CD8αα intraepithelial lymphocytes arise from two main thymic precursors

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TCRαβ+CD4−CD8α+CD8β− intestinal intraepithelial lymphocytes (CD8αα IELs) are an abundant population of thymus-derived T cells that protect the gut barrier surface. We sought to better define the thymic IEL precursor (IELp) through analysis of its maturation, localization and emigration. We defined two precursor populations among TCRβ+CD4+CD8− thymocytes by dependence on the kinase TAK1 and rigorous lineage-exclusion criteria. Those IELp populations included a nascent PD-1+ population and a T-bet+ population that accumulated with age. Both gave rise to intestinal CD8αα IELs after adoptive transfer. The PD-1+ IELp population included more strongly self-reactive clones and was largely restricted by classical major histocompatibility complex (MHC) molecules. Those cells localized to the cortex and efficiently emigrated in a manner dependent on the receptor S1PR1. The T-bet+ IELp population localized to the medulla, included cells restricted by non-classical MHC molecules and expressed the receptor NK1.1 and the chemokine receptor CXCR3. The two IELp populations further differed in their use of the T cell antigen receptor (TCR) α-chain variable region (Vα) and β-chain variable region (Vβ). These data provide a foundation for understanding the biology of CD8αα IELs.

Intestinal intraepithelial lymphocytes (IELs) are a heterogeneous T cell population embedded within the intestinal epithelial layer, where they carry out numerous effector, regulatory and protective functions1,2. How IELs exert these functions is not fully understood. IELs include memory TCRαβ+CD4+ cells and TCRαβ+CD8αβ+ cells generated from naive T cells during an immune response (induced IELs), and TCRγδ+ cells and TCRαβ+ CD4+CD8ααCD8β− cells (CD8αα IELs) that differentiate in the thymus (natural IELs)3. The development of natural CD8αα IELs has been the subject of extensive research, yet their precise thymic precursor has remained unknown. Post-selection IEL precursors (IELps) can be found within the CD4+CD8− double-negative (DN) thymocyte population as cells expressing the negative regulator CD5 and the T cell antigen receptor β-chain (TCRβ)3–5, although within this population there is still substantial heterogeneity. Various molecules, including the transcription factor T-bet, the costimulatory molecule PD-1, integrin α4β7 and CD103 (integrin αε) have been linked to IEL development6–7. However, other features of IEL development are not yet known, including thymic localization, emigration patterns and TCR specificity. Published approaches have suggested variability among CD8αα IELs in their MHC restriction4,5.

Published studies of IELps have relied on NK1.1 as an exclusion marker for invariant natural killer T cells (iNKT cells). That approach, however, does not exclude the thymic iNKT cell subsets that are NK1.1−: NKT2 cells and NKT17 cells8. Here we investigated the development and emigration of thymic IELps in detail through the use of a tetramer linked to the antigen-presenting molecule CD1d (CD1d+tet) to specifically exclude iNKT cells. We discovered two distinct mature DN T cell subsets of different phenotype that were able to differentiate into CD8αα IELs. One was PD-1+ and showed enrichment for self-reactive cells, which confirmed in a polyclonal setting what had previously been found in a regenetic model5. The second subset was a previously unrecognized PD-1− T-bet+ population with high expression of NK1.1. This population did not show signs of overt self-reactivity, in contrast to the PD-1+ population, and included non-classical MHC class I–restricted cells. The two subsets further differed in their use of TCRα and TCRβ chains and underwent emigration from the thymus with different efficiency. Even though T-bet+ IELps had a more mature ‘IEL-like’ phenotype, the main emigrating IELps were PD-1+ and expressed integrin α4β7. Both IELp populations were able to seed the gut, where they expressed CD8αα, and since the PD-1+ IELp population downregulated the expression of PD-1 and increased the expression of T-bet, cells arising from the two progenitor populations were largely indistinguishable in the gut environment through the use of conventional markers. Our study reconciles variability in phenotypes and MHC restriction reported for IELps, in addition to revealing previously unknown aspects of IELp development and functionality.

RESULTS

A mature IELp population among thymic TCRβ+ DN cells

Published data have suggested that IELps can be found within the TCRβ+CD5+CD4−CD8− DN gate3,5. We used that gating strategy and further excluded iNKT cells (using a CD1d+tet loaded with the cognate antigen analog PBS57) and CD25+ regulatory T cells (Fig. 1a).

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Within this DN population, approximately one third had a mature phenotype, as indicated by increased expression of the MHC class I molecule H-2Kβ (ref. 9), and H-2Kβ+ cells had high expression of the cytokine receptor subunit CD122 (IL-2Rβ); shared with the receptor for the cytokine IL-15 (Fig. 1b). TCRβ+ mature T cells require the kinase TAK1 for maturation. Analysis of Tak1fl/flCd4cre mice (in which loxp-flanked Tak1 alleles (Tak1fl/fl) are deleted by Cre recombinase expressed from the promoter of the gene encoding the monomorphous co-receptor CD4 (Cd4cre)) confirmed the selective absence of CD8αε IELs, but not of TCRγδ+ IELs, in the small intestine (Fig. 1c), as previously reported (10). Correspondingly, we observed fewer CD122+H-2Kβ+ DN thymocytes in Tak1-deficient mice than in Tak1-sufficient (Tak1+/+ or Tak1fl/flCd4cre+) mice (Fig. 1b), which suggested that this population represented mature precursor cells. Wild-type mature CD122+ (H-2Kβ+) cells among the total CD5TCRβ+ DN population also had little expression of the C-type lectin Cds6 (Fig. 1d), analogous to mature conventional thymocytes. These results indicated that within the CD25+CD1dtet−CD5+TCRβ+ DN population, mature cells, including mature IELs, could be identified as CD122+H-2Kβ+ cells. We used this gating strategy for mature IELs throughout the studies reported here, unless indicated otherwise.

