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CD155 Is Involved in Negative Selection and Is Required To Retain Terminally Maturing CD8 T Cells in Thymus

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During their final maturation in the medulla, semimature single-positive (SP) thymocytes downregulate activation markers and subsequently exit into the periphery. Although semimature CD4+ SP cells are sensitive to negative selection, the timing of when negative selection occurs in the CD8 lineage remains elusive. We show that the abundance of terminally matured CD8+ SP cells in adult thymus is modulated by the genetic background. Moreover, in BALB/c mice, the frequency of terminally matured CD8+ SP cells, but not that of CD4+ SP cells present in thymus, varies depending on age. In mice lacking expression of the adhesion receptor CD155, a selective deficiency of mature CD8+ SP thymocytes was observed, emerging first in adolescent animals at the age when these cells start to accumulate in wild-type thymus. Evidence is provided that the mature cells emigrate prematurely when CD155 is absent, cutting short their retention time in the medulla. Moreover, in nonmanipulated wild-type mice, semimature CD8+ SP thymocytes are subjected to negative selection, as reflected by the diverging TCR repertoires present on semimature and mature CD8+ T cells. In CD155-deficient animals, a shift was found in the TCR repertoire displayed by the pool of CD8+ SP cells, demonstrating that CD155 is involved in negative selection. The Journal of Immunology, 2010, 184: 1681–1689.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: DC, dendritic cell; DP, double-positive; ISP, immature single-positive; KO, knockout; mLN, mesenteric lymph node; mTEC, medullary epithelial cell; pLN, peripheral lymph node; RTE, recent thymic emigrant; S1P, sphingosine 1-phosphate; SP, single-positive; WT, wild-type.

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accumulate in an age-dependent fashion in wild-type (WT) BALB/c thymus, offering an explanation for why the paucity of CD8\(^+\) SP cells triggered by CD155 deficiency starts to emerge in adolescent mice. Because we failed to observe defects in maturation and the degree of emigration or apoptosis regarding CD155-deficient CD8\(^+\) SP thymocytes, our findings suggest that newly matured CD8\(^+\) thymocytes cannot be retained in the medulla when CD155 expression is missing. This demonstrates that the adhesive mechanisms keeping SP cells inside the medulla differ between CD4\(^+\) and CD8\(^+\) SP cells, semimature and mature CD8\(^+\) SP cells, and mature CD8\(^+\) SP cells produced in young versus adult mice.

**Materials and Methods**

**Mice**

WT BALB/c (H2\(^d\)) and C57BL/6 (H2\(^b\)) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) or bred in Hannover Medical School’s animal facility. DBA/1 (H2\(^b\)), C3H (H2\(^b\)), and SJL (H2\(^b\)) mice were purchased from Janvier (Le Genest St. Isle, France). CD155 knockout (KO) mice were described before (37). Mice were crossed onto the BALB/c background for at least six generations. All experiments including animals were conducted according to the regulations of the local government and institutional guidelines.

**Flow cytometry**

Single-cell suspensions of thymi were obtained by mincing the organs through a cell strainer. Cells were washed in FACS buffer (PBS/2% FBS), counted, and stained in 96-well plates according to standard procedures, in 50 \(\mu\)l reaction volume. After three final washes, FAPi was added to exclude dead cells from analysis. The Annexin-V-Fluos kit (Roche, Basel, Switzerland) was used to analyze apoptosis.

The following Abs were used: \(\text{\textit{V}}\text{\textit{B}}\text{\textit{V}} 2,3,4,5,6,7,8,11,12\text{ TCR (BD Biosciences, San Jose, CA); CD24-FITC, CD69-PerCp-Cy5.5, CD45.2 PerCp-Cy5.5, Ly51, and SA-PerCP (all from BD Biosciences); CD24-Alexa Fluor 488, CD44-Alexa Fluor 647, CD44-APC, CD44-APC, CD11b-PE, DX5-bio, oβTcR, and SA-Pacific Orange (all from Invitrogen, Karlsruhe, Germany); CD17-PE; (eBioscience, Frankfurt, Germany); Ep CAM-PE (eBioscience); and CD24-PE-Cy7 (Biolegend, Uithoorn, The Netherlands). The following homemade Abs were used: CD3-bio (17A2), CD4-bio (RMCD4-2), CD8-bio (RMCD8-2), CD19 (1D3), TER119, GR1, β2 TCR-bio (GL3), CD26L (MEL-14), B220 (TIB146), CD155 (4G3), and CD226 (3B3); they were either used directly labeled as indicated or detected with fluorochrome-labeled secondary Abs. Flow cytometry was performed on an LSRII flow cytometer (BD Biosciences); the data were evaluated using WinList 5.0.

