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In vivo blockade of T cell development reveals alternative pathways for generation of intraepithelial lymphocytes in mice

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ABSTRACT

Intraepithelial lymphocytes (IELs) are resident cells localized within the intestinal epithelia and play an important role in regulating gut inflammations and host defense against pathogens. $CD8\alpha^+$ $TCR\alpha\beta^+$ IELs are heterogeneous populations that are generated from T cell precursors including $CD4^-$ $CD8\alpha^-$ double-negative (DN) cells and $CD4^+$ $CD8\alpha^+$ double-positive (DP) cells. However, developmental pathways of $TCR\alpha\beta^+$ IELs remained unclear. To gain insight into the mechanisms, we generated mice (Bcl11b$^{\Delta DN2}$ mice) that lack thymic precursors (DN $CD5^+$ $TCR\beta^+$ cells) for $CD4^-$ $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs. Unexpectedly, we found that, in the absence of the precursors in thymi of Bcl11b$^{\Delta DN2}$ mice, $CD4^-$ $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs were still present in the intestine though the number was reduced. Adoptive transfer experiment showed that their precursors were highly enriched in $CD8\alpha^+$ $TCR\beta^-$ thymocytes. The $CD4^-$ $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs in Bcl11b$^{\Delta DN2}$ mice are distinguished by Thy1.2 expression and are indeed present in WT mice. Taken together, our study reveal a novel developmental pathway for $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs.

1. Introduction

Intestine in mammals is one of the mucosal tissues that are continuously exposed to challenges by pathogenic organisms in ingested food and water. Intraepithelial lymphocytes are localized within the epithelium of the intestine, and play an important roles in maintaining homeostasis by fighting against pathogens and regulating excessive inflammations [1–3]. Indeed, these populations are highly conserved in vertebrates including fish and mammals [4]. Therefore, elucidation of full pictures of developmental pathways of IELs provides us novel insights into T cell biology involved in mucosal immunity.

Bcl11b is a transcription factor essential for T cell development [5–7]. T cell development is initiated after migration of haematopoietic stem cells to the thymus. Most immature T cell precursors are identified by the lack of CD4 and $CD8\alpha$ expressions, known as double-negative (DN) cells. DN cells are further classified into 5 populations, DN1, DN2a, DN2b, DN3 and DN4 cells, as they mature. T cell identity is fixed at the DN2b stage at which Bcl11b has an indispensable role. Indeed, in Bcl11b-deficient mice, T cell development is completely blocked at the DN2a stage. DN4 cells become DP cells at which $TCR\alpha$ rearrangement occurs and then give rise to conventional $\alpha\beta$ T cells and unconventional $\alpha\beta$ T cells including invariant natural killer T (iNKT) cells and $CD4^-$

$CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs [8–11]. During the intrathymic development of $CD4^-$ $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs, DN $CD5^+$ $TCR\beta^+$ cells were identified as their precursors in the thymus after the DP stage [9]. In contrast to $\alpha\beta$ T cell development through the DP stage, iNKT cell subsets bypassing DP stage have been identified [12]. Thus, Bcl11b-deficient mice could be a novel tool for analysis of T cells derived from DN stages.

To gain insights into developmental pathways of $TCR\alpha\beta^+$ IELs, we generated mice in which T cell development was blocked before the DP stage by inducing T cell-specific disruption of Bcl11b (designated as Bcl11b$^{\Delta DN2}$ mice hereafter), because Bcl11b-deficient mice were shown to be fatal around perinatal period [13,14]. Here we found that DN $CD5^+$ $TCR\beta^+$ thymocytes were absent in Bcl11b$^{\Delta DN2}$ mice. Unexpectedly, however, $CD4^-$ $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs were still present in the small intestine of Bcl11b$^{\Delta DN2}$ mice. Thy1.2 expression was higher in $CD4^-$ $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs from Bcl11b$^{\Delta DN2}$ mice than those from WT mice. Adoptive transfer experiment suggested that precursors for Thy1.2$^+$ $CD4^-$ $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IELs were enriched in $CD8\alpha^+$ $TCR\beta^-$ thymocytes. Thus, we identify a novel developmental pathway for $CD8\alpha\alpha^+$ $TCR\alpha\beta^+$ IEL subset.