Two main subsets of mature TCRβ+ DN cells

We reasoned that within the mature DN population, IELs might express molecules critical for the development of CD8αε IELs, such as the transcription factor T-bet (encoded by Tbx21) (9). Within the mature TCRβ+ DN population from mice expressing a green fluorescent protein (GFP) as a reporter for T-bet (Tbx21GFP), distinct GFP− and GFP+ cells were present (Fig. 2a). IELs have been suggested to arise from precursor cells diverted from clonal deletion in response to strong TCR signaling (2,12). PD-1 expression is upregulated by TCR signaling, and PD-1 has been shown to be expressed on DN thymocytes in mice expressing retinogenic IEL TCRs (3). Notably, only the DN cells lacking T-bet had abundant expression of PD-1 (Fig. 2b). Furthermore, in mice expressing GFP as a reporter for the orphan nuclear receptor Nur77 (encoded by Nr4a1; Nr4a1GFP), only the PD-1+ cells had high expression of GFP (Fig. 2b), indicative of recent and strong TCR stimulation. Consistent with the proposal of clonal deletion as an alternative fate for IELs, the frequency and number of PD-1− DN cells was 35-fold greater in the thymus of mice deficient in the proapoptotic protein Bim (Bim−/−); a model in which normally deleted T cells are rescued (13) than in that of Bim+ mice (Fig. 2c). It is possible that the PD-1− cells were precursors to PD-1− T-bet− cells and had experienced strong TCR signaling only transiently before upregulating their T-bet expression. Although Bim−/− mice had more PD−1− mature DN cells than Bim+ mice had, this difference was much smaller than that for the PD-1+ population (Fig. 2c). In addition, mice deficient in the costimulatory receptor CD28 (Cd28−/−), in which self-reactive thymocytes are diverted into the CD8αε IEL lineage (11), had only more PD−1− mature DN cells, not more PD−1+ mature DN cells, than Cd28+ mice had (Fig. 2d). These characteristics suggested that the PD−1+ (T-bet+) and PD−1− (T-bet−) cells might represent separate lineages; thus, we called these ‘type A’ IELs and ‘type B’ IELs, respectively.

The integrins αβ1 and αε (CD103) have been associated with homing to and retention in gut (14–16); thus, we assessed the expression of these molecules on thymic progenitors. Type A IELs were the predominant αβ1−, expressing cells, while only type B IELs expressed CD103 (Fig. 2e). As T-bet expression in the thymus is high in NKT cells (9,17), we also searched for other NKT cell–related molecules. Indeed type B IELs were NK1.1+, but type A IELs were not

Figure 1. A subset of TCRβ+DN cells is phenotypically and functionally mature. (a) Flow cytometry for the identification of TCRβ+ DN cells in the thymus. Numbers in plots indicate percent cells in outlined area: CD25−CD1dtet− cells (left), CD4−CD8− (DN) cells among the cells gated at left (middle) and CD5+ TCRβ+ cells among the cells in the middle plot (right). (b) Flow cytometry of TCRβ+ DN cells (gated as in a) from Tak1fl/fl or Tak1fl/flCd4cre+ mice (left), and summary of the frequency and number of mature CD122+H-2Kβ+ cells gated as at left (right). Numbers in plots (left) indicate percent CD122+H-2Kβ+ cells. (c) Quantification of CD8αε (TCRβ+)CD4−CD8− (CD8β−) IELs (left) and TCRβ+ IELs (right) in the small intestine of Tak1fl/fl or Tak1fl/flCd4cre+ mice, assessed by flow cytometry. (d) Flow cytometry of total thymic wild-type CD5−TCRβ+ DN cells. Number in plot indicates percent CD69+ in CD122+H-2Kβ+ cells. Each symbol (b,c) represents an individual mouse; small horizontal lines indicate the mean. *P < 0.05 and ***P < 0.001 (Student’s t-test).

Data are representative of more than ten experiments (a), three experiments with six Tak1fl/fl mice or five Tak1fl/flCd4cre+ mice (b, left) or two experiments with three mice (d) or are pooled from three (b right middle) and c or two (b far right) independent experiments.
types were negative for CCR7 (Fig. 2) and Supplementary Fig. 1k), a chemokine receptor associated with the migration of developing T cells from the thymic cortex to the medulla20,21. In conclusion, we identified two main subtypes within the mature TCRβ⁺DN cell population distinguished by expression of PD-1 or of T-bet and NK1.1.