**Ab treatment of mice**

WT BALB/c mice were injected i.v. with 400 \(\mu\)g anti-CD155 mAb 4G3 (37) or an isotype control mAb every 5 d. The animals were sacrificed 28 d following the first injection.

**FTY720 application**

Mice were administered sterile water or FTY720 (40 \(\mu\)g/mouse; Calbiochem, San Diego, CA) in drinking water or by oral gavage every 3 d. Ten to 12 d later, mice were sacrificed and analyzed. Drug effectiveness was confirmed by analyzing lymphopenia induction in the blood of treated animals (data not shown).

**Intrathymic FITC injection**

Animals were anesthetized, and the chest was disinfected with 70% ethanol. A 5-mm longitudinal incision was made into the skin directly above the sternum, then the area was cleaned with 70% ethanol by wiping carefully with a lint-free cloth, followed by scanning the sternal/rib area for proper injection (the thymus is located underneath the second rib, close to the sternum). A 27G needle was used to inject 10 \(\mu\)l 1 mg/ml FITC solution (Sigma-Aldrich, St. Louis, MO) per lobe. The injection depth was ∼5 mm. The skin incision was closed with surgical clips. The spleen and lymph nodes were harvested for analysis ∼40 h postinjection.

**Bone marrow chimeras**

To enable the analysis of bone marrow chimeras at an age when thymus development culminates, newborn mice no older than 1 d were subjected to two irradiations of 2.5 Gy each, separated by 4 h, followed by bone marrow transfer. Bone marrow was harvested from donor mice and depleted for differentiated cells by applying a lineage mixture of rat anti-mouse Abs recognizing Ter119, CD4 (RMCD4-2), CD86 (RMCD6-2), CD11b (M1/70, eBioscience), CD19 (1D3), DX5 (Biolegend), and Gr1, followed by incubation with sheep anti-rat IgG coupled to Dynabeads (Invitrogen). Magnetic separation was performed according to the manufacturer’s instructions. Lineage negative cells were concentrated in PBS to allow intrahepatic injection of 2 \(\times\) 10\(^7\) cells in a total of 25 \(\mu\)l. Mice were analyzed 8–10 wk following the bone marrow transfer. All animals exerted normal behavior, yet they were distinguished by a substantially reduced weight compared with age-matched controls.

**Real-time PCR analysis**

Cells were prepared from thymi pooled from five WT or CD155-deficient mice. CD8 SP cells were enriched by negative selection, removing CD4\(^+\) cells via anti CD4-biotin/M-280 Streptavidin Dynabeads (Invitrogen). Then staining with anti CD8α-Cy5/CD24-PECy7 was done, followed by flow cytometric sorting into CD8\(^+\)CD24\(^+\) and CD8\(^+\)CD24\(^-\) fractions. Successful separation was controlled by reanalysis. Up to 5 \(\times\) 10\(^5\) cells were used for RNA isolation (Absolutely RNA Microprep kit, Stratagene, La Jolla, CA); subsequently, synthesis of the first strand of cDNA (SuperScript II reverse transcriptase; Invitrogen) was done using random hexamer primers. The expression of GAPDH and S1P\(_2\) was analyzed using a Lightcycler 2.0 (Roche) and the Fast Start DNA Master plus SYBR Green Kit (Roche) or the SYBR Premix Ex Taq Kit (Takara Shuzo, Otsu, Japan). The primers applied and the relative comparative quantification (2\(^{-}\Delta\Delta CT\)) expression levels was done based on GAPDH expression, as described earlier, except that a cloned PCR product representing the GAPDH amplification was used for standardization (39).