Fig. 1. Precursors for CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs are completely absent in Bcl11b$^{\Delta DN2}$ mice.

Thymocytes in WT and Bcl11b$^{\Delta DN2}$ mice were analyzed after gating on total lymphocytes (A) or DN cells (B). (C) Bar graph shows absolute numbers of TCRβ$^+$ IEL precursors (TCRβ$^+$CD5$^+$) in thymus from WT (n = 8) and Bcl11b$^{\Delta DN2}$ (n = 7) mice. Asterisks indicate statistical significance as determined by Student's *t*-test (***, p < 0.001). Data are representative of more than five independent experiments.

2. Materials and methods

2.1. Mice

Bcl11b$^{flox/flox}$ mice were provided by R. Kominami (Niigata University, Niigata, Japan). *Rag1-Cre* mice were provided by T.Rabbits (Leeds Institute of Molecular Medicine, Leeds, United Kingdom). *CD3ε$^{\Delta5/\Delta5}$* mice were kindly provided by Bernard Malissen (Centre d'Immunologie de Marseille- Luminy, France). Bcl11b$^{\Delta MatT}$ mice were generated by crossing *Bcl11b$^{flox/flox}$* mice with *MaT-Cre* mice which were kindly provided by S Yamasaki (Kyushu University, Fukuoka, Japan) [15]. 8–12 week-old mice were used throughout the experiments. Experiments were carried out in accordance with Guidelines for Animal Experiments. This study was approved by Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University.

2.2. Cell preparation from various tissues

Small intestine tissues were dissected and Peyer's Patches were carefully removed. After flushing of fecal contents with ice-cold HBSS, small intestines were opened longitudinally, gently rinsed several times of the same ice cold buffer and cut laterally into small pieces, and placed in 50 ml plastic tubes, incubated in 199 media buffer for 30 min at 37 °C with constant shaking in water bath. After centrifugation at 440*g* for 5 min, pellets were resuspended in RPMI 1640 containing 10% FCS. Dissociated cells were filtered through the gauze mesh and cells were further purified using 40/70% Percoll gradient centrifugation for 20 min at 2200 rpm. Single cell suspensions of thymocytes were prepared by grinding the organs by ends of two glass slides.

2.3. Antibodies and flow cytometric analysis

The following antibodies were purchased from Biolegend (San Diego, CA): anti-TCRβ (H57-597), anti-CD8α (53–6.7), anti-CD8β (YTS156.7.7), anti-CD5 (53–7.3), Anti-CD4 (RM 4–5), anti-CD103 (2E7), anti-B220 (RA3-6B2) and anti-Thy1.2 (53–2.1). Anti-I-A/I-E (M5/114.15.2) mAb was obtained from eBioscience (San Diego, CA). FITC-conjugated anti-Bcl11b (Clone 25B6) mAb was obtained from Abcam. Dead cells were excluded adding propidium iodide. Intracellular staining was performed according to the manufacturer's instructions (BD Biosciences). Stained cells were acquired on FACS Verse flow cytometer (BD Biosciences). The data were analyzed using FlowJo software version 9.9.4 (Tree Star).

2.4. Adoptive transfer experiment

After fluorescence-activated cell sorting by BD FACS Aria (BD Biosciences), cells (1 × 10^5 cells/mouse) were transferred into gender-matched *CD3e$^{\Delta5/\Delta5}$* mice. At 7–8 weeks after transfer, mice were sacrificed for the analysis.