Both type A IELPs and type B IELPs give rise to CD8α⁺ IELs

To assess the ability of each mature DN subtype to develop into CD8α⁺ IELs, we adaptively transferred sorted type A IELPs or type B IELPs together with congenically distinct total CD4⁻CD8⁻ DN control thymocytes into immunodeficient (Rag2⁻/⁻) recipient mice and recovered cells from the spleen and the epithelium of the small intestine of the host mice 5–10 weeks later. While TCRβ⁺ control competitor cells gave rise to heterogeneous CD4⁺ or CD8⁺ cells in both the IEL compartment as well as the spleen, type A IELPs and type B IELPs gave rise almost exclusively to CD8α⁺ IELs in the intestine (Fig. 3a and Supplementary Fig. 2a). That strong fate determination was tissue specific, as the spleen did not show significant enrichment for CD8α⁺ descendants of either IELP subset (Supplementary Fig. 2b). Type A IELPs and type B IELPs competed...
equivalently with the control cells, and experiments in which type A IELs were transferred together with type B IELs resulted in similar outcomes (data not shown), which suggested that both progenitor cells had a robust potential to seed the gut, at least in Rag2−/− recipient mice.

**Type A IELs are nascent; type B IELs accumulate with age**

CD103 expression is associated not only with IELs and IELPs but also with tissue-resident T cells. We were therefore interested in determining if type B IELPs were retained in the thymus. For this, we made use of Rag2GFP mice, in which thymocytes at the DP stage have high expression of GFP and this protein degrades over time after positive selection. While type A IELPs exhibited Rag2GFP expression similar to that of developing CD4SP thymocytes, type B IELPs were Rag2GFP− (Fig. 3b), which suggested that the latter population was older or had proliferated extensively. As GFP would be diluted by proliferation, we assessed cell-cycle activity via the proliferation marker Ki67. Type B IELPs had much lower expression of Ki67 than that of type A IELPs (Fig. 3c), which indicated that the depletion of GFP was not due to recent proliferation. In addition, type B IELPs had high expression of the maturation marker Qa2, with its intensity far exceeding that in type A IELPs (Fig. 3d). Furthermore, while the absolute number of type A IELPs decreased, the type B IELP population accumulated, with increasing age of mice (Fig. 3e). Of note, those findings were not due to recirculation of type B IELPs, as indicated by adoptive transfer and parabiosis studies (data not shown). In summary, type A IELPs seemed to be developmentally younger than type B IELPs, with the latter expressing markers indicative of retention in the thymus. Nevertheless both types of cells, once in circulation, had an inherent ability to seed an open niche and become CD8αα+ IELs.

**Type A IELPs and type B IELPs localize differently in the thymus**

The chemokine receptors CXCR4 and CCR9 are expressed predominantly by DP thymocytes and are thought to be involved in the cortical localization of those cells. On the other hand, expression of CCR4 and CCR7 is induced after positive selection and is associated with migration of thymocytes from the cortex to the medulla. Both IELp types (A and B) were devoid of surface expression of CCR9 and had negative or low expression of CXCR4 (Fig. 4a), consistent with their post-selection maturation status. However, neither IELp type expressed CCR4 or CCR7. CXCR3 expression has been suggested to retain iNKT cells in the medulla. Type B IELPs were CXCR3+, consistent with our SPADE results, but type A IELPs were not. Given these patterns, it was not easy to predict where each precursor would be localized within the thymus.

To directly investigate that, we devised an immunofluorescence staining strategy. Flow-cytometry-based back-gating showed that among total thymocytes, PD-1hiNrs4a1GFPhi cells were predominantly CD5+TCRβ+ DN cells (Supplementary Fig. 3a). Total Tbx21GFP+CD1dtet− thymocytes were largely CD5+TCRβ− DN cells that included mainly type B IELPs (Supplementary Fig. 3b). Thus, we employed immunofluorescence-based quantitative histocytochemistry using PD-1, Nrs4a1GFP, Tbx21GFP and CD1dtet to determine the localization of type A and type B IELPs in the thymus. Comparison of flow cytometry to quantitative immunofluorescence revealed that imaging identified a greater proportion of both IELP types than did flow cytometry (Fig. 4b,e). That was in agreement with a publication reporting that cell isolation and flow cytometry can underestimate the number of T cells. We next quantified the localization of gated events: 70% of PD-1hiNrs4a1GFPhi (type A) IELPs were in the cortex, while the remaining 30% were typically in the cortico–medullary junction (Fig. 4b–d and not shown). In contrast, 75% of Tbx21GFP+CD1dtet− (type B) IELPs were present in the medulla (Fig. 4e–g).