**Results**

**The number of CD8\(^+\) SP cells is severely reduced in thymus of CD155-deficient mice**

In our earlier analyses of CD155-deficient mice, we noticed regular numbers of peripheral T cell subsets in all secondary lymphoid organs investigated (37). Moreover, we also failed to observe any differences in size or obvious anatomical and morphological aberrations in the lymphoid organs (37). When investigating CD155-deficient thymi of adult BALB/c mice (8–10 wk) by immune histology, regularly sized and distributed cortico-medullary areas were detected (not shown). In addition, determination of overall thymic cell counts failed to reveal significant differences compared with WT mice. However, when analyzing the cellular composition of thymocytes by flow cytometry, we found a significant reduction in the frequency of CD8\(^+\) SP cells in mutant thymi (Fig. 1A; WT: 5.08% ± 1.06%; KO: 3.23% ± 0.60%), which was paralleled by a slight increase in the percentage of DP cells (data not shown). In contrast, the frequency of CD4\(^+\) SP cells remained unchanged (WT: 10.28% ± 1.17%; KO: 9.39% ± 1.50%). A considerable proportion of the flow cytometric determinations of CD8\(^+\) SP frequencies were also done more stringently, including markers revealing other cell types that are present in the thymus, and therefore, may contribute to the observed imbalance. However, when gating was done to exclude memory cells (CD44\(^+\), regulatory T cells (CD25\(^+\)), TCR\(\alpha\beta\) T cells (GL3\(^+\)), and NK T cells (CD3\(^+\)DX5\(^-\)), the same degree of discrepancy between WT and KO thymocyte numbers was found, confirming that regular CD8\(^+\) T cells are affected by CD155 deficiency (data not shown).

We recently established a panel of anti-CD155 mAbs; two of these (3F1 and 4G3) block the interaction of CD155 with its ligands CD96 and CD226 (37, 40) and data not shown). Therefore, mAb 4G3 was injected repeatedly into WT BALB/c mice for 4 wk, and the thymus and the peripheral lymph nodes (PLNs) were analyzed. mAb 4G3 was able to provoke a loss of CD8\(^+\) SP cells to an extent seen in CD155-deficient mice (Fig. 1B). The frequencies of peripheral T cells were not influenced by mAb treatment, demonstrating that T cells were not depleted. This suggested that the observed deficiency in CD8\(^+\) SP cell numbers can be triggered instantaneously by interrupting CD155 adhesion.
CD155 deficiency affects primarily mature CD8⁺ SP thymocytes

We next sought to characterize the CD8⁺ SP thymocytes in more detail with regard to their maturation stage. It is known that SP cells downregulate CD69 and CD24 during their final maturation. CD24 staining revealed a characteristic distribution pattern among WT CD8⁺ SP cells. The narrow majority of cells displayed intermediate levels of CD24, representing semimature CD8⁺ SP cells. A considerable proportion of cells was CD24⁺ (mature CD8⁺ SP cells), whereas a minor fraction was CD24⁻ (Fig. 1C). The latter population is composed of immature SP cells (TCRαβ negative, data not shown), which develop into DP cells. When comparing this pattern with that obtained from CD155-deficient cells, it was evident that the lack of CD155 almost exclusively affects the most mature CD24⁺ cells because, to a large extent, these cells were missing in the KO thymi (Fig. 1C). The CD24-staining profiles of the CD4⁺ SP WT and mutant cells did not diverge significantly from each other, in accordance with the finding that the cell frequencies were identical. As mentioned above, injection of anti-CD155 mAb 4G3 caused a deficiency of thymic CD8⁺ SP cells. Analysis of the CD24 expression profile of mAb-treated CD8⁺ SP cells found that they displayed the same phenotype as seen in the classical KO cells, with the mature CD24⁺ cells almost completely missing in the pool of CD8⁺ SP thymocytes (data not shown).

Mature CD4⁺ SP thymocytes upregulate their S1P₁ expression, rendering them competent for emigration (19). Therefore, we investigated the expression of this receptor by real-time PCR in immature and mature CD8⁺ SP cells separately (Fig. 1C). In both, WT and CD155 mutant CD8⁺ SP S1P₁ expression was virtually absent in immature cells. A pronounced upregulation of S1P₁ expression was observed in the mature subpopulation to an extent that did not differ between WT and CD155-deficient cells. This indicates that the lack of mature CD8⁺ SP cells in CD155-deficient thymi is not caused by an aberrantly regulated S1P₁ expression.