2.5. Statistical analysis

Statistical analysis was calculated by the Student *t*-test using Prism software (GraphPad Software, San Diego, CA). Differences with values of p < 0.05 were considered to be statistically significant.
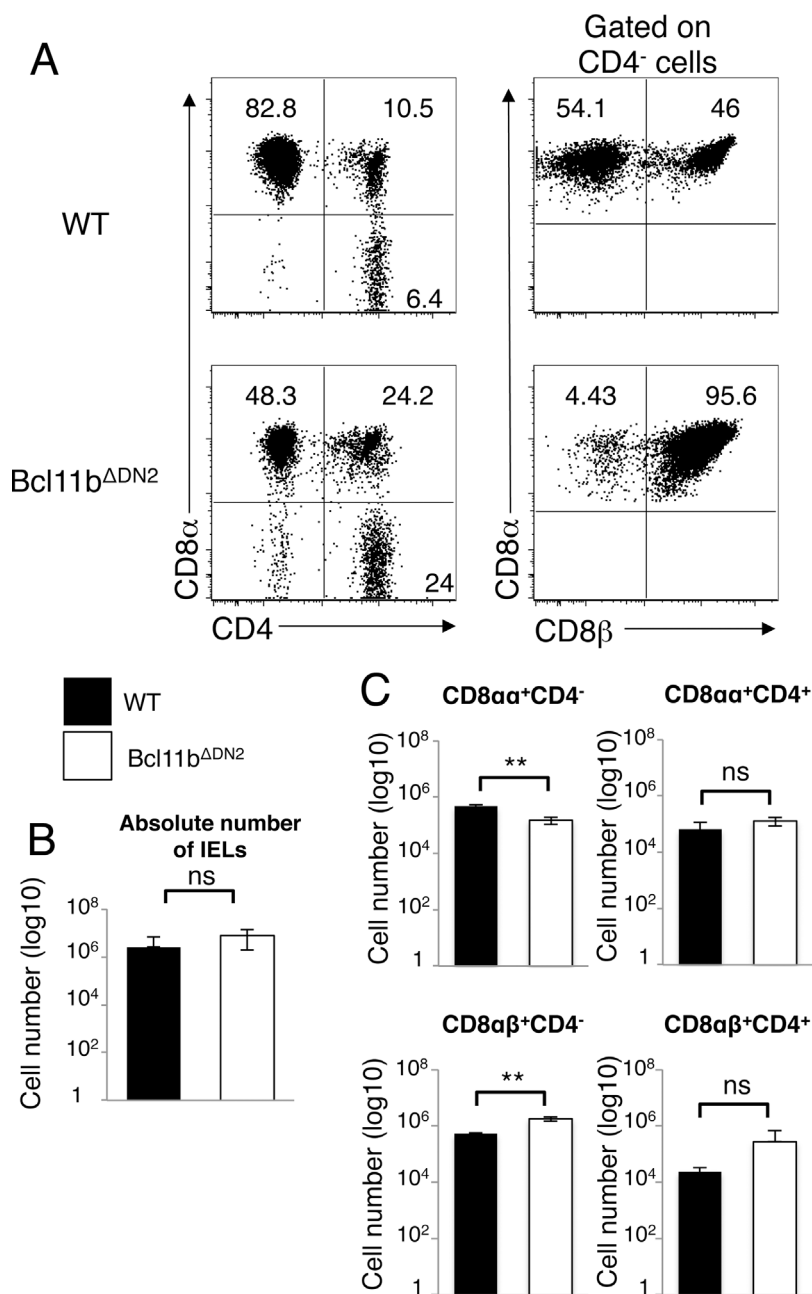
Fig. 2. TCRαβ$^+$ IEL subsets in Bcl11b$^{\Delta DN2}$ mice.
(A) Representative dot plots of IELs from small intestine of WT and Bcl11b$^{\Delta DN2}$ mice are shown after gating on TCRαβ$^+$ cells (left) or CD4$^-$ TCRαβ$^+$ cells (right). Numbers in quadrants indicate percentage of each cell subsets. (B, C) Bar graphs show absolute numbers of indicated IEL populations of WT (n = 8) and Bcl11b$^{\Delta DN2}$ (n = 8) mice are shown. Data are representative of four independent experiments. Asterisks indicate statistical significance as determined by Student's *t*-test (**, p < 0.01; ns, not significant).