As IL-15 is a cytokine that can induce T-bet expression, and iNKT cells in the thymus are suggested to depend on IL-15 trans-presented by medullary thymic epithelial cells, we assessed the effects of IL-15 deficiency on IELPs. Lethally irradiated Il15−/− mice reconstituted with Tbx21GFP bone marrow cells were found to be depleted of type B IELPs, while type A IELPs were still present in these mice (Supplementary Fig. 3c). Notably, CD122 expression by mature IELPs was not affected in those Il15−/− chimeras (Supplementary Fig. 3c). These results suggested ‘preferential’ localization of type A IELPs to the cortex of the thymus versus ‘preferential’ localization of type B IELPs to the medulla of the thymus.
Figure 4  Thymic localization of type A and type B IELps. (a) Flow cytometry of type A and type B IELps and CD4SP and DP thymocytes (key), stained for various chemokine receptors (horizontal axes). (b,c) Immunofluorescence microscopy (top) of thymic sections of Nr4a1<sup>GFP</sup> mice, stained for PD-1 (red) and the cortical-thymic-epithelial-cell-associated proteasomal subunit β5t (purple) and with the DNA-binding dye DAPI (gray); arrow (b) indicates a GFP<sup>hi</sup>PD-1<sup>+</sup> (type A) IELp. Yellow outline at left (b) indicates area enlarged at right; yellow outlines (c) indicate the borders of the medulla. C, cortex; M, medulla. Scale bars, 100 µm. Below, analysis of images by histo-cytometry (b, right) and analysis of thymocytes by flow cytometry (b, left), or localization of type A IELps (red dots), as determined by histo-cytometry (c). Numbers adjacent to outlined areas (b, bottom) indicate percent Nr4a1<sup>GFP</sup>PD-1<sup>+</sup> cells. (d) Frequency of type A IELps in the cortex and medulla (horizontal axis) of the Nr4a1<sup>GFP</sup> mouse thymus. (e,f) Immunofluorescence microscopy (top) of thymic sections of Tbx21<sup>GFP</sup> mice, stained for CD1dtet (red) and RORγt (blue) and with DAPI (gray); arrow (e) indicates a GFP<sup>hi</sup>CD1dtet<sup>−</sup> (type B) IELp (yellow outlines, as in b,c). Scale bars, 100 µm. Below, analysis of images above, as in b (e), and localization (as in c) of type B IELps (green dots) (f). Numbers adjacent to outlined areas (e, bottom) indicate percent CD1dtet<sup>−</sup>Tbx21<sup>GFP</sup> cells. (g) Frequency of type B IELps in the cortex and medulla (horizontal axis) of the Tbx21<sup>GFP</sup> mouse thymus. Each symbol (d,g) represents an individual image area; small horizontal lines indicate the mean. ***P < 0.001 (Student’s t test). Data are representative of two experiments with three individual mice (a) or one experiment with three mice per genotype (b,c,e,f) or are from one experiment with three mice (pooled) (d,g).
Emigration of type A IELps is dominant over that of type B IELps

We next investigated the emigration properties of IELp. The emigration of conventional T cells and IELps from the thymus is mediated by the G-protein-coupled receptor S1PR1 targeted by the transcription factor KLF2 (refs. 33–35). Among the mature IEL population, 14% of the cells were Klf2GFP+S1PR1+ (Supplementary Fig. 4a). Reciprocally, most of the Klf2GFP+S1PR1+ cells among total CD4–CD8α–CD5+TCRß+ IELps were CD122+HI-2Kb+ (Supplementary Fig. 4b). The Klf2GFP+S1PR1+ mature IEL population contained mainly type A IELps, which included a significantly higher proportion of αCD8+ cells than did the total mature type A IELp population (Fig. 5a). This suggested that type A IELps expressed αCD8+ as they became emigration-competent.

Intravenous labeling with phycoerythrin-conjugated antibody to CD4 has been used to define CD4SP thymocytes that are emigrating, as they are thought to do so from perivascular spaces at the cortico-medullary boundary36. We employed a similar technique for IELps and found that among the labeled fraction, type A IELps were more abundant than type B IELps and showed further enrichment relative to the abundance of the unlabeled paracortical IELps (Fig. 5b).

In a further approach, we defined peripheral recent thymic emigrants (RTEs) by ultrasound-guided intrathymic biotin labeling (Supplementary Fig. 5a) and analysis of streptavidin-positive cells in the spleen 24 h later (Supplementary Fig. 5b). Type A IELps showed enrichment among the splenic RTEs, relative to their proportion in the thymus tissue (Fig. 5c), in agreement with the enrichment for type A IELps in the intravenously labeled fraction (Fig. 5b). In contrast, RTE populations had a lower proportion of type B IELps than the proportion of type B IELps in the thymus tissue (Fig. 5c).

To determine if type A IELps emigrated in an S1PR1-dependent fashion, we treated Rag2GFP mice with FTY720, a functional antagonist of S1PR1, for 6 consecutive days. The frequency of GFP+ splenic CD1d tet–CD25+ TCRß+ cells was much lower in FTY720-treated mice than in untreated mice, as expected (Supplementary Fig. 5c). DN cell populations within this fraction were almost entirely depleted of mature-phenotype IELps (Supplementary Fig. 5c). Of note, the mature IELps in untreated mice were predominantly PD-1αCD8+ (Supplementary Fig. 5c), consistent with the enrichment for these cells in the Klf2GFP+S1PR1+ compartment of mature IELps. These results suggested that type A IELps were the main population exiting the thymus at steady state and that their emigration was S1PR1 dependent. However, our data also indicated that despite being retained in the thymus, a small fraction of type B IELps also emigrated.

Type B IELps do not arise from type A IELps

We analyzed IELps in FTY720-treated mice to investigate the effects of retention on their phenotype. Notably, type A IELps trapped in the thymus via treatment with FTY720 gradually lost their surface expression of PD-1 and began to express Tbx21GFP (Supplementary Fig. 6a). Bin gating on PD-1hi (gate 1), PD-1int (gate 2), PD-1lo (gate 3) and PD-1– (gate 4) mature IELps revealed an increase in numbers at all these stages (Supplementary Fig. 6a). Notably, we also found an increase in αCD8+ expression at the PD-1hi, PD-1int and PD-1– stages (Supplementary Fig. 6b), consistent with the accumulation of a trapped type A IELp population that began to differentiate toward a type B IELp phenotype. However, the population of Tbx21GFP+ type B IELps remained αCD8– and constant in number despite administration of FTY720 for 6 d (Supplementary Fig. 6a,c).

