigrating IL-17–producing iNKT cells (9), this is the first study to identify a marker for emigration expressed within the NKTR1, NKTR2, and NKTR17 sublineages. In this context, our primary analysis of differentially expressed mRNAs within emigrating iNKT lymphocytes corroborates the finding that mRNA for neuropilin-1 is upregulated within iNKT RTEs (Supplemental Table II); however, the fold-change observed for neuropilin-1 was 10-fold less than that observed for Cx3cr1 mRNA within iNKT RTEs (Supplemental Table II). In addition, we also demonstrate that CX3CR1 expression within NKTR1 sublineage cells is differentially regulated by development of the thymic microenvironment. In this study, NKTR1α thymocytes fail to upregulate CX3CR1 within thymic that lack a fully differentiated stromal cell compartment, but become developmentally redirected to traffic toward CX3CR3 ligands such as CXCL10 (10). Surprisingly, this developmental niche is only observed for the NKTR1α subset, implying that a causal relationship exists between stromal cell differentiation and trafficking of NKTR1α thymocytes. Whether this reflects a more fundamental relationship between thymic organogenesis and the retention of mature T cells in the organ remains to be determined; however, it is interesting to note that NKTR1α thymocytes constitute the bulk of CX3CR3+ iNKT cells retained in the thymus organ (Supplemental Figs. 1B, 5B). Although the noncell-autonomous regulation of CX3CR3 expression within the iNKT lineage has, to our knowledge, not been previously reported, upregulation of CX3CR3 within the conventional CD4 T cell lineage has been shown to occur in animals lacking the transcription factor Kruppel-like factor 2 (46). More specifically, noncell-autonomous upregulation of CX3CR3 expression within the CD4 T cell compartment required the T box transcription factor Eomesoderm and IL-4. It is, however, unlikely that a similar mechanism regulates CX3CR3 upregulation on bystander NKTR1α thymocytes in the absence of LTβ, as Eomesoderm is not expressed within the iNKT cell lineage (47).

In the context of thymus physiology, expression of the chemokine CX3CL1 within vasculature at the CMJ would support a role for CX3CR1-mediated chemotaxis during intrathyMIC iNKT cell trafficking; however, the absence of an iNKT cell developmental defect in CX3CR1-deficient mice clearly shows a limited role for CX3CR1-dependent trafficking during iNKT cell export in naive mice. Nevertheless, we propose that CX3CR1 expression can be used as a marker for iNKT RTEs in the peripheral compartment, and that the distribution of CX3CR1+ iNKTs in different tissues represents the degree of homeostatic export to those organs under naive conditions. In this context, it is tempting to speculate that, under conditions of inflammation, the requirement for a CX3CL1/CX3CR1 migratory axis may have a more pronounced role during trafficking of iNKT cells in the thymus and peripheral compartment. For example, CX3CR1-dependent lymphocyte chemotaxis and iNKT cells have both been implicated in the augmentation of inflammatory diseases such as atherosclerosis (48, 49).

To summarize, we show that the trafficking and development of NKT sublineages in the murine postnatal thymus can be characterized by G protein–coupled transmembrane receptors distinct from those described for development of the conventional T cell lineage. In addition, fractalkine receptor expression can be used to specifically identify RTEs for the NKTR1, NKTR2, and NKTR17 sublineages. Importantly, the development and trafficking of the NKTR1 sublineage require LTβR–dependent differentiation of the thymic microenvironment. More specifically, seeding of the thymic anlage by IL-7Rα hematopoietic precursors promotes differentiation of thymic stroma in a manner that promotes development of the NKTR1 sublineage. Collectively, we propose that differentiation of the NKTR1 sublineage is intrinsically linked to development of the thymus organ.

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Disclosures

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References