3. Results

3.1. CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs develop in the absence of DN CD5$^+$ TCRβ$^+$ thymocytes

As expected, due to the blockage of T cell development at the DN2a stage, DP cells were almost completely absent in the thymus of Bcl11b$^{\Delta DN2}$ mice (Fig. 1A) and thereby DN CD5$^+$ TCRβ$^+$ precursors for CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs were not observed (Fig. 1B and C). However, in the intestine of Bcl11b$^{\Delta DN2}$ mice, CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs were still able to develop in Bcl11b$^{\Delta DN2}$ mice though the number was significantly reduced (Fig. 2A and C). In contrast, numbers of other IEL subsets were increased in Bcl11b$^{\Delta DN2}$ mice. We confirmed that

Bcl11b expressions were not observed in both TCRαβ$^+$ thymocytes and TCRαβ$^+$ IELs of Bcl11b$^{\Delta DN2}$ mice (Fig. S1), suggesting that they were not leaky populations due to insufficient expression of Cre recombinase. To examine cell-intrinsic effects of Bcl11b which regulates T cell development [16], we generated mice in which Bcl11b was deleted in mature T cells (designated as Bcl11b$^{\Delta MatT}$ mice). However, proportional changes in TCRαβ$^+$ IEL subsets were not evident in Bcl11b$^{\Delta MatT}$ mice suggesting less impact of Bcl11b deficiency after the development of TCRαβ$^+$ IELs (Figs. 3 and S2). These results suggested that, in WT mice, Bcl11b-dependent DN CD5$^+$ TCRβ$^+$ cells were the most dominant precursors for CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs. Moreover, Bcl11b$^{\Delta DN2}$ mice revealed the presence of as-yet-unidentified precursors for CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs.
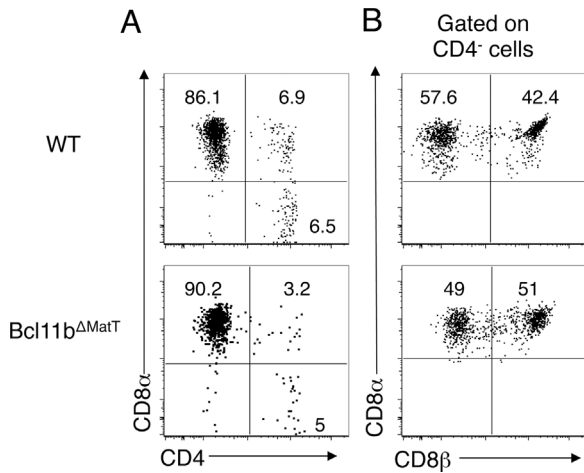
Fig. 3. No impacts of TCRαβ$^+$ IEL subsets by Bcl11b deletion after DP stage. TCRαβ$^+$ IEL subsets from WT and Bcl11b$^{\Delta MatT}$ mice were analyzed after gating on total lymphocytes (A) and CD4$^-$ lymphocytes (B). Data are representatives of three independent experiments.
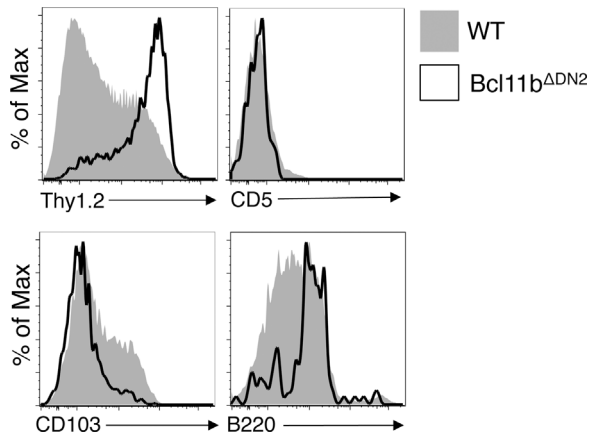

Fig. 4. Identification of surface markers for CD8αα$^+$ TCRαβ$^+$ IELs. (A) Histograms show expressions of CD5, Thy1.2, CD103 and B220 on CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs from WT (filled) or Bcl11b$^{\Delta DN2}$ (open) mice. Data are representative of at least four independent experiments.

3.2. Thy1.2 is a marker for CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs in Bcl11b$^{\Delta DN2}$ mice

Next, we searched for the markers to identify CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs in Bcl11b$^{\Delta DN2}$ mice. Consistent with previous reports [17,18], in WT mice, CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs were mostly negative for Thy1.2 (Fig. 4). In contrast, in Bcl11b$^{\Delta DN2}$ mice, CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs were mostly positive for Thy1.2 similar to other TCRαβ$^+$ IEL subsets (Figs. 4 and S3). CD5 expression on CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs was negative in both WT and Bcl11b$^{\Delta DN2}$ mice. B220 and CD103 (also known as aE integrin) were shown to be additional markers for identification of CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs

[19,20] and B220 expression but not CD103 expression was slightly higher in Bcl11b$^{\Delta DN2}$ mice. These results suggested that CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs in Bcl11b$^{\Delta DN2}$ mice were distinguished by expressions of Thy1.2.