To more rigorously investigate the possibility of an immediate precursor–product relationship between type A IELps and type B IELps, we performed ‘time-stamp’ experiments. For this we used reporter mice obtained from the crossing of Cd4CreERT2 mice (with tamoxifen-inducible expression of Cre from Cd4) with mice Rosa26Rosa26-STOP mice (in which a loxp-flanked STOP cassette (floxedSTOP) precludes expression the red fluorescent protein tdTomato (TdT)). Treatment of these Cd4CreERT2 × Rosa26Rosa26STOP mice with tamoxifen results in permanent labeling of every DP or CD4+ cell with tdTomato at the time of exposure. Although neither type A IELps nor type B IELps expressed Cd4, they were both derived from DP progenitor cells (data not shown). Therefore, this approach allowed the labeling of a cohort of DP progenitor cells and tracking through differentiation thereafter. After intraperitoneal administration of tamoxifen, tdTomato expression was detected only in DP+ or CD4+ cell with tdTomato at the time of exposure. Although neither type A IELps nor type B IELps expressed Cd4, they were both derived from DP progenitor cells (data not shown). Therefore, this approach allowed the labeling of a cohort of DP progenitor cells and tracking through differentiation thereafter. After intraperitoneal administration of tamoxifen, tdTomato expression was detected only in DP+ or CD4+ cell with tdTomato at the time of exposure. Although neither type A IELps nor type B IELps expressed Cd4, they were both derived from DP progenitor cells (data not shown). Therefore, this approach allowed the labeling of a cohort of DP progenitor cells and tracking through differentiation thereafter. After intraperitoneal administration of tamoxifen, tdTomato expression was detected only in DP+ or CD4+ cell with tdTomato at the time of exposure. Although neither type A IELps nor type B IELps expressed Cd4, they were both derived from DP progenitor cells (data not shown). Therefore, this approach allowed the labeling of a cohort of DP progenitor cells and tracking through differentiation thereafter. After intraperitoneal administration of tamoxifen, tdTomato expression was detected only in DP+ or CD4+ cell with tdTomato at the time of exposure. Although neither type A IELps nor type B IELps expressed Cd4, they were both derived from DP progenitor cells (data not shown). Therefore, this approach allowed the labeling of a cohort of DP progenitor cells and tracking through differentiation thereafter.
the investigation of a small number of clones in retrogenic mice, it has been established that CD8αα IELs can be restricted by various MHC classes. Similarly to published results, we found that β2-microglobulin-deficient (B2m−/−) mice had considerably fewer IELs than wild-type mice (Fig. 7a). That finding was recapitulated by the presence of fewer type A IELp thymocytes and type B IELp thymocytes in B2m−/− mice than in wild-type mice, although the reduction was much larger in the gut (100-fold) than in the thymus (3- to 6-fold) (Fig. 7a). Conversely, only type A IELps were affected by deficiency in the classical MHC molecules H2-Kb and H2-Db (Fig. 7a), consistent with restriction of type B IELps by non-classical MHC class I molecules. The number of type B IELps (even after gating exclusively on PD-1+ NK1.1−) was also lower in Cd1−/− mice, which lack expression of CD1d, than in wild-type mice (Fig. 7b), suggestive of the existence of type 2 NKT cells within this population. Deficiency in the MHC class II molecule I-Aβ did not significantly affect type A IELps but slightly diminished the number of type B IELps (Fig. 7c).

Together our data suggested differential MHC restriction of type A IELps relative to that of type B IELps and indicated greater involvement of β2-microglobulin-dependent molecules in the maintenance of IELs in the gut than in selection in the thymus.

In addition to their differential dependence on MHC, the two IELp types also varied in use of the TCR α-chain. In a comparison of α-chain variable region 2 (Vα2) and Vα3.2, type A IELps showed the greatest use of Vα2, while type B IELps were dominated by use of Vα3.2 (Fig. 7d). Notably, the proportion of both Vα2+ IELps and Vα3.2+ IELps was intermediate between that of thymic type A IELps and type B IELps (Fig. 7d). Given the lower thymic output of type B IELps, this might reflect either greater population expansion of Vα3.2+ IELps within the gut or a greater ability of type B IELps to seed the IEL.

Figure 6 No immediate precursor–product relationship between type A IELps and type B IELps. (a) Flow cytometry of type A and type B IELp thymic populations (left margin) obtained from Cd4CreERT2 × Rosa26STOP/tdT mice on day 2, 5 or 10 (above plots) after injection of tamoxifen, pre-gated on mature phenotype IELps and defined as PD-1+(type A) or PD-1−NK1.1+(type B). Numbers above outlined areas indicate percent tdTomato+ cells. SSC, side scatter. (b) Frequency of tdTomato+ DP, CD8SP, type A or type B cells in mice as in a. Each symbol represents an individual mouse. *P < 0.05 and ***P < 0.001 (ANOVA with Bonferroni post-test). Data are pooled from three experiments with three mice (2 d and 5 d) or four mice (10 d) (mean ± s.d. in b).