Abundance of CD8⁺ SP cells is strain dependent

In general, C57BL/6 thymi harbor a lower percentage of CD8⁺ SP cells compared with the BALB/c background, indicating that the abundance of SP cells varies among different mouse strains. When analyzing the cellular composition of the CD8⁺ SP cell pool, it was evident that only a small proportion of mature CD8⁺ CD24⁻ cells is present in adult thymus of C57BL/6 mice (Fig. 2A). We selected additional inbred mouse strains of differing MHC class I haplotypes (see Materials and Methods) and investigated the composition of their CD8⁺ SP cell populations in more detail. To this end, the expression analysis of the CD155 counter-receptor CD226 was included. CD226 is expressed in immature and mature, but not immature, CD8⁺ SP cells, thus allowing a better discrimination of immature and semimature CD8⁺ SP cells (36). In addition, CD226 expression declines in mature CD8⁺ SP cells of BALB/c origin. By and large, the investigated strains fall into two categories: DBA/1, SJL, and BALB/c mice possess considerable amounts of mature CD8⁺ SP cells in their thymi as illustrated by their low ratio of semimature to mature CD8⁺ SP cells, whereas C3H and C57BL/6 mice largely lack that cell type (Fig. 2B, 2C). In addition, a characteristic CD226 expression pattern could be found in each strain, with SJL CD8⁺ SP cells expressing this CD155 counter-receptor at a low level and DBA/1 mice exhibiting the biggest difference in CD226 expression between semimature and mature CD8⁺ SP cells. This is an interesting observation considering that BALB/c thymocytes express slightly more CD155 than C57BL/6 thymocytes (data not shown). We analyzed heterozygous BALB/c animals to test whether the level of CD155 expression is critical for modulating the abundance of mature CD8⁺ SP cells. CD155 is expressed in

FIGURE 1. Lack of CD155 causes a deficiency in mature CD8⁺ thymocytes. A. Flow cytometric analysis of thymocytes and peripheral T cells of WT and CD155⁻/⁻ origin showing the separation according to CD4 and CD8 expression. Dead cells (DAPI⁺) and doublets were excluded. For peripheral cells (pLNs), a pregate was set to include DAPI⁺ CD62L⁺ CD3⁺ cells only. The lower panels depict the percentages of CD4⁺ SP and CD8⁺ SP cells. Each circle represents one animal. Closed circles represent WT mice; open circles denote CD155-deficient mice. B. Distribution of SP cells and peripheral T cells in mice treated with anti-CD155 mAb 4G3 caused a deficiency of thymic CD8⁺ SP cells. Analysis of the CD24 expression profile of mAb-treated CD8⁺ SP cells found that they displayed the same phenotype as seen in the classical KO cells, with the mature CD24⁺ cells almost completely missing in the pool of CD8⁺ SP thymocytes (data not shown).

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a gene dosage-dependent fashion, resulting in half-maximum CD155 levels on CD155+/- cells (data not shown). However, CD155+/-CD8+ SP cells resembled their WT counterparts, whereas the CD155−/−CD8+ SP cells of BALB/c origin mimicked the CD57BL/6 WT phenotype with regard to the ratio of semimature to mature CD8+ SP cells (Fig. 2C).

All data presented in the following sections refer to BALB/c mice.

**Paucity of mature CD8+ SP cells is age dependent**

Thymus represents a highly dynamic organ that grows tremendously in size after birth. At ~3 wk of age, thymi have grown to their maximal size, which remains constant during the following weeks until atrophy begins (41). When analyzing the emergence of SP cells in WT BALB/c thymus, we found that the frequency of CD4+ SP cells reached a plateau at ~3 wk of age, concurring with thymic growth dynamics (Fig. 3A). In contrast, expansion of the CD8+ SP cell pool was delayed (peaking at ~6–8 wk; Fig. 3B), confirming earlier reports (41). Considering this, it was of interest to monitor the progression of the observed CD8+ SP phenotype, caused by CD155 deficiency, from suckling to advanced adulthood that is distinguished by thymus involution. The frequencies of CD155-deficient CD4+ SP cells did not differ significantly from those of WT cells at any of the ages investigated (Fig. 3A). In contrast, the percentage of mutant CD8+ SP cells remained remarkably constant over the entire study (i.e., not reproducing WT characteristics) (Fig. 3B). Until ~3–4 wk of age, only a small percentage of the WT CD8+ SP cells are of the mature (CD24lo) phenotype (Fig. 3C). Shortly thereafter, these cells seem to accumulate in thymus, reaching a plateau ~6–10 wk after birth, before their fraction starts to gradually decline again. In contrast, the separate analysis of CD24lo versus CD24int/hiCD8+ mutant SP cells revealed that, by and large, the percentage of mature CD8+ SP cells remained at a low level, because the age-dependent accumulation of these cells does not take place when CD155 is missing (Fig. 3C).

**Paucity of mature CD155−/− CD8+ SP cells is triggered by premature exit**

The selective absence of mature CD8+ SP cells in mutant thymi of BALB/c mice may be caused by a defective maturation, a premature exit, or an increased apoptotic rate of these particular cells. Determination of the latter did not reveal a significant difference between WT and mutant thymocytes (Fig. 4), even though apoptotic rates seemed to be slightly elevated in the KO mice.