3.3. Two types of CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs develop from distinct intrathymic precursors

To test the precursor activity, total thymocytes were transferred into CD3ε$^{\Delta 5/\Delta 5}$ mice devoid of all T cells. After 8 weeks post transfer, appreciable level of CD4$^-$ CD8αα^{++} TCRαβ$^+$ IELs was observed (Fig. 5A). To further characterize the precursors, we transferred DN cells of Bcl11b$^{\Delta DN2}$ mice into CD3ε$^{\Delta 5/\Delta 5}$ mice. As shown previously [9,21], we confirmed that WT DN cells gave rise to CD8α^{++} TCRαβ$^+$ IELs (Fig. S4) and the most dominant IEL subsets were CD4$^+$ cells. However, after transfer with DN thymocytes from Bcl11b$^{\Delta DN2}$ mice, we could not observe TCRαβ$^+$ IELs (Fig. S4). Since CD8α$^+$ TCRβ$^-$ thymocytes were observed in Bcl11b$^{\Delta DN2}$ mice (Fig. 1A), we next examined their precursor activity. After reconstitution with the CD8α$^+$ TCRβ$^-$ thymocytes from Bcl11b$^{\Delta DN2}$ mice, CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs were observed and expressed Thy1.2 and importantly, these populations were also generated from CD8α$^+$ TCRβ$^-$ thymocytes from WT mice (Figs. 5 B, C and S5). These results suggested that CD8α$^+$ TCRβ$^-$ thymocytes contain precursors for Thy1.2$^+$ CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs in both WT and Bcl11b$^{\Delta DN2}$ mice.

4. Discussion

In the present study, we identified alternative pathways for the development of CD8αα$^+$ TCRαβ$^+$ IELs. Our data suggest that Bcl11b$^{\Delta DN2}$ mice are the useful model to dissect the developmental pathways in TCRαβ$^+$ IELs as well as γd T cells [13].

Currently accepted developmental process about TCRαβ$^+$ IELs was made by fate mapping approach clearly demonstrating the presence of DP cell-derived TCRαβ$^+$ IELs [22]. However, the nature of DN cell-derived TCRαβ$^+$ IELs remained to be elucidated [23]. The data using Bcl11b$^{\Delta DN2}$ mice suggest the presence of DN cell-derived TCRαβ$^+$ IELs. These results are reminiscent of recent study showing that DN cell-derived iNKT cells [12]. In this sense, it is interesting to see whether DN cell-derived iNKT cells develop in Bcl11b$^{\Delta DN2}$ mice. Nevertheless, since a tiny DP population was observed in Bcl11b$^{\Delta DN2}$ mice, we could not exclude the possibility that Thy1.2$^+$ CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs developed from the DP stage.

Under normal condition, DP cell-derived Thy1.2$^-$ CD8αα$^+$ TCRαβ$^+$ IELs predominate and Thy1.2$^+$ CD8αα$^+$ TCRαβ$^+$ IELs are a minor population among total CD8αα$^+$ TCRαβ$^+$ IELs. However, under lymphopenic condition, it was previously shown that DN cells were migrated to the intestine where CD8αα$^+$ TCRαβ$^+$ IELs were generated [17]. These data support the idea that Thy1.2$^+$ CD8αα$^+$ TCRαβ$^+$ IELs could be generated from DN cells. However, underlying mechanism for the lack of competitiveness of Thy1.2$^+$ CD8αα$^+$ TCRαβ$^+$ IELs remains unknown.