Figure 7 Type A IELps and type B IELps have different antigen-receptor specificities. (a) Absolute number of CD8αα IELs and type A or type B IELps in wild-type mice (WT), and in mice deficient in classical MHC class I (H2-Kb and H2-Db; Kd−/−; Kβ−/−) or β2-microglobulin (B2m−/−), in which mature IELps were defined by CD122 expression only, due to the lack of H-2Kb. (b) Quantification of type A and NK1.1+ type B IELps in the thymus of wild-type and Cd1-deficient (Cd1−/−) mice. (c) Absolute number of cells as in a in wild-type mice and mice deficient in MHC class II (I-A−/−). (d) Frequency of Vα2+ cells (left) and Vα3.2+ cells (right) among CD8αα IELps and type A or type B IELps (key). *P < 0.05, **P < 0.01 and ***P < 0.001 (ANOVA with Bonferroni post-test (a) or Student’s t-test (b–d)). Data are pooled from six experiments with 6 mice (WT or Kd−/−) or 7 mice (B2m−/−) (a; IELs), eleven experiments with 27 mice (WT), 12 mice (Kd−/−) or 20 mice (B2m−/−) (a; type A and B IELps), four experiments with 5 mice (WT) or 9 mice (Cd1−/−) (b), five experiments with 5 mice (WT) or 7 mice (I-A−/−) (c; IELs), ten experiments with 27 mice (WT) or 14 mice (I-A−/−) (c; type A and B IELps), eleven experiments with 15 mice (d; IELs) or seven experiments with 7 mice (d; type A and B IELps) (median (center line) with 25th and 75th percentiles (box limits) and largest and smallest values (extensions above and below)).
niche despite their absence of αβ. We also investigated the β-chain variable region (Vβ) repertoire of type A and type B IELps. Among 14 Vβ chains analyzed, we found significantly less use of Vβ5.1-Vβ5.2 and Vβ9 and significantly more use of Vβ6 by type B IELps than by type A IELps (Supplementary Fig. 7). Thus, in addition to their phenotypic differences, type A IELps and type B IELps also varied in their MHC dependence and TCR use, suggestive of distinct antigen specificities and potential functions of these two subtypes.

**DISCUSSION**

Thymic precursors of intestinal CD8αα IELs and their maturation, localization and emigration have not been fully characterized. In this study, we investigated these parameters and have provided detailed analysis to this end. Our findings suggested the existence of two distinct subsets of mature IELps that we called ‘type A’ IELps and ‘type B’ IELps. The type A IELps were localized in the cortex and showed signs of strong agonist signaling. Thus, they had the expected characteristics of cells undergoing ‘clonal diversion’ at the DP stage. In contrast, type B IELps resided in the medulla and included few or no self-reactive cells. The majority of type B IELps were NK1.1+ and would have been excluded from analysis in previously published studies using NK1.1 as an exclusion marker. Our data support a model in which in addition to the ‘mainstream’ type A IELps, type B IELps with potentially different antigen specificity and function diversify the gut IEL repertoire.

In terms of the developmental relationship, we considered the possibility that type B IELps were derived from type A IELps. However, the ‘rescue’ of type A IELps, but not that of type B IELps, in Bim−/− mice and Cd28−/− mice, as well as the distinct TCR use and MHC dependence of these cells, challenge that proposal. Though we cannot exclude the possibility that a proportion of the type B IELps might have been derived from cells in the type A IELp pool, we favor the idea that the majority of type A IELps and type B IELps are on parallel developmental pathways rather than linear developmental pathways, a proposal further supported by our time-stamp experiments.

Concordantly, SPADE placed type B IELps in close relationship with CD4SP and βNKT cells, while type A IELps branched off from the DP pool. Our data also showed a differential effect of MHC deficiency on IELps. The number of type A IELps was affected most by deficiency in classic MHC class I and β2-microglobulin, while the number of type B IELps was affected by deficiency in MHC class II, CD1d and β3-microglobulin. Those findings were in line with published reports showing restriction of individual retrogenic IEL clones by various classical and non-classical MHC molecules. Due to a lack of specific markers on IELs derived from type A IELps and type B IELps, we were unable to determine the relative proportion of gut-resident lymphocytes derived from each. However, thymic type A IELps and B IELps showed different use of TCR Vα and their use of TCR Vβ was intermediate among that of IELs, which suggested that progeny of both were present in the gut.

The thymic localization and emigration properties versus the retention properties of the two precursor populations was clearly distinct. About 25–30% of type A IELps were located in proximity to the cortico–medullary junction, and we think it likely that these cortico–medullary IELps were about to emigrate from the thymus, similarly to conventional T cells. How type B IELps, which were CCR4+ and CCR7+, migrated toward and were retained in the medulla was not clear. We considered the possibility that like NKT cells, CXCR3+ type B IELps were retained by CXCR3 ligands derived from medullary thymic epithelial cells. However, we did not find differences in the proportion of type A IELps or type B IELps in Cx3cr1−/− mice relative to that in their wild-type littermates (data not shown). In addition, medullary IL-15 might have a role in this: in agreement with a study showing that IELps can develop without thymic IL-15 (ref. 38), Il15−/− chimeras reconstituted with Tbx21GFP bone marrow cells had a considerably lower proportion of type B IELps, but not of type A IELps, than that of wild-type chimeras reconstituted with Tbx21GFP bone marrow cells. It is therefore possible that type B IELps might accumulate in the medulla due to their dependence on TCR. Type B IELps were similar to NK1T1 cells in that they were IL-15 dependent and T-bet+CD44hiCXCR3+IFN-γ+ were largely retained in the thymus and also ‘preferentially’ localized to the medullass, that similarity would indicate a mutual, albeit unidentified, function in the thymus. Notably, differential MHC restriction and thymic localization also indicate selection by different cell types rather than by one specific antigen-presenting cell. These aspects should be explored further in future studies.