To address thymic maturation and the exit of mutant CD8 T cells, animals were treated for 10–12 d with FTY720, an immunomodulatory drug. FTY720 selectively interferes with S1P receptor activity, blocks...
the exit of mature T cells from thymus and pLNs, and induces phenotypic maturation of semimature SP thymocytes (18, 19, 42). Upon FTY720 application, the CD8+ SP cell frequency became indistinguishable between WT and mutant thymi, whereas in both cases, the overall number of SP cells increased considerably (Fig. 5 A). This is most likely owed to the exit block triggered by the presence of FTY720. Subsequently, the profiles of expression of CD24, CD44, CD69, and CD62L were determined by flow cytometry (Fig. 5 B and data not shown). The vast majority of SP cells in FTY720-treated thymi represent mature CD69loCD24loCD62hi cells. Most strikingly, WT and mutant cells gave rise to identical expression patterns of these maturation markers. In conjunction with the identical frequencies of CD8+ SP cells mentioned above, this indicates that CD155-deficient semimature SP thymocytes are able to mature and accumulate to an extent comparable to WT thymocytes. This suggests that the rates of SP cell production are similar, if not identical, in CD155-deficient thymi.

We then analyzed the frequency of recent thymic emigrants (RTEs) by injecting the fluorescent dye FITC into WT and KO thymi. The dye unspecifically labels thymocytes that can be tracked as RTEs 40 h later when analyzing secondary lymphoid organs. For unknown reasons, the frequency of labeled mutant thymocytes was slightly less pronounced compared with WT thymocytes (Fig. 6A), yet we failed to note significant differences in the rates of RTEs in all secondary lymphoid organs analyzed. Although the numbers of RTEs are identical, the maturation stage may differ between WT and CD155-deficient RTEs. To test whether the latter are more or less mature compared with their WT counterparts, we analyzed the expression of CD44, CD62L, and CD24 on both types of RTEs. We observed that the levels of CD62L did not differ between RTEs and FITC peripheral CD8+ T cells, whereas RTEs had not yet completely downregulated CD44 and, in particular, CD24 to the levels found on the peripheral cells (Fig. 6B). This is in accordance with data published by other investigators (43, 44). More importantly, RTEs of CD155−/− and WT origin displayed an identical profile with regard to the expression of CD44, CD62L, and CD24. We conclude that the rate of production of mature SP thymocytes, as well as their rate of emigration, is not affected by CD155 deficiency.
Taking into account that mutant CD8+ SP thymocytes did not differ from their WT counterparts, with regard to the critical parameters of emigration, maturation, and sensitivity to apoptosis, the most likely explanation for the observed paucity of mature CD8+ SP cells in mutant thymus is a defect in the capacity to retain these cells in the medulla during their final maturation. This is in line with the finding that not only SP thymocytes themselves express CD155 and its ligands CD226 and CD96 [(37, 40) and data not shown]. In addition, mTECs and thymic DCs are positive for CD155 (data not shown), thus providing a dense CD155-driven adhesive network in the medulla.

**Shift in the TCR Vβ repertoire used by CD8+ SP cells in the absence of CD155**

The finding that CD4+ SP cells and semimature CD8+ SP cells are not affected in mutant thymi suggests that positive selection proceeded normally in CD155-deficient cells. In general, positive selection determining lineage fate is assumed to precede negative selection. Passing through their final differentiation steps, thymocytes transit from the cortex to the medulla with semimature CD4+ SP cells commencing negative selection (2, 6). However, doubts were raised regarding the general validity of this selection schedule (45). In addition, virtually nothing is known about at which stage(s) negative selection occurs in the CD8 lineage. Provided that CD155 is involved in establishing or maintaining contacts with medullary cells, this may influence the dwell time of CD8+ SP cells as well as indirectly influence TCR-driven selection by decreasing the average TCR-mediated signal intensity (possibly also in the case of CD4+ SP cells). Therefore, it was of interest to investigate whether CD155 deficiency correlates with a shift in the TCR repertoire present in the pool of mutant SP cells. To this end, the expression rate of several TCR Vβ chains (Vβ2, 3, 4, 5, 6, 7, 8, 11, and 12) was determined by flow cytometry in 8–10-wk-old mice (Fig. 7A). The panel of chains analyzed was also guided by the earlier finding that a profound alteration in the frequency of diverse Vβ subsets especially prone to

![Diagram](http://www.jimmunol.org/)

**FIGURE 6.** RTEs do not differ in frequency and phenotype between WT and CD155-deficient cells. A. FITC dye was injected intrathymically as described in Materials and Methods. The thymi, pLN, and mLN were analyzed 42 h later for CD8+ RTEs identified as FITC+ cells among unlabeled T cells. Each circle represents one animal. Closed circles: WT mice; open circles: CD155-deficient animals. B. Representative graphs show the expression pattern of CD62L, CD24, and CD44 in WT and CD155 deficient-CD8+ RTEs. The corresponding expression profile of peripheral naive CD8+ T cells that are FITC+ is also shown.