Precursors for CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs were identified in DN CD5$^+$ TCRβ$^+$ thymocytes [9]. More recently, these populations were further classified into two sublineages based on requirement of TAK1 for their development [21]. Here we found that CD8αα$^+$ TCRαβ$^+$ IELs were observed in mice that lack DN CD5$^+$ TCRβ$^+$ thymocytes and transfer experiment showed that CD8α$^+$ TCRβ$^-$ thymocytes were
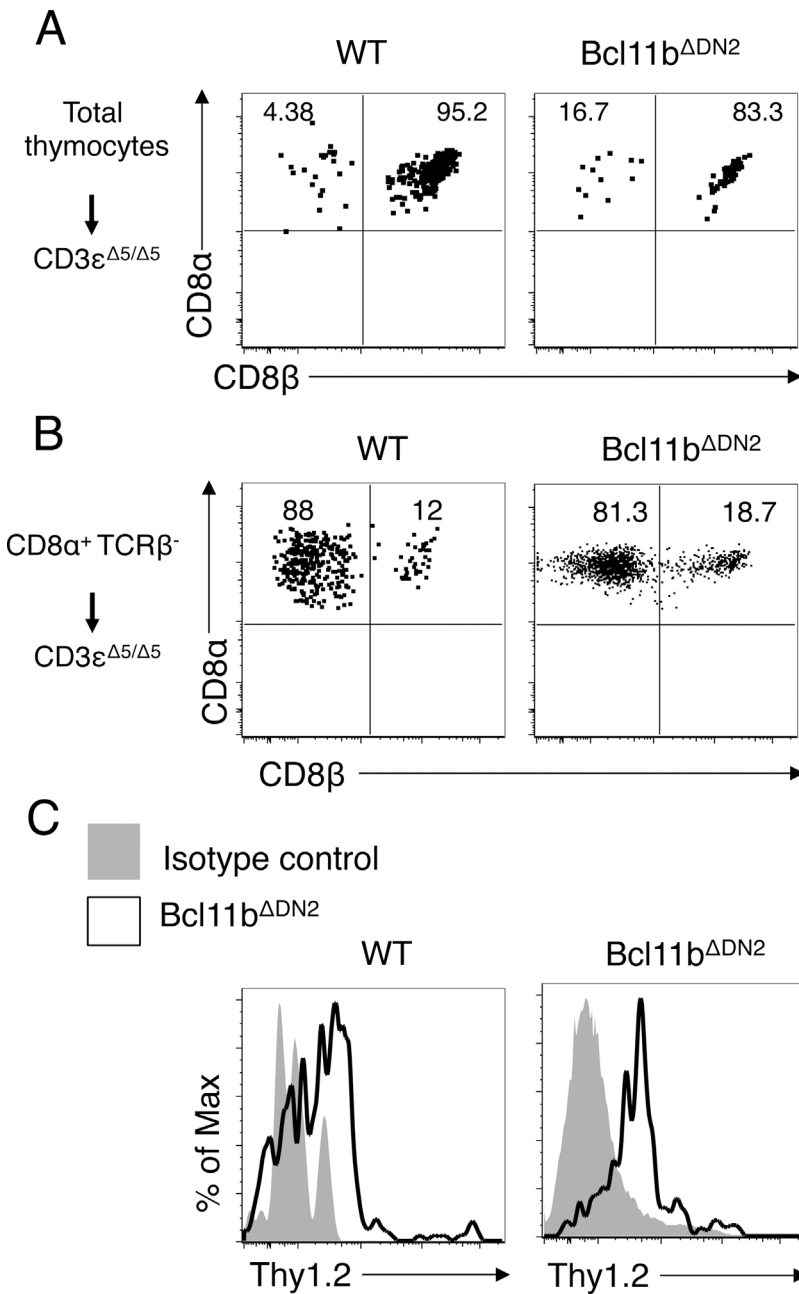
Fig. 5. Thy1.2$^+$ CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs are thymus-derived. (A, B) Whole thymocytes (A) or CD8α$^+$ TCRβ$^-$ thymocytes (B) from WT and Bcl11b$^{\Delta DN2}$ mice were transferred into *CD3ε$^{\Delta5/\Delta5}$* mice. After 8 weeks post transfer, IELs from recipient mice were analyzed after gating on CD4$^-$ CD8α$^+$ TCRαβ$^+$ cells. Thy1.2 expression was analysed after gating on CD4$^-$ CD8α$^+$ TCRαβ$^+$ cells after transfer of CD8α$^+$ TCRβ$^-$ thymocytes from WT and Bcl11b$^{\Delta DN2}$ mice (C). Grey filled histogram shows isotype control. Numbers in quadrants indicate percentage of each cell subsets. Data are representatives of three independent transfer experiments.

identified as precursors for Thy1.2$^+$ CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs. These results suggest that at least two independent pathways for the development of CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs in the thymus. To understand differences between Thy1.2$^-$ and Thy1.2$^+$ CD8αα$^+$ TCRαβ$^+$ IELs, expression levels of markers related to functions such as NK markers (NK1.1, NKG2D, CD49b) and Granzyme B were compared, but we have not seen any differences so far (data not shown).