SIPR1 expression, under control of the transcription factor KLF2, has been established as a critical factor for the emigration of conventional T cells. While the contribution of SIPR1 to the trafficking of IELps from thymus to gut has been previously investigated, its involvement specifically in the egress of IELps from the thymus has remained controversial. Our findings suggested that type A IELps required this molecule for exiting the thymus. Of note, populations of SIPR1+ type A IELps and RTEs showed further enrichment for β2−expressing cells that promote homing to mucosal tissues. Indeed, the complete absence of type A IELps in the spleen of mice treated for 6 d with an SIPR1 antagonist indicated swift ‘parking’ in the gut and possibly other mucosal sites, or death, rather than extended circulation. Although we did not investigate this for the minor population of emigrating type B IELps, they might have the same SIPR1-dependent mechanism for exit from the thymus.

In summary, we have identified two subsets of mature thymocytes distinguished by phenotype, TCR specificity and MHC restriction: type A IELps and type B IELps. Both were able to give rise to CD8αα IELs in the small intestine. Given their disparities, functional differences between type A IELps and type B IELps are likely. Thus, the findings of this study should affect future expression-profiling studies and elucidation of the roles of IELs in homeostasis and immunity and how they relate to human IEL populations.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

R.R. and K.A.H. designed experiments; R.R., R.L.K. and Y.J.L. performed experiments and analyzed data; R.R. wrote the manuscript; S.C.J. provided input for interpretation; and K.A.H. conceptualized and directed the study and edited the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.
Quantifying memory CD8 T cells reveals regionalization of αSphingosine 1-phosphate dependence in the regulation of Tissue-specific distribution of iNKT cells impacts their cytokine αLymphocyte egress from thymus and peripheral lymphoid Thymic T cell development and progenitor localization depend

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ONLINE METHODS

Mice. C57BL/6 (B6) and B6.SJL mice were purchased from the National Cancer Institute. Tak1βfl/fl, Tak1βCreERT2, Cd4CreERT2 (T-betGFP), Nr4a1GFP (Nur77GFP), B2mCd28−/−, Rag2GFP /KIfloxedSTOP and B2mCd28−/− were obtained from Jackson Laboratories. Il15−/− mice and mice deficient in H-2K and H-2D were obtained from Taconic. TgCd4R2/− mice were obtained from Jackson Laboratories and bred with B6.SJL mice to establish a CD4.5.1 × CD4.5.2 Rag2−/− line. For chimeras, lethally irradiated recipients were reconstituted with donor bone marrow depleted of T cells and were provided water supplemented with neomycin and polymyxin B for 2 weeks. Chimeras were analyzed at a minimum of 8 weeks after reconstitution. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

IEL isolation. IELs were isolated similarly to a previously published protocol.42 Changes were made as follows: DTt was used instead of DTt, and samples were placed into a 37 °C shaking incubator for 30 min for incubation with the DTt solution. Samples were resuspended in 40% Percoll and overlaid onto 80% Percoll and spun, and the interlayer was collected and labeled for flow cytometry.

Flow cytometry. Single-cell suspensions of tissues were labeled with antibodies from Biolegend, Biolegend or BD. Antibodies to the following molecules were used (clone numbers in parentheses; dilutions follow): CD4 (RM4-5) 1:200, CD25 (PC61) 1:200, TCRβ (GL3) 1:200, αβ (DATSK2) 1:200, NK1.1 (PK136) 1:100, T-bet (B101) 1:100, K67 (16A8) 1:200,CCR4 (2G12) 1:200, CCR7 (4B12) 1:200, CXCR3 (CXC3-173) 1:200, CXCR4 (L7E6F12) 1:200, CD69 (HI2F6) 1:200, Qq2 (69H19-9) 1:200, CD45.1 (A20) 1:200, CD45.2 (104) 1:100, Vq2 (B20.1) 1:200, and Vq3.2 (RR3-16) 1:200, all from Biolegend; CD8α (53-6.7) 1:200, CD103 (M290) 1:200, TCRβ (H57-597) 1:200, H-2K β7 (A6F-88.5) 1:200, Vq2 (B20.6) 1:200, Vq3 (K25) 1:200, Vq4 (KT4) 1:200, Vq5.1/5.2 (MR9-4) 1:200, Vq6 (RR4-7) 1:200, and Vq9 (MR10-2) 1:200, all from BD; and CD44 (IM7) 1:200, CD3 (53-7.3) 1:200, PD-1 (1D3) 1:200, CD122 (TM-b1) 1:100, and CCR9 (CW-1.2) 1:100, all from eBi. Biotinylated CD1d–PBPS7 monomers were obtained from US National Institutes of Health tetramer facility and were incubated with PE, APC or PC/CF7 streptavidin to fluorochrome-labeled multimers. Cells were stained for 20 min at 4 °C. For staining for CCR4 or CCR7, the cells were collected for 30 min at 4 °C. For SIiPR1 staining thymic samples were enriched for IELp by depletion of CD4α cells (Milteny, manual MACS). Enriched cell populations were labeled with 10 μg rat IgG2 anti-mSIiPR1 (R&D Systems, clone 714312) per 1 x 106 cells for 30 min at 4 °C. The samples were then labeled with biotin anti-rat IgG (BD clone G28-5, 1:200) for 20 min on ice and subsequently incubated with streptavidin-fluorochrome (BioLegend cat.# 405229, 1:100). Before staining with other markers, the cells were blocked with 5% rat serum and 1% free streptavidin.