**FIGURE 7.** Negative selection of CD8+ SP cells in WT and CD155-deficient mice. A. A series of Vβ chain-specific mAbs was exploited to determine the repertoire of TCRs expressed by WT and CD155-mutant DP cells, CD4+ SP cells, and CD8+ SP cells. Each circle represents one thymus. B. Separately analyzed Vβ repertoire in thymocytes of WT animals according to their CD24 expression. This was compared with the corresponding usage found in the CD8+CD62L+ fraction of mLN cells. A total of eight mice were analyzed in two separate experiments. Statistical analysis refers to the comparison of the CD24low fraction with the CD24int or mLN fraction. *p < 0.05; **p < 0.01; ***p < 0.001.
recognizing endogenous retroviral Ags is indicative of defective negative selection in the CD4<sup>+</sup> cell pool. In adult BALB/c animals, the strain-specific retroviral Ags cause massive deletion of CD4<sup>+</sup> T cells expressing Vβ3, 5, 11, and 12 (8, 9). When comparing the TCR Vβ repertoire between WT and mutant thymocytes, we observed moderate, but highly statistically significant, differences in the frequencies of Vβ2, 3, 4, 5, and 12 present on CD8<sup>+</sup> SP cells, whereas only a marginal difference in Vβ8 usage was found for CD4<sup>+</sup> SP cells. Likewise, the subchain composition in the DP fraction was virtually identical, suggesting that, up to this stage, T cell development is regular in CD155-deficient mice. These findings correlate well with the above-described selective deficiency in CD8<sup>+</sup> SP cells, whereas CD4<sup>+</sup> SP cells are largely unaffected; this supports the idea that adhesion receptor-mediated dwell time and negative selection represent coupled phenomena.

Surprisingly, a different result was obtained when analyzing peripheral naive T cells (Supplemental Fig. 1). Some of the observed shifts displayed by the mutant thymocytes appear attenuated in the periphery. CD8<sup>+</sup> T cells isolated from mesenteric lymph nodes (mLNs) resemble their thymic counterparts with respect to Vβ4 but not Vβ2, 3, and 12 (no difference). Interestingly, the increased presence of Vβ5 on CD8<sup>+</sup> SP thymocytes is opposed by a reduced frequency of this chain among peripheral CD8<sup>+</sup> T cells.

**Semimature and mature WT CD8<sup>+</sup> SP cells and peripheral CD8<sup>+</sup> T cells display divergent TCR Vβ patterns, indicating an ongoing selection**

In theory, the biases in the Vβ repertoire selection observed in the CD155-deficient CD8<sup>+</sup> SP pool may represent a numerical artifact caused by the highly divergent contributions of the semimature CD24<sup>int</sup>CD8<sup>+</sup> and mature CD24<sup>lo</sup>CD8<sup>+</sup> SP cell numbers in the KO scenario compared with the WT one. Such an assumption anticipates that the TCR Vβ distributions among semimature and mature CD8<sup>+</sup> SP cells differ from each other, consequently postulating that negative selection operates predominantly in the semimature or mature stage. Following the concept that semimature CD24<sup>int</sup>CD8<sup>+</sup> SP cells are sensitive to negative selection, as suggested for the CD4 lineage (6), it would be more appropriate to compare the CD24<sup>int</sup> fractions when determining whether negative selection is impaired in thymus of CD155-deficient mice. Likewise, the repertoire of the peripheral CD8<sup>+</sup> T cells should be compared with that of the mature CD24<sup>lo</sup>CD8<sup>+</sup> thymocytes. Therefore, we analyzed the Vβ chain repertoire of semimature CD24<sup>int</sup>CD8<sup>+</sup> and mature CD24<sup>lo</sup>CD8<sup>+</sup> SP cells in WT animals separately. Unfortunately, a corresponding investigation in CD155-deficient thymi was affected by the paucity of mature cells, rendering such an analysis statistically insignificant for low-frequency Vβ chains in the mature compartment. The results obtained from WT cells sustain the supposition of ongoing selection in the semimature CD24<sup>int</sup>CD8<sup>+</sup> SP stage, because statistically significant differences in the usage of Vβ chains 3, 4, 5, 6, 7, and 11 were found compared with mature CD8<sup>+</sup> SP cells (Fig. 7B). In support of this, the frequency patterns of CD24<sup>int</sup>CD8<sup>+</sup> SP cells and peripheral naive CD8<sup>+</sup> T cells resemble each other more closely than do those of CD24<sup>lo</sup>CD8<sup>+</sup> and CD24<sup>hi</sup>CD8<sup>+</sup> SP thymocytes. This is in accordance with the above-mentioned model. Nevertheless, the minor divergences in Vβ usage found between the mature CD24<sup>lo</sup>CD8<sup>+</sup> thymocytes and peripheral CD8<sup>+</sup> T cells (Vβ2, 6, 7, 8, 11, and 12) indicate an ongoing shaping of the TCR repertoire finally engaged by peripheral naive CD8<sup>+</sup> T cells. However, it is uncertain whether such fine-tuning occurs in the pool of mature thymocytes and/or in the periphery.

When comparing the WT CD24<sup>int</sup> fraction with the CD155-deficient CD8<sup>+</sup> SP population that is dominated by the CD24<sup>int</sup> pool, it is evident that the original differences in Vβ3, 5, and 12 frequency found were largely caused by the biased contributions of the semimature and mature cells to the pool of mutant CD8<sup>+</sup> SP cells (Supplemental Fig. 2). However, this does not apply for Vβ2, and particularly not for Vβ4. These discrepancies in the repertoire selection between WT and KO CD8<sup>+</sup> thymocytes must have come into existence before cells completed their transition to the mature stage.

This assumption is supported by another observation. As shown above, following treatment with FTY720, the composition of the CD8 SP compartment no longer differed between WT and CD155-deficient thymi (Fig. 5). When analyzing the frequencies of Vβ2, 3, 4, 5, and 12-expressing T cells, we observed that FTY720 application equalized the different frequencies in Vβ3, 5, and 12 but not in Vβ2 and 4 (Supplemental Fig. 3). Remarkably, however, Vβ4 was slightly increased by FTY720 treatment in KO animals, whereas it was reduced in untreated mice compared with the corresponding WT controls. Such a phenomenon may relate to the finding that FTY720 can interfere with negative selection (46).

Considering this, the shortened residency of mature CD8<sup>+</sup> SP cells caused by CD155 deficiency and the concomitant bias in the repertoire selection may not always share a common origin (i.e., negative selection and length of stay do not trace back to the same adhesive events); thus, they may be mediated by different cell types contacted by the thymocytes. However, a defect in adhesion may account for both phenomena, and both seem to overlap chronologically; the results presented above admit the interpretation that negative selection continues to shape the TCR repertoire to a certain extent beyond the semimature stage. Thus, a prolonged residency of CD8<sup>+</sup> SP cells in medulla beginning with adolescence may influence the TCR repertoire of naive CD8<sup>+</sup> cells by increasing the probability of productive encounters between selectors and CD8<sup>+</sup> thymocytes.

**Discussion**

When analyzing thymi of CD155-deficient mice, we found a significant reduction in the frequency of CD8<sup>+</sup> SP but not CD4<sup>+</sup> SP cells. Inclusion of maturation markers, such as CD24, CD69, or CD62L, in the flow cytometric analyses allowed assignment of the observed phenotype to the most mature CD8<sup>+</sup> cell population present in thymus: CD24<sup>hi</sup>CD69<sup>hi</sup>CD62L<sup>hi</sup> cells ready to exit into the periphery. A further refinement of the investigations of this cell type including thymi of various ages revealed that the frequency profile of mature WT CD8<sup>+</sup> SP cells is subject to significant variations, whereas that of semimature CD8<sup>+</sup> SP cells remained remarkably constant. Beginning at ~3–4 wk after birth, mature CD8<sup>+</sup> SP cells accumulate in thymus, reaching a plateau at 6–10 wk. In contrast, the frequencies of mature CD8<sup>+</sup> SP cells remained invariably low in CD155-deficient mice of all ages analyzed. Regarding these dynamics, the BALB/c CD155<sup>−/−</sup> thymocytes displayed characteristics resembling mature WT C57BL/6 CD8<sup>+</sup> SP cells that failed to accumulate during adolescence (Fig. 2 and data not shown). Interestingly, the genetic background seems to exert a profound influence on the capability of mature CD8<sup>+</sup> SP cells to accumulate in thymus, but conclusive statements await more detailed analyses for various strains as done in this study for BALB/c animals. A strain-dependent modulation of SP frequencies and population dynamics (potentially also affecting CD4<sup>+</sup> SP cells) represents a thus-far neglected parameter in the discussion of experimental results addressing, for example, the dwell time of SP cells in thymus (see Ref. 47 for a concise overview). Therefore, considering this aspect may help to reconcile conflicting interpretations of studies that used different mouse model strains.

CD155 represents an adhesion receptor. Together with the results obtained from other experiments presented in this study (FITC
pressed by mature thymic versus peripheral CD8+ T cells would interpret the results is complicated further by the concept that potentially autoreactive cells escape into the periphery. Whatever applies, the premature release of cells distinguished by uncompleted thymic selection may cause problems in developing thymocytes depending on the T cell subset as well as their developmental stage. The end of weaning and the concomitant discontinuation of the protective maternal Ig shield coincide with structural alterations/maturation in GALT and remote lymphoid organs, a phenomenon that was found to correlate with communal microflora now present in the gut (54–56). As outlined above, newly released RTEs refresh the precedent peripheral pool of T cells, possibly facilitating to adopt to the requirement of changing immunological challenges met during subsequent stages in life (52). Therefore, it is tempting to speculate that the generation of CD8+ T cells starting to settle the periphery at ∼3–4 wk must fulfill different criteria that manifest as an extended residency in the medulla.

Taken together, the discrepancies in repertoire selection as well as age-dependent dwell time of CD8+ SP thymocytes, but not CD4+ SP thymocytes, related to CD155 function helped disclose hidden aspects of the life cycle of terminally maturing CD8+ T cells. To our knowledge, the provoked abnormalities due to CD155 deficiency are not sufficient to elicit macroscopic phenotypes, such as overt signs of autoimmunity, even in old mice (data not shown). However, final conclusions regarding these aspects await more detailed analyses of CD155-deficient mice.

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Disclosures
The authors have no financial conflicts of interest.

References
**Supplementary Figure S1.** Vβ chain usage among adult peripheral naive CD8⁺ T cells. CD4⁺ and CD8⁺ T cells from mesenteric LN of 8-10 week old mice were analyzed using Vβ chain specific antibodies as indicated. Closed circles: wild type mice. Open circles: CD155 deficient mice. Each dot represents one mouse.

**Supplementary Figure S2.** Frequencies of CD155 deficient thymic CD8⁺ T cells compared to those of wild type CD8⁺ T cells of different maturation. Shown are the frequencies as given in Figure 7A for the CD155 deficient CD8⁺ SP (KO) along with the distribution pattern for wild type CD8⁺ T cells as seen in Figure 7B for the Vβ chains 2, 3, 4, 5, and 12. ***: p<0.001; ns: not significant.

**Supplementary Figure S3.** Vβ chain usage among adult thymic CD8⁺ T cells following FTY720 treatment. Mice were fed with FTY720 for 12 days before thymic CD8⁺ T were analyzed using Vβ chain specific antibodies as indicated. Closed circles: wild type mice. Open circles: CD155 deficient mice. Each dot represents one mouse.

**Supplementary Figure S4.** Analysis of bone marrow chimeras. Adult chimeric mice (8-10 weeks of age) were generated following intra-hepatic injection of bone marrow into newborn mice. The determination of the CD8⁺ SP frequency (A) and the ratio of semi-mature to mature CD8⁺ SP (B) is based on the analysis of 10 animals (wt to wt), 7 animals (wt to ko), 7 animals (ko to wt), and 10 animals (ko to ko), respectively. Analysis for CD8⁺ SP frequency was done as described in Figure 1, that for the ratio as described in Figure 2. ko: CD155 deficient mouse. Shown are the means and the SD. *: p<0.05; **: p<0.01; ***: p<0.001.

**Supplementary Figure S5.** Vβ chain usage among juvenile peripheral naive CD8⁺ cells. CD4⁺ and CD8⁺ T cells from mesenteric LN of 3-4 week old mice were analyzed using Vβ chain specific antibodies as indicated. Closed circles: wild type mice. Open circles: CD155 deficient mice. Each dot represents one mouse.
Supplementary Figure S1

Vβ2

Vβ3

Vβ4

Vβ5

Vβ6

Vβ7

Vβ8

Vβ11

Vβ12
Supplementary Figure S2
Supplementary Figure S3

![Graphs showing frequency of Vβ2, Vβ3, Vβ4, Vβ5, and Vβ12 in CD4, CD8, and DP cells.](image-url)
Supplementary Figure S4

A  Frequency of CD8 SP

B  semi-mature to mature CD8 SP

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<th>Group</th>
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Supplementary Figure S5