5. Conclusion

In conclusion, among heterogeneous IEL populations including CD4$^-$ CD8αα$^+$ TCRαβ$^+$ IELs, CD4$^-$ CD8αβ$^+$ TCRαβ$^+$ IELs and CD4$^+$ TCRαβ$^+$ IELs, our study provides us additional insights into the development of CD8αα$^+$ TCRαβ$^+$ IELs as depicted in the graphical figure (Fig. 6).
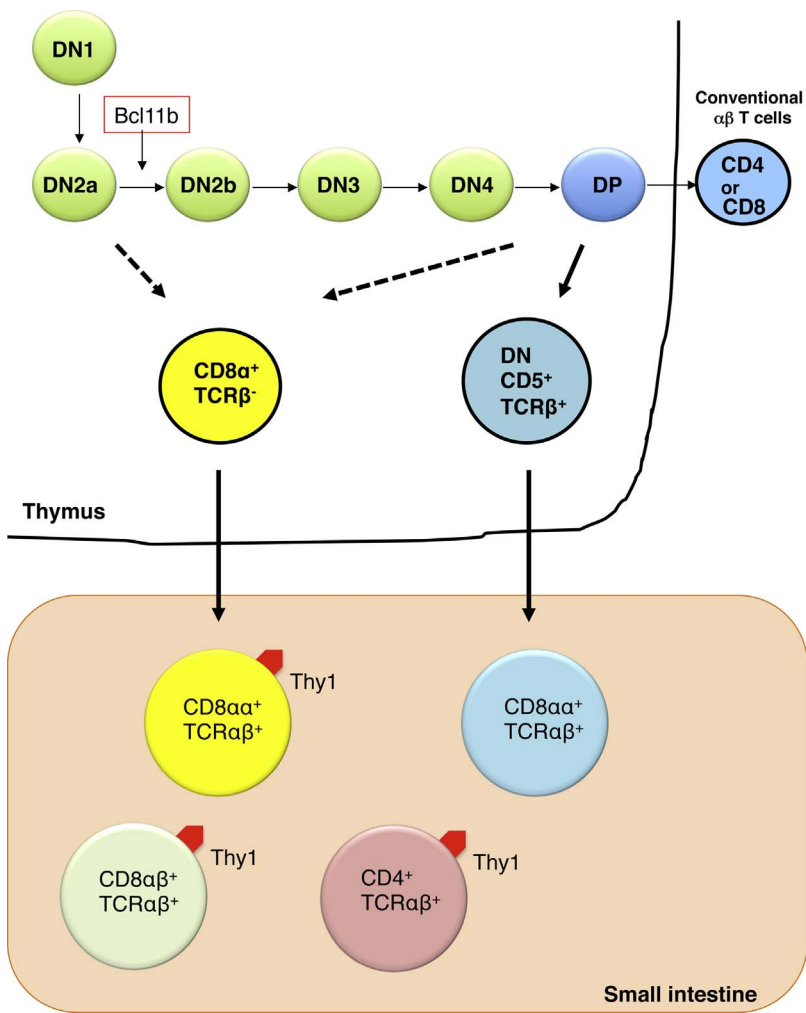
Fig. 6. Developmental pathways of TCRαβ$^+$ IELs.
In addition to well-known precursors (CD5$^+$ TCRβ$^+$ cells) for CD8αα$^+$ TCRαβ$^+$ IELs, CD8α$^+$ TCRβ$^-$ thymocytes were identified as novel precursors. After migration to the small intestine, Thy1.2$^+$ and Thy1.2$^-$ CD8αα$^+$ TCRαβ$^+$ IELs are generated.

Conflicts of interest

The authors have no financial conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2017.09.008.

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