SPADE. Spanning-tree analysis was performed using the Cytobank Premium online platform (https://premium.cytobank.org/cytobank/) and according to previous publications.18,19 Heat maps were created with the Matrix2png version 1.2.1 online platform (http://www.chibi.ubc.ca/matrix2png/).

Adaptive-transfer studies. Pooled thymic single-cell suspensions were enriched for IELps by depletion of CD8α and/or CD4α cells by manual MACS (Milteny). IELps were then purified by flow cytometry. Within the live CD25 CD1d/tetramer-TCRβ−/− CD4−/− CD8α− population, CD5+TCRβ+ cells were divided and purified as CD122+PDL1− (type A) IELps or CD122−PDL1− (type B) IELps. Between 5 x 105 and 28 x 106 cells were transferred into recipients by intravenous injection. Total DN competitor cells were purified by MACS-mediated depletion of CD4α and CD8α cells (Milteny).

Immunofluorescence. Immunofluorescence analysis of Tbx2CreERT2 thymus was described previously.17 In brief, thymi were incubated overnight in PBS containing PE labeled CD1d-tetramer loaded with PBS57 at 4 °C. Thymi were then washed, fixed with 4% paraformaldehyde (PFA) for 1 h and snap frozen. 5-μm sections were blocked with PBS containing 5% bovine serum albumin (BSA) and goat serum (Jackson Laboratory) before staining. Clone RORG2 (Millipore, 1:200) was used for staining of RORγt. Processing of Nr4a1GFP thymi up to labeling of the tissue with the primary antibody rabbit anti-mouse β5t (MBL, cat. # PD021, 1:100) was performed as previously described13 with the exception of the use of 3-μm sections. In brief, thymi were fixed in 4% PFA overnight, followed by immersion in 15% (wt/vol) sucrose in PBS overnight. Thymi were then frozen. For immunofluorescence, sections were treated with 0.1% Triton-X100 in PBS (PBST) for 5 min at 20 °C and then were incubated in PBS 3% BSA for 1 h at 20 °C before staining. For staining of PD-1, goat anti-mouse PD-1 (R&D Systems, cat. # AF1021, reconstituted at 0.2 mg/ml, used at 1:50) was added with the anti-β5t (MBL, cat. # PD021, 1:200) for overnight labeling. Secondary antibody staining was done with AF647 donkey anti-rabbit IgG (Jackson ImmunoResearch, cat. # 711-605-152, 1:200) and AF550 donkey anti-goat IgG (Invitrogen, cat. # A-21432, 1:300) for 2 h at 20 °C before DAPI staining. The sections were then covered with Prolong anti-fade mounting medium (Life Technologies) and images were obtained 1 to 3 d later with a Leica DM6000B Epi-Fluorescent microscope.

Histo-cytometry. Histo-cytometry was performed as described previously.17 The quantified values represent the frequency after normalization to the total average cortical and medullary areas as previously described.17

Intravenous labeling of cells in the perivascular space. PE-conjugated anti-CD45.2 (BioLegend, clone 104) was injected intravenously (1 μg per mouse), and mice were euthanized for tissue collection 3–4 min after injections. Initially, perfused mice were compared with non-perfused mice. As no substantial differences were observed, subsequent analyses used non-perfused mice. Labeled cells were isolated from single-cell suspensions with anti-PE beads (Milteny) and manual MACS before further labeling for flow cytometry. Potential contamination of the labeled fraction by cells in circulation was negligible, as indicated by the largely different proportions of various cell types in the blood (Supplementary Fig. 4c, d), and by different ratios of CD8SP cells to CD4SP cells or mature IELps to CD4SP cells in the blood (Supplementary Fig. 4c).

FTTY720 treatment. 25 μg FTTY720 (Calbiochem) in PBS was injected intraperitoneally into mice for 6 consecutive days, and tissues were harvested on day 7. Mock-treated and untreated mice were used as controls.

Ultrasound-guided intrathymic injection (RITE assay). Intrathymic injection of 10–20 μl EZ-Link Sulfo-NHS biotin (ThermoFisher Scientific) was performed as described previously43 and with the Vevo 2100 System. Two factors substantiated the accuracy of this method: First, the proportion of streptavidin-positive B220+ cells was less than 5% (Supplementary Fig. 3b). Second, in analyses of Rag2GFP mice, over 90% of streptavidin+ TCRβ+ cells were GFP (Fig. 5c and Supplementary Fig. 5b), indicative of their recent thymic origin.

Time-stamp labeling. Cd4CreERT2 mice were bred with Rosa26flloxstop mice. The offspring were given intraperitoneal injection of 2 mg tamoxifen (Sigma-Aldrich) in sunflower oil 2% EtOH on 1 or 2 consecutive days. The thymus of tamoxifen-treated recipients was analyzed on various days after injection.

Statistical analysis. Prism 5.0 and 6.0 were used for data analysis and arrangement. For comparison of two data sets, the unpaired two-tailed Student’s t test was used. For comparison of three or more data sets, one-way ANOVA with Bonferroni post-test was performed. Details on sample size, experimental replicates and statistics are included in the figure legends.

Data availability statement. The data used to support the conclusions of our study are available from the corresponding author upon request.

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