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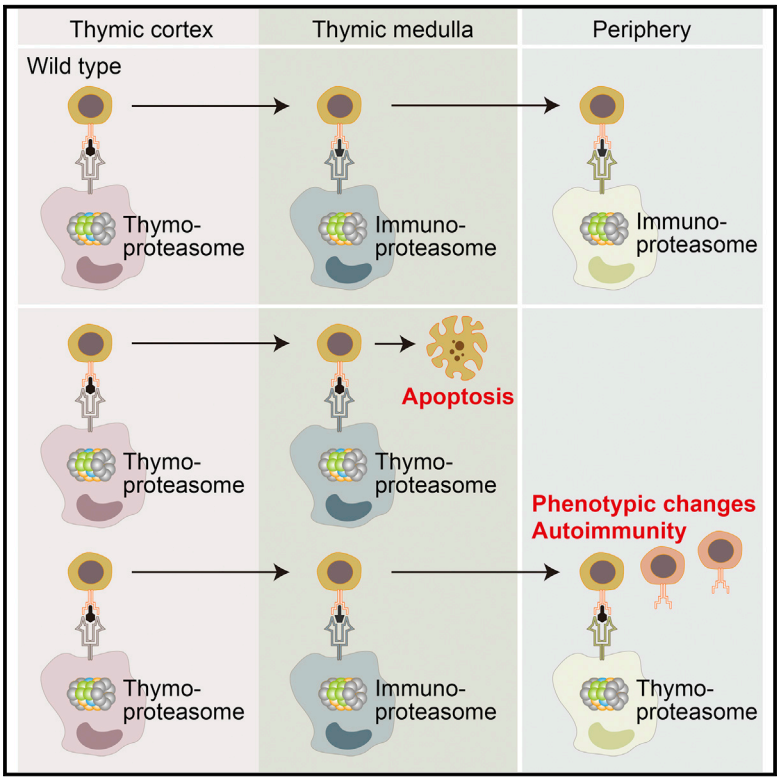
Restricted Expression of the Thymoproteasome Is Required for Thymic Selection and Peripheral Homeostasis of CD8$^+$ T Cells

Graphical Abstract



Highlights

- Mice expressing β5t in both cTECs and mTECs show decreased CD8$^+$ lineage thymocytes
- Extra-thymic expression of β5t accumulates memory or exhausted CD8$^+$ T cells
- Extra-thymic expression of β5t causes autoreactive T cell responses *in vivo*

Authors

Utano Tomaru, Saori Konno, Syota Miyajima, ..., Shigeo Murata, Akihiro Ishizu, Masanori Kasahara

Correspondence

tomaruu@med.hokudai.ac.jp

In Brief

Tomaru et al. show that aberrant expression of self-peptides generated by thymoproteasomes, which are expressed exclusively in the thymic cortex, affects CD8$^+$ T cell homeostasis. Restricted expression of thymoproteasomes is crucial for thymic selection, maintenance of the peripheral naive pool of CD8$^+$ T cells, and avoiding autoimmune responses.

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Restricted Expression of the Thymoproteasome Is Required for Thymic Selection and Peripheral Homeostasis of CD8$^+$ T Cells

Utano Tomaru,[1,4,*] Saori Konno,[1] Syota Miyajima,[1] Rikuto Kimoto,[2] Mari Onodera,[2] Shizuka Kiuchi,[1] Shigeo Murata,[3] Akihiro Ishizu,[2] and Masanori Kasahara[1]

[1]Department of Pathology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan
[2]Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido University, Sapporo 060-0812, Japan
[3]Laboratory of Protein Metabolism, Graduate School of Pharmaceutical Science, University of Tokyo, Tokyo 113-0033, Japan
[4]Lead Contact
*Correspondence: tomaruu@med.hokudai.ac.jp
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SUMMARY

The thymoproteasome subunit β5t is specifically expressed in cortical thymic epithelial cells (TECs) and generates unique peptides to support positive selection. In this study, using a mouse model ubiquitously expressing β5t, we showed that aberrant expression of self-peptides generated by β5t affects CD8$^+$ T cell homeostasis, including thymic selection and maintenance of the peripheral naive pool of CD8$^+$ T cells. In mice in which β5t was expressed both in cortical and medullary TECs, the abundance of CD8$^+$ lineage thymocytes was reduced, and extra-thymic expression of β5t caused accumulation of CD8$^+$ T cells with the memory or exhausted phenotype and induced autoreactive T cell responses. We found that thymoproteasomes are essential for positive selection but that the subsequent change in peptide repertoire in the medulla is also crucial for thymic selection and that β5t-derived peptide must be confined to the thymus to avoid autoimmunity in peripheral tissues.

INTRODUCTION

In the immune system, proteasomes generate peptides presented by major histocompatibility complex (MHC) class I molecules (Rock et al., 1994; Kloetzel, 2001) and play an essential role in adaptive immunity. Three types of proteasomes have been identified in jawed vertebrates: the constitutive or housekeeping proteasome, which is highly conserved from yeast to man; the immunoproteasome, which is induced by stimulation with interferon γ (IFN-γ) in most tissues and is constitutively expressed in immune tissues such as the spleen and thymus (Gaczynska et al., 1993; Hisamatsu et al., 1996; Tanaka and Kasahara, 1998); and the thymoproteasome, which is expressed exclusively in the thymic cortex (Murata et al., 2007; Tomaru et al., 2009). The immunoproteasome contains the IFN-γ-inducible subunit β5i and shows strong chymotrypsin-like activity, which is required for MHC class I ligand production. In contrast, the β5t-containing thymoproteasome displays weak chymotrypsin-like activity and generates specialized peptides that promote positive selection of CD8 single positive (CD8SP) cells in the thymus (Murata et al., 2007, 2008; Nitta et al., 2010; Xing et al., 2013; Sasaki et al., 2015). However, it remains unclear how thymoproteasomes regulate CD8$^+$ T cell homeostasis. In the thymus, β5t is selectively expressed in cortical thymic epithelial cells (cTECs), whereas medullary TECs (mTECs) and dendritic cells (DCs) express β5i. Outstanding questions about the mechanisms of thymoproteasome-mediated T cell selection include the following. Are low-affinity peptides provided by thymoproteasomes themselves essential for positive selection of CD8$^+$ T cells? Is the difference in peptides involved in positive versus negative selection also important for thymic selection (Klein et al., 2009; Murata et al., 2018)? In addition, in the context of systemic immune homeostasis, it remains unclear why expression of β5t-containing proteasomes is restricted to the thymus.

In this study, we generated transgenic mice ubiquitously expressing β5t and examined the thymic differentiation and phenotype of peripheral CD8$^+$ T cells. The results demonstrated that restricted expression of β5t in cTECs is important for thymic selection and maintenance of the peripheral pool of CD8$^+$ T cells. Peripheral expression of self-peptides generated by β5t induced autoreactive T cell responses.

RESULTS

Aberrant Expression of β5t Induces Defects of CD8SP Cells in the Thymus

The thymoproteasome is specifically expressed in cTECs because its unique catalytic subunit β5t is selectively transcribed in these cells (Murata et al., 2007, 2008). To investigate the significance of restricted expression of β5t in cTECs, we generated mice expressing β5t in systemic tissues. We previously generated β5t transgenic mice (β5t-Tg) expressing mouse β5t cDNA under the control of the cytomegalovirus immediate-early enhancer and the chicken actin promoter with an enhancer element (Tomaru et al., 2012). In β5t-Tg mice, β5t is ubiquitously expressed and preferentially incorporated into the 20S proteasome, whereas β5i is partially detected in lymphoid tissues. In this study, *β5i*$^{-/-}$ mice were crossed with β5t-Tg mice to generate *β5i*$^{-/-}$β5t-Tg mice, in which β5t but not β5i was detected in all tested tissues (Figures S1A and S1B). In *β5i*$^{-/-}$β5t-Tg mice, β5t was expressed in mTECs and DCs that

physiologically express β5i (Figures S1C and S1D; Table S1). Mice tested in the present study (8–12 weeks old) displayed no apparent histological abnormalities (data not shown).

Thymocytes from *β5i*$^{-/-}$β5t-Tg mice were analyzed by flow cytometry. To determine the effect of the genetic modification (transduction or deletion) of the β5t and β5i genes, thymocyte differentiation was examined in *β5i*$^{-/-}$, *β5t*$^{-/-}$, β5t-Tg, and *β5i*$^{-/+}$β5t-Tg mice. Development of CD8SP cells was impaired in *β5i*$^{-/-}$β5t-Tg mice in comparison with wild-type (WT) mice (Figure 1A). The number and percentage of CD8SP cells expressing high levels of T cell receptor β (TCRβ^{high}) were significantly reduced in *β5i*$^{-/-}$β5t-Tg mice (Figures 1B, 1C, and 1E), although there was no remarkable change in CD4SP cells (Figures 1B, 1D, and 1F). *β5i*$^{-/+}$β5t-Tg mice exhibited a slight decrease in the proportion of TCRβ^{high} CD8SP cells compared with the WT, although β5t-Tg mice exhibited no significant change. Together, these observations indicated that the remarkable reduction of CD8SP cells in *β5i*$^{-/-}$β5t-Tg mice was not caused by the transgene itself. Regarding the expression levels of MHC class I, previous studies show that cells lacking β5i express approximately 50% lower levels of MHC class I than WT mice (Fehling et al., 1994). In this study, MHC class I expression was 2-fold lower in *β5i*$^{-/-}$ and *β5i*$^{-/-}$β5t-Tg mice than in WT mice (Figure S1E). CD8SP cell development and TCRβ levels were comparable between WT and *β5i*$^{-/-}$ mice; therefore, the decreased levels of MHC class I did not affect the differentiation of CD8SP cells in *β5i*$^{-/-}$β5t-Tg mice. *β5t*$^{-/-}$ mice show significantly reduced numbers of CD8SP cells, as reported previously (Murata et al., 2007). We found that the proportion of TCRβ^{high} CD8SP cells in *β5i*$^{-/-}$β5t-Tg mice was slightly higher than that in *β5t*$^{-/-}$ mice (Figure 1E). Despite a significantly lower frequency of certain Vβ regions of CD8SP cells in *β5i*$^{-/-}$β5t-Tg mice than in WT mice, CD8SP cells from *β5i*$^{-/-}$β5t-Tg mice expressed all TCRβ variable regions tested (Vβ2, Vβ3, Vβ4, Vβ5.1 or 5.2, Vβ6, Vβ7, Vβ8.1 or 8.2, Vβ8.3, Vβ9, Vβ10b, Vβ11, Vβ12, Vβ13, and Vβ14) (Figure S2A). The frequency of Vβ regions in CD4SP cells was comparable between WT, *β5t*$^{-/-}$, and *β5i*$^{-/-}$β5t-Tg mice (Figure S2B).

In light of the observation that CD4SP cells developed normally in *β5i*$^{-/-}$β5t-Tg mice, the decrease in CD8SP cells in *β5i*$^{-/-}$β5t-Tg mice was unlikely to be caused by an abnormality in thymocytes themselves because of expression of β5t. Instead, the reduction may depend on the interaction between the TCR and altered self-peptides generated by aberrant β5t expression in thymic stromal cells. To confirm this hypothesis, we created bone marrow (BM) chimeras by transferring BM from EGFP mice, which are phenotypically WT except for the systemic expression of EGFP, into *β5i*$^{-/-}$β5t-Tg or WT hosts. As expected, the development of EGFP^{+}CD8SP cells was impaired in *β5i*$^{-/-}$β5t-Tg hosts in comparison with WT hosts (Figures 1G–1J). The percentage of TCRβ^{high} cells in CD8SP was significantly reduced in *β5i*$^{-/-}$β5t-Tg hosts (Figure 1J), although there was no remarkable change in the development of CD4SP cells in BM chimeras (Figures 1G, 1H, and 1K).

Restricted Expression of β5t in cTECs Is Important for Thymic Selection

To analyze the differentiation of CD8SP cells in *β5i*$^{-/-}$β5t-Tg mice in detail, CD8SP cells were divided into four subsets according to the expression of TCRβ and the activation marker CD69; thymocyte maturation occurred in the order TCRβ^{low}CD69^{-}, TCRβ^{low}CD69^{+}, TCRβ^{high}CD69^{+}, and TCRβ^{high}CD69^{-} (Lawson et al., 2010). Representative sort gates are shown in Figure 2A. In WT mice, the abundant CD8SP cells (around 7 × 10^{6} cells, >80% of CD8SP cells) were TCRβ^{high}CD69^{+} or TCRβ^{high}CD69^{-}, representing post-selected or mature thymocytes. By contrast, pre-selected immature TCRβ^{low}CD69^{-} CD8SP cells accumulated significantly in *β5i*$^{-/-}$β5t-Tg mice (Figures 2A and 2B). The proportion of TCRβ^{low}CD69^{-} cells was lower and that of TCRβ^{high}CD69^{-} cells was higher in CD8SP cells from *β5i*$^{-/-}$β5t-Tg mice than in those from *β5t*$^{-/-}$ mice. Next we analyzed differentiation of CD8^{+} lineage double positive (DP) cells using the cell surface marker CD103, an integrin expressed on CD8^{+} lineage cells in the thymus (Grueter et al., 2005; Egawa and Littman, 2008). To distinguish CD8^{+} lineage cells at an early stage of thymic selection, we analyzed the population of TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells. Representative sorting gates are shown in Figure 2C. Although there was no significant change in the total number of TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells (population encircled by the thin solid line in Figure 2C), TCRβ^{low}CD69$^{-/+}$CD103^{+}CD4^{+}CD8^{low} cells (population encircled by the thick solid line in Figure 2C) were less abundant in *β5t*$^{-/-}$ mice but not in *β5i*$^{-/-}$β5t-Tg mice (Figures 2D and 2E). Because DP cells in the process of positive selection upregulate their expression of the activation marker CD69 and the TCR (Yamashita et al., 1993; Davey et al., 1998) while downregulating expression of CD8, this suggests that positive selection is affected in *β5t*$^{-/-}$ mice but not in *β5i*$^{-/-}$β5t-Tg mice. Thymic selection is primarily mediated by a TCR-induced program, and Bim and Nur77 are involved in proapoptotic functions via TCR signaling (Palmer, 2003). Both Bim and Nur77 are ultimately dispensable for negative selection; however, combined deficiency of Bim and Nur77 impairs induction of clonal deletion by ubiquitous self-antigens (Hu et al., 2009; Hu and Baldwin, 2015). Bim- or Nur77-expressing cells were more abundant in TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells in *β5t*$^{-/-}$ mice (Figures 2F, S3A, and S3E). Interestingly, aberrant expression of CCR7 was elevated in TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells of *β5t*$^{-/-}$ mice (Figure S4). During the normal process of thymic selection, CCR7 is upregulated following positive selection (Ueno et al., 2004); this may relate to abnormal selection of *β5t*$^{-/-}$ mice in the absence of β5t-containing proteasomes in the thymic cortex.

We next considered the possibility that a thymocyte defect could be occurring after positive selection in *β5i*$^{-/-}$β5t-Tg mice. We analyzed the population of TCRβ^{high}CD69^{+}CD103^{+} cells, which included a transitional subset of late post-selected DP cells. Representative sorting gates are shown in Figure 2G. TCRβ^{high}CD69^{+}CD103^{+} DP cells were less abundant in *β5i*$^{-/-}$β5t-Tg mice (Figure 2H). Those cells were also less abundant in *β5t*$^{-/-}$ mice, possibly because of the influence of a defect at an earlier stage or thymocyte deletion, which also occurs at this stage. In accordance with the reduction in the number of TCRβ^{high}CD69^{+}CD103^{+} DP cells, the percentage of Bim- or Nur77-expressing cells in this population was elevated in *β5i*$^{-/-}$β5t-Tg mice (Figures 2I and S3B). The ratio of Bim- or Nur77-expressing cells was also elevated in TCRβ^{high} CD8SP cells, but not in TCRβ^{high} CD4SP cells, in *β5t*$^{-/-}$ and *β5i*$^{-/-}$β5t-Tg

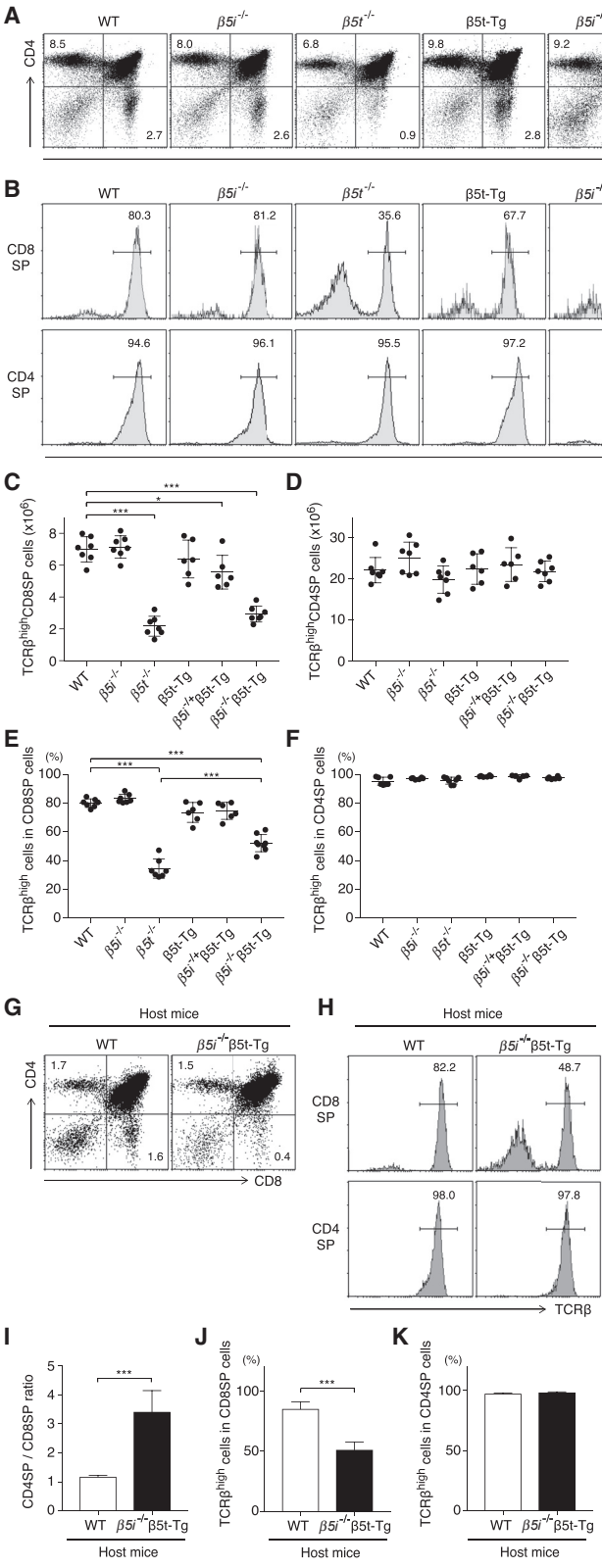
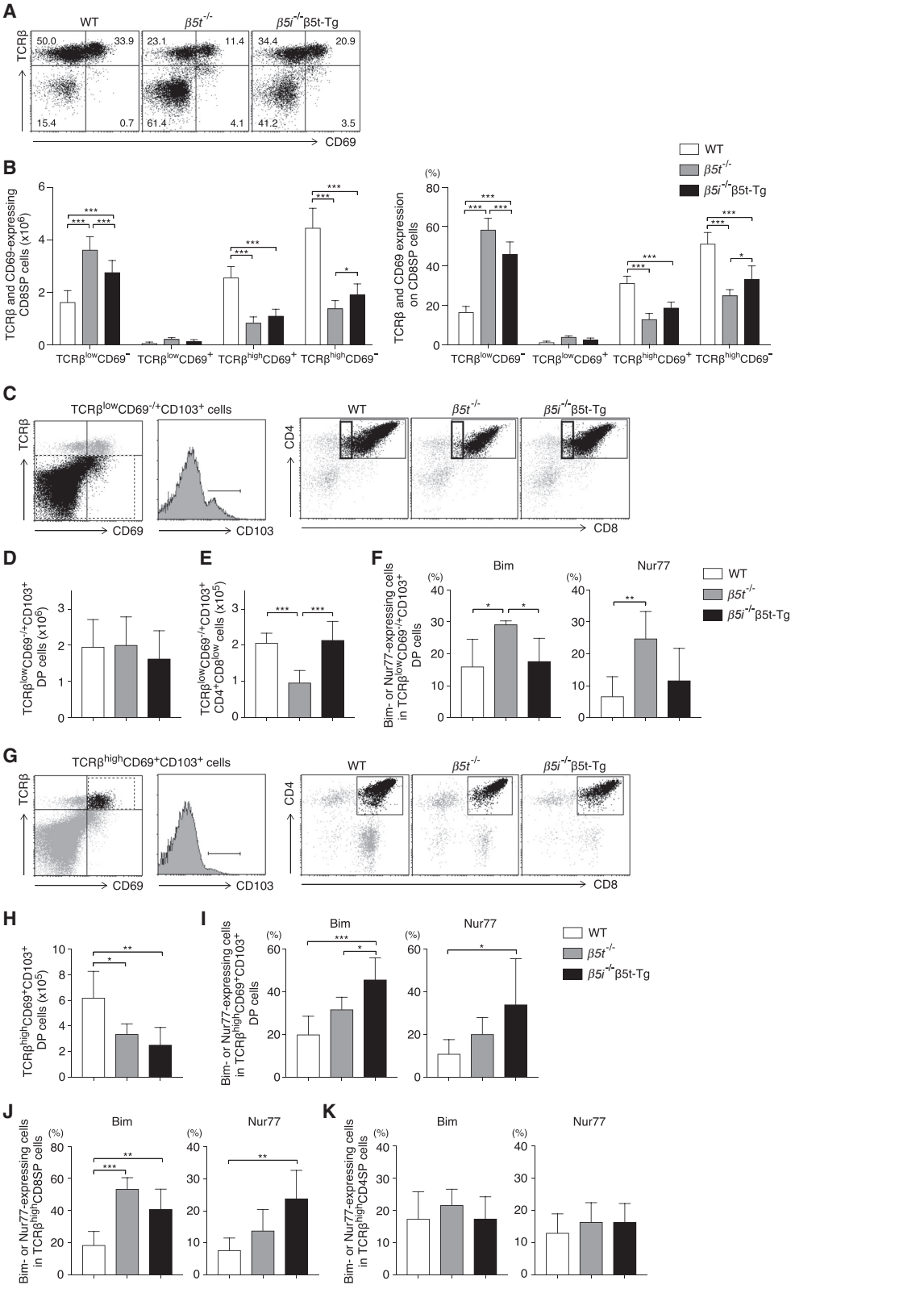


Figure 1. Thymic CD8SP Cells Are Less Abundant in Mice that Aberrantly Express β5t

(A–F) Flow cytometric analysis of thymocyte differentiation. Representative data of dot plot quadrants (A) and cell numbers of TCRβ^{high} CD8SP (C) and TCRβ^{high} CD4SP (D) cells in the indicated mice. Shown are (B, E, and F) expression of TCRβ on CD8SP and CD4SP cells. Also shown are representative data of histograms (B) and percentages of TCRβ^{high} cells among CD8SP cells (E) and CD4SP cells (F).

(G–K) BM chimeras created by transferring BM from EGFP mice into WT or *β5i*$^{-/-}$β5t-Tg host mice. The abundance of CD8SP cells was reduced in *β5i*$^{-/-}$β5t-Tg host mice, as was the proportion of TCRβ^{high} cells among CD8SP cells. Representative data of dot plot quadrants (G) and CD4SP/CD8SP ratio (I) in the indicated host mice. Also shown are representative data of histograms (H) and percentages of TCRβ^{high} cells among CD8SP cells (J) and CD4SP cells (K). There was no change in CD4SP cells (K).

Numbers in dot plot quadrants and histograms indicate percentages (A, B, G, and H). Each symbol represents an individual mouse; the long horizontal line represents the mean, and short horizontal lines represent SD (C–F). Data are expressed as means ± SD (I–K). Data were pooled from at least three independent experiments. Statistical significance was analyzed by Student's t test and one-way ANOVA with multiple comparisons post-test: *p < 0.05, ***p < 0.001.



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mice (Figures 2J, 2K, S3C, and S3D). Because the total numbers of TCRβ^{high}CD69^{+}CD103^{+} DP cells as well as TCRβ^{high} CD8SP cells were reduced in *β5i*$^{-/-}$β5t-Tg mice, there was no significant increase in the absolute numbers of Bim- or Nur77-expressing cells in those populations (Figures S3F and S3G). The numbers of Bim- or Nur77-expressing TCRβ^{high} CD4SP cells were comparable between WT, *β5t*$^{-/-}$, and *β5i*$^{-/-}$β5t-Tg mice, as were the relative proportions of those cells (Figure S3H).

The observation that the expression of proapoptotic molecules in CD4SP did not differ among the mice tested suggested that the reduced abundance of mature CD8SP cells in *β5i*$^{-/-}$β5t-Tg was not caused by an abnormality in thymocytes themselves. In *β5t*$^{-/-}$ mice, deficiency of β5t in the thymic cortex affects positive selection of CD8SP cells and decreases the abundance of post-selected thymocytes at the DP or cortical stage (Murata et al., 2007; Xing et al., 2013). In contrast, β5t-mediated thymic selection by cTECs in *β5i*$^{-/-}$β5t-Tg mice was not affected because expression of β5t was retained in cTECs in the transgenic animals as in WT mice. A reduction in the number of thymocytes was observed after the TCRβ^{high}CD69^{+} DP stage in *β5i*$^{-/-}$β5t-Tg mice, suggesting that the maturation of CD8SP cells was affected during a transitional process from late post-selection DP to early SP cells, possibly during negative selection.

Restricted Expression of β5t in the Thymus Is Important for Maintaining a Peripheral Naive Pool of CD8^{+} T Cells

Splenic CD8^{+} T cells from *β5i*$^{-/-}$β5t-Tg mice were analyzed by flow cytometry and compared with splenic CD8^{+} T cells from WT, *β5i*$^{-/-}$, *β5t*$^{-/-}$, β5t-Tg, and *β5i*$^{-/+}$β5t-Tg mice. Splenic CD8^{+} T cells, but not CD4^{+} cells, were less abundant in *β5i*$^{-/-}$β5t-Tg mice, consistent with the reduction in the number of thymic CD8SP cells (Figures 3A–3C). As previously reported, *β5t*$^{-/-}$ mice also showed a significant decrease in CD8^{+} T cells (Murata et al., 2007). The proportions of TCRβ^{high} CD8^{+} T cells were almost identical among the mice tested (Figure 3D). The phenotype of CD8^{+} T cells was analyzed by classifying them according to the expression of CD44 and CD122. Naive CD8^{+} T cells express low levels of CD44 and CD122, whereas memory CD8^{+} T cells are characterized by high levels of both proteins (Walzer et al., 2002). Because CD8^{+} T cells were much less abundant in *β5i*$^{-/-}$β5t-Tg mice, the number of naive CD44^{low}CD122^{low} and memory CD44^{high}CD122^{high} CD8^{+} T cells was lower than in WT mice. However, the percentage of naive CD44^{low}CD122^{low} T cells was reduced, and the percentage of memory CD44^{high}CD122^{high} CD8^{+} T cells was significantly elevated, in *β5i*$^{-/-}$β5t-Tg mice (Figures 3E–3H). In all mice tested, more than 90% of CD8SP cells in the thymus had a CD44^{low}CD122^{low} phenotype (Figure 3I), indicating that CD8SP cells in *β5i*$^{-/-}$β5t-Tg mice change phenotype from naive CD44^{low}CD122^{low} to memory CD44^{high}CD122^{high} after migrating to the periphery. *β5t*$^{-/-}$ mice exhibited a significantly elevated proportion of memory CD8^{+} T cells, as reported previously (Xing et al., 2013). There was no alteration of naive and memory phenotypes in CD4^{+} T cells (Figure S5). Naive T cells rely on TCR signals from self-peptide and MHC complexes in peripheral lymphoid tissues to maintain a naive pool and physiological function (Takada and Jameson, 2009). *β5i*$^{-/-}$β5t-Tg mice expressed the β5t subunit in both cTECs and DCs (Figures S1C and S1D; Table S1). Hence, CD8^{+} T cells in the periphery may re-associate with self-peptides involved in positive selection.

To confirm this hypothesis, we transferred sorted naive CD44^{low}CD122^{low} CD8^{+} T cells from EGFP mice into *β5i*$^{-/-}$β5t-Tg, WT, and *β5t*$^{-/-}$ mice and then monitored phenotypic changes 5 and 12 days after the transfer. CD8^{+} T cells from EGFP mice exhibited the WT phenotype except for systemic expression of EGFP, indicating that they underwent thymic selection by β5t-expressing cTECs. Transferred EGFP^{+}CD8^{+} T cells changed phenotype from CD44^{low}CD122^{low} to CD44^{high}CD122^{high} in *β5i*$^{-/-}$β5t-Tg recipient mice; approximately 40% and 80% of EGFP^{+}CD8^{+} T cells exhibited a CD44^{high}CD122^{high} phenotype 5 and 12 days after the transfer, respectively (Figures 4A and 4B). However, the transferred EGFP^{+}CD8^{+} T cells retained the CD44^{low}CD122^{low} phenotype in WT and *β5t*$^{-/-}$ recipient mice. Because *β5i*$^{-/-}$β5t-Tg mice, but not WT and *β5t*$^{-/-}$ mice, express β5t in peripheral tissues, these results suggested that transferred naive EGFP^{+}CD8^{+} T cells may respond to self-peptides generated by β5t that are already present during positive selection. Memory T cells accumulate when mature T cells are placed in a lymphopenic environment. However, this effect can be excluded because the phenotypic alterations were not observed when naive EGFP^{+}CD8^{+} T cells were transferred into *β5t*$^{-/-}$ mice with decreased CD8^{+} T cells. In mice, CD4^{+} T cells can be divided into naive and memory subtypes based on expression of the adhesion molecules CD44 and CD62L. Naive CD4^{+} T cells express high levels of CD62L and low levels of CD44, whereas memory CD4^{+} T cells are characterized by high CD44 and low CD62L expression (Zhao and Davies, 2010). When sorted naive CD44^{low}CD62L^{high} CD4^{+} T cells from EGFP mice were transferred

Figure 2. Thymic Selection of CD8SP Cells Is Impaired in Mice with Aberrant Expression of β5t

(A and B) Flow cytometric analysis of TCRβ and CD69 on CD8SP cells in WT, *β5t*$^{-/-}$, and *β5i*$^{-/-}$β5t-Tg mice. Shown are representative dot plot data (A) and numbers and percentages of TCRβ^{low}CD69^{-}, TCRβ^{low}CD69^{+}, TCRβ^{high}CD69^{+}, and TCRβ^{high}CD69^{-} cells among CD8SP cells (B).

(C–F) Flow cytometric analysis of TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells in the indicated mice. Shown are a representative profile of flow cytometric analysis and dot plot data for TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells (C), numbers of TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells (D), TCRβ^{low}CD69$^{-/+}$CD103^{+}CD4^{+}CD8^{low} cells (E), and percentages of Bim-expressing or Nur77-expressing cells among TCRβ^{low}CD69$^{-/+}$CD103^{+} DP cells (F).

(G–I) Flow cytometric analysis of TCRβ^{high}CD69^{+}CD103^{+} DP cells in the indicated mice. Shown are a representative profile of flow cytometric analysis and dot plot data for TCRβ^{high}CD69^{+}CD103^{+} DP cells (G). Also shown are numbers of TCRβ^{high}CD69^{+}CD103^{+} DP cells (H) and percentages of Bim-expressing or Nur77-expressing cells among TCRβ^{high}CD69^{+}CD103^{+} DP cells (I).

(J and K) Percentages of Bim-expressing or Nur77-expressing cells in TCRβ^{high} CD8SP (J) and TCRβ^{high} CD4SP cells (K).

Numbers in dot plot quadrants indicate percentages (A). Data are expressed as means ± SD (B, D–F, and H–K). Data were pooled from at least three independent experiments. Statistical significance was analyzed by one-way ANOVA with multiple comparisons post test: *p < 0.05, **p < 0.01, ***p < 0.001.

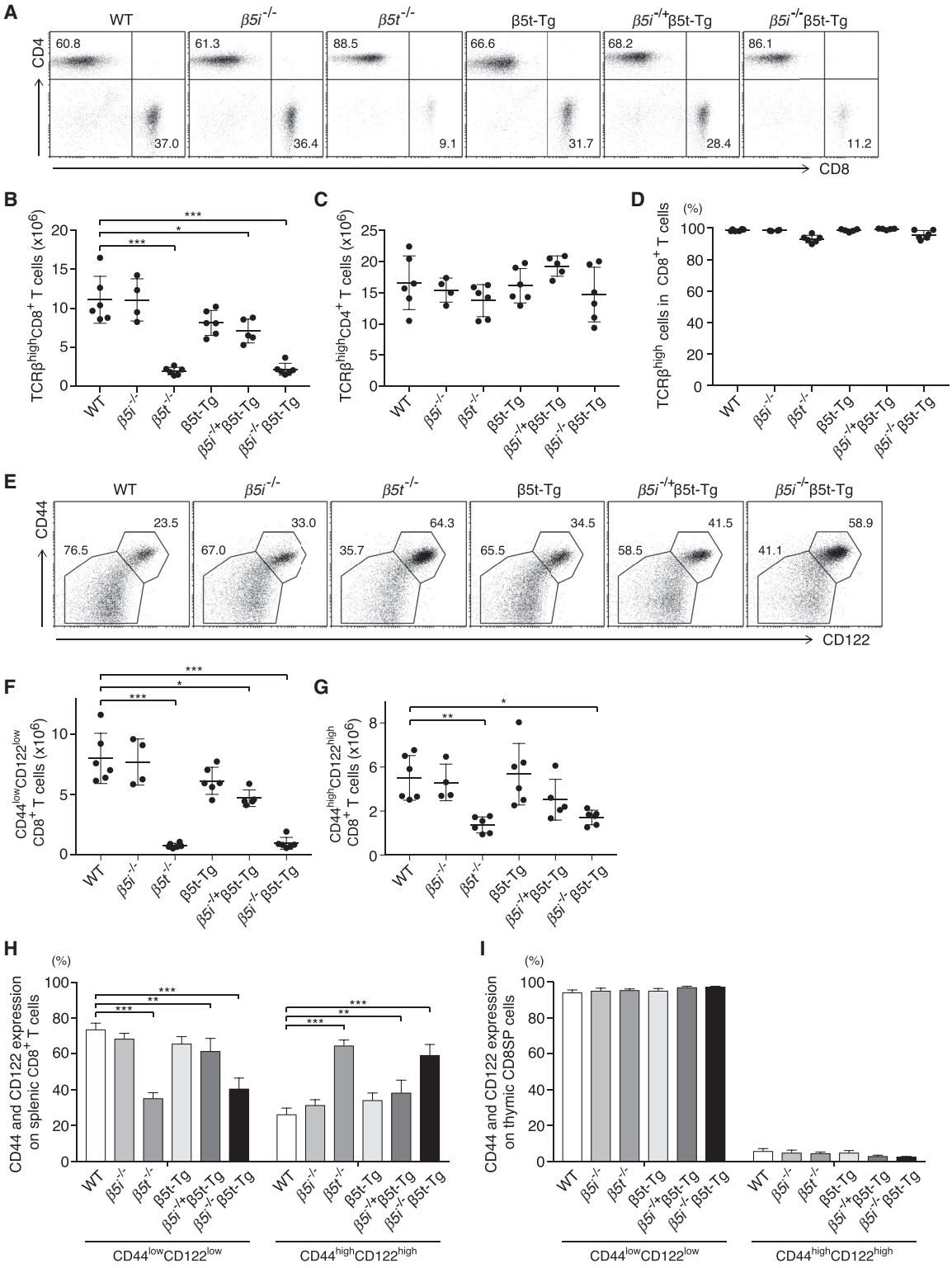

Figure 3. The Peripheral Pool of Naive CD8$^+$ T Cells Is Impaired in Mice with Aberrant Expression of β5t

(A–D) Flow cytometric analysis of peripheral T cells. Shown are representative data of dot plot quadrants (A), numbers of TCRβ^{high} CD8$^+$ T cells (B), and numbers of TCRβ^{high} CD4$^+$ T cells (C) in the indicated mice. The percentage of TCRβ^{high} cells among CD8$^+$ T cells is shown in (D).

(E–H) Flow cytometric analysis of CD44 and CD122 on peripheral CD8$^+$ T cells in the indicated mice. Shown are representative data of dot plot profiles (E), numbers of CD44^{low}CD122^{low} (F) or CD44^{high}CD122^{high} CD8$^+$ T (G) cells, and percentages of CD44^{low}CD122^{low} or CD44^{high}CD122^{high} CD8$^+$ T cells in CD8$^+$ T cells (H).

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into $\beta5i^{-/-}$β5t-Tg mice, the change to the memory phenotype was not observed (Figure S6).

In $\beta5i^{-/-}$β5t-Tg mice, lymphoid tissues express proteasomes containing β5t with β1i and β2i (thymoproteasomes) that are the same as those expressed in the thymus, whereas other tissues (such as liver, kidney, and muscle) express proteasomes containing β5t with β1 and β2 that do not exist physiologically. One possible explanation for the phenotypic alteration is that the transferred naive $CD8^+$ cells were stimulated by peptides produced by thymoproteasomes with which they had already reacted in the thymus during positive selection. Alternatively, these cells may have reacted with unique peptides produced by proteasomes containing β5t with β1 and β2 that they had not encountered in the thymus as foreign antigens. To investigate these possibilities, we created BM chimeras by transferring BM from EGFP mice into $\beta5t^{-/-}$ or WT host mice. Following BM transplantation, naive $EGFP^+CD8^+$ T cells in host mice were sorted and transferred into $\beta5i^{-/-}$β5t-Tg mice. As shown in Figure 4C, the change to the memory phenotype was more abundant in transferred $EGFP^+CD8^+$ T cells from WT hosts than in those from $\beta5t^{-/-}$ hosts. These results suggested that the transferred naive $CD8^+$ T cells were stimulated more abundantly by peptides with which they had already reacted than by those they had not encountered during positive selection. However, because a slight alteration to the memory phenotype was observed in transferred $EGFP^+CD8^+$ T cells from $\beta5t^{-/-}$ hosts, it remains possible that some of the transferred $CD8^+$ T cells may have reacted with peptides they had never encountered in the thymus as foreign antigens.

Bcl-6 plays important roles in TCR signaling to generate antigen-induced memory $CD8^+$ T cells (Ichii et al., 2002). Bcl-6 was upregulated in transferred $EGFP^+CD8^+$ T cells showing a phenotypic change to $CD44^{high}CD122^{high}$ (Figure 4D), indicating that this phenotypic change of transferred $CD8^+$ T cells depends on the interaction between peptide-MHC and the TCR. Moreover, transferred $EGFP^+CD8^+$ T cells with a $CD44^{high}CD122^{high}$ phenotype showed increased expression of PD-1 and Tim-3 (Figures 4E–4H). The sustained presence of antigenic peptides induces accumulation of PD-1- or Tim-3-expressing $CD8^+$ T cells (Ferris et al., 2014). These results demonstrate that aberrant peripheral expression of self-peptides generated by β5t disrupts the maintenance of a naive pool of $CD8^+$ T cells.

Aberrant Expression of β5t in the Periphery Induces Autoreactive T Cell Responses

Finally, we analyzed whether aberrant expression of self-peptides derived from β5t induced autoreactive T cell responses. $\beta5i^{-/-}$β5t-Tg mice showed few inflammatory lesions; this could be attributed to negative selection of autoreactive T cells by β5t-expressing mTECs or to an insufficient number of autoreactive T cells to induce inflammation in $\beta5i^{-/-}$β5t-Tg mice. We therefore performed cell transfer experiments using splenic cells from WT mice (WT-SpCs). WT-SpCs (1.5×10^7) were intraperitoneally transferred into $\beta5i^{-/-}$β5t-Tg mice four times at intervals of 3 days, and recipient mice were subjected to laboratory analysis and histological examination 28 days after the first transfer. The controls consisted of WT or $\beta5i^{-/-}$β5t-Tg mice receiving WT-SpCs or $\beta5i^{-/-}$β5t-Tg-SpCs. Serum glutamic oxaloacetic transaminase (GOT) and lactate dehydrogenase (LDH) levels were higher in $\beta5i^{-/-}$β5t-Tg mice receiving WT-SpCs than in the controls (Figure 5A). Histologically, $\beta5i^{-/-}$β5t-Tg mice receiving WT-SpCs exhibited a significant increase in inflammatory foci in the liver, kidney, and lung (Figures 5B, 5C, and S7). To clarify whether the transferred WT-SpCs in $\beta5i^{-/-}$β5t-Tg mice showed autoreactive T cell responses against self-peptides generated by β5t, we performed *in vitro* experiments using immunized WT-SpCs transferred into $\beta5i^{-/-}$β5t-Tg mice. After performing the transfer using the protocol described, the SpCs of recipient $\beta5i^{-/-}$β5t-Tg mice containing immunized WT-SpCs were co-cultured with primary vascular endothelial cells (pVECs) derived from $\beta5i^{-/-}$β5t-Tg mice or pVECs derived from WT mice as a control (Figure 5D). After 8 days, the expression of CD69, which indicates a recently activated and antigen-experienced phenotype, and IFN-γ production in incubated $CD8^+$ T cells were analyzed by flow cytometry. $CD69^+$ $CD8^+$ T cells and IFN-γ-producing $CD8^+$ T cells were significantly increased when SpCs from recipient $\beta5i^{-/-}$β5t-Tg mice were incubated with the pVECs from $\beta5i^{-/-}$β5t-Tg mice (Figures 5E and 5F). These results indicated that a population of $CD8^+$ T cells reacted against self-peptides generated by β5t in WT-SpC-transferred recipient $\beta5i^{-/-}$β5t-Tg mice, suggesting that autoreactive T cell responses against self-peptides in the periphery are induced when $CD8^+$ T cells recognize similar self-peptides presented during positive selection.

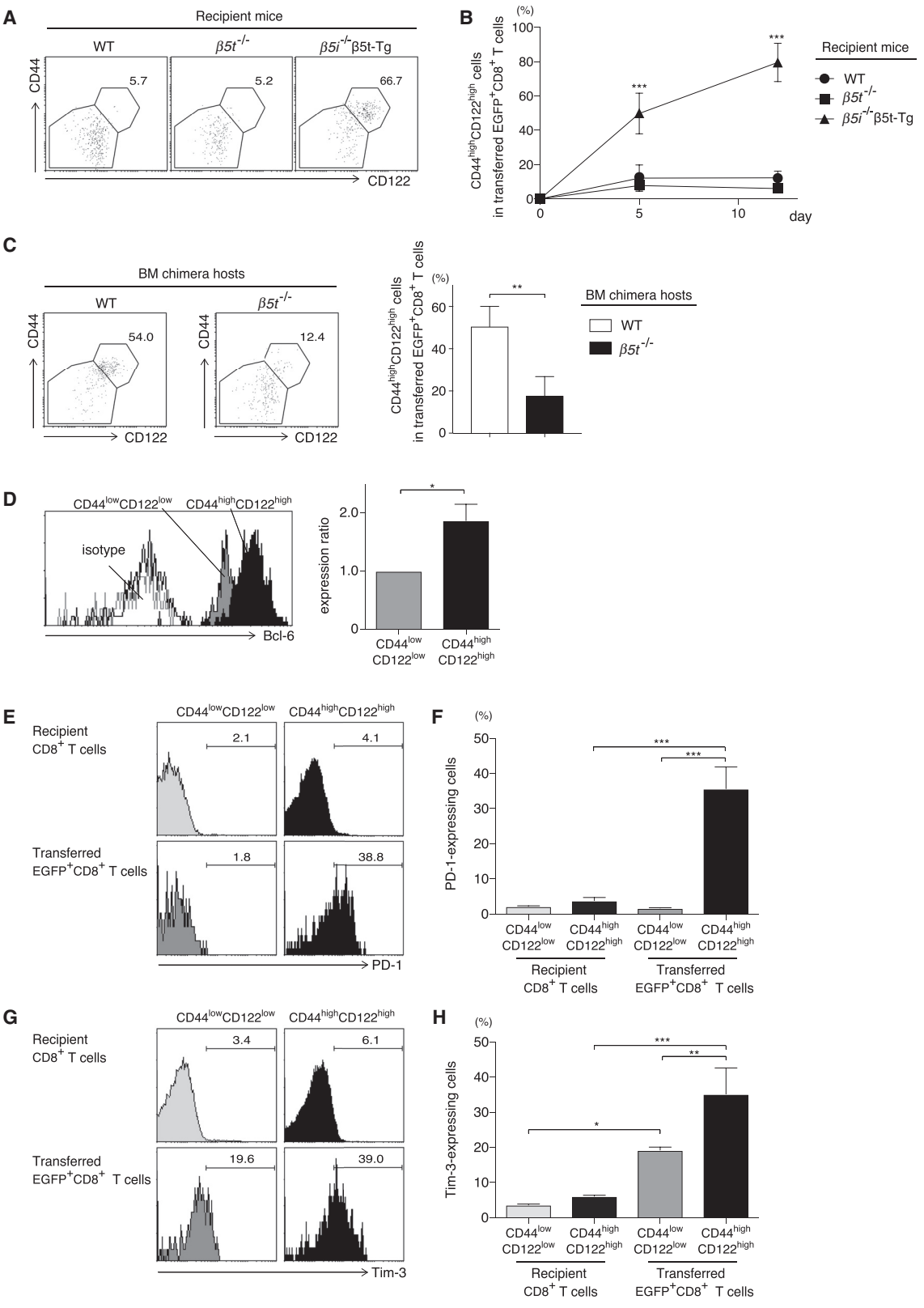
DISCUSSION

The self-peptides presented by MHC class I are generated by proteasomes. During thymic selection, cTECs express unique peptides produced by the thymoproteasome subunit β5t. The thymoproteasome supports positive selection by generating peptides that are optimized for the selection (Murata et al., 2007, 2008; Nitta et al., 2010; Xing et al., 2013; Sasaki et al., 2015). However, it remains unclear how thymoproteasomes regulate the development of $CD8^+$ T cells or affect homeostasis of $CD8^+$ T cells. In this study, we showed that aberrant expression of self-peptides generated by β5t affects $CD8^+$ T cell homeostasis, including thymic selection of CD8SP cells and the maintenance of a peripheral naive pool of $CD8^+$ T cells.

Thymic selection processes are compartmentalized in the thymic cortex and medulla. Positive selection is largely driven by interactions between DP thymocytes and cTECs in the cortex. The medulla is considered a specialized site for negative selection because of autoimmune regulator (AIRE)-mediated

(I) Flow cytometric analysis of CD44 and CD122 on thymic CD8SP T cells in the indicated mice.

Numbers in dot plot quadrants and adjacent to outlined areas indicate percentages (A and E). Each symbol represents an individual mouse; the long horizontal line represents the mean, and short horizontal lines represent SD (B–D, F, and G). Data are expressed as percentages (mean ± SD) (H and I). Data were pooled from at least three independent experiments. Statistical significance was analyzed by one-way ANOVA with multiple comparisons post test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



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expression of tissue-restricted antigens (Andersen et al., 2007; Kyewski and Klein, 2006). Mice that lack the thymoproteasome subunit β5t had substantially fewer CD8SP cells; it is likely that thymoproteasome-generated self-peptides expressed by cTECs contribute to the positive selection of $CD8^+$ T cells (Murata et al., 2007). However, given that $\beta5t^{-/-}$ mice express immunoproteasomes in cTECs as well as mTECs, two hypotheses have been proposed to explain the mechanisms of thymoproteasome-mediated T cell selection. First, thymoproteasomes contribute to positive selection of $CD8^+$ T cells by providing self-peptides for low-affinity TCR interactions, and deletion is caused by high-affinity peptides expressed by immunoproteasomes in cTECs of $\beta5t^{-/-}$ mice. Second, because immunoproteasomes were expressed both in cTECs and mTECs of $\beta5t^{-/-}$ mice, overlap between peptides involved in positive and negative selection causes enhancement of negative selection (i.e., deletion by re-encounter of self-peptides between the cortex and medulla). Xing et al. (2013) demonstrated that the thymoproteasome supports positive selection by generating peptides that are optimized for the selection; the animals used in these studies were generated by crossing $\beta5t^{5i}$ knockin mice and $\beta5i^{-/-}$ mice, yielding mice in which β5i was exclusively expressed in cTECs, whereas β5 was expressed in other cells. On the other hand, an analysis using mice that lack all four subunits (β5t, β5i, β1i, and β2i) (Kincaid et al., 2016) showed that peptide switching between positive and negative selection is important for the establishment of a broad TCR repertoire. In this study, we established and analyzed mice expressing β5t in both cTECs and mTECs. In $\beta5i^{-/-}$β5t-Tg mice, β5t-mediated thymic selection by cTECs was not affected because expression of β5t was retained in cTECs in transgenic animals as in WT mice. $CD8^+$ lineage cells in $\beta5i^{-/-}$β5t-Tg mice were less abundant, and the proapoptotic molecules Bim and Nur77 were induced during the transitional process from late post-selection DP to early SP cells, possibly during negative selection; these observations support the second hypothesis. By contrast, $CD8^+$ lineage DP cells in $\beta5t^{-/-}$ mice started to disappear much earlier, between the pre-selected and positive selection stages, suggesting that deletion occurred during positive selection because of the defect in β5t. The decrease in the abundance of $CD8^+$ lineage cells was prolonged to the late post-selected phase in $\beta5t^{-/-}$ mice, implying that additional deletion (as described by the second hypothesis) might occur in $\beta5t^{-/-}$ mice as well. Taken together, low-affinity peptides generated by thymoproteasomes are essential for positive selection, but the difference in presented peptides between the cortex and medulla is also crucial for $CD8^+$ T cell development in the thymus. Interestingly, according to the results of a peptide digestion analysis using purified thymoproteasomes or immunoproteasomes, 60% of the detected peptides are thymoproteasome-specific, and peptides carrying thymoproteasome-dependent motifs are enriched in low-affinity TCR ligands that effectively induce positive selection (Sasaki et al., 2015). However, 40% of peptides are common to thymoproteasomes and immunoproteasomes; in other words, some peptides produced by thymoproteasomes in cTECs provide high-affinity TCR interactions, as do immunoproteasomes in mTECs. Therefore, it is reasonable to assume that some CD8SP cells with high affinity for thymoproteasome-derived peptides are positively selected but subsequently deleted when they re-encounter mTECs during negative selection.

It is also possible that alternate mechanisms are involved in the abnormal development of $CD8^+$ T cells in $\beta5i^{-/-}$β5t-Tg mice. Negative selection can take place in both the cortex and medulla. Negative selection in the thymic cortex is specific for ubiquitous self-antigens, and clonal deletion is induced at the DP stage upon interaction with cortical DCs (Baldwin et al., 2005; McCaughtry et al., 2008). In the thymic cortex, DCs physiologically express the proteasome subunit β5i, and β5i-expressing DCs may induce high-affinity TCR signaling to delete thymocytes that respond to ubiquitous self-antigens (McCaughtry et al., 2008). DC-presenting low-affinity peptides generated by β5t may promote abnormal proapoptotic cascades in thymocytes. Another possibility is that, because proteasomes play important roles in protein homeostasis, including proteolysis and metabolism, the abnormal activity of proteasomes in $\beta5i^{-/-}$β5t-Tg mice might affect the functions of the thymocytes themselves. However, CD4SP cells developed normally in $\beta5i^{-/-}$β5t-Tg mice, and BM chimeras created by transferring BM from EGFP mice into $\beta5i^{-/-}$β5t-Tg hosts had significantly fewer CD8SP cells. Therefore, the reduced abundance of CD8SP cells in $\beta5i^{-/-}$β5t-Tg mice was not caused by an intrinsic abnormality in thymocytes themselves because of expression of β5t; instead, it depended on the interaction between the TCR and altered self-peptides generated by aberrant β5t expression in thymic stromal cells.

Figure 4. $CD44^{low}CD122^{low}$ $CD8^+$ T Cells of EGFP Mice Change Phenotype to $CD44^{high}CD122^{high}$ after Transfer into $\beta5i^{-/-}$β5t-Tg Mice

(A and B) Flow cytometric analysis of CD44 and CD122 on transferred $EGFP^+CD8^+$ T cells. $CD44^{low}CD122^{low}$ $CD8^+$ T cells of EGFP mice were transferred into WT, $\beta5t^{-/-}$, and $\beta5i^{-/-}$β5t-Tg mice. Representative data of dot plot profiles 12 days after the transfer are shown in (A), and the chronological changes of CD44 and CD122 expression on transferred $EGFP^+CD8^+$ T cells are shown in (B).

(C) Flow cytometric analysis of CD44 and CD122 on transferred $EGFP^+CD8^+$ T cells from BM chimeras. BM chimeras were created by transferring BM from EGFP mice into $\beta5t^{-/-}$ or WT mice, and $CD44^{low}CD122^{low}$ $CD8^+$ T cells from BM chimera hosts were transferred into $\beta5i^{-/-}$β5t-Tg mice. Flow cytometry analysis was performed 10 days after the transfer.

(D) Bcl-6 expression in transferred $EGFP^+CD8^+$ T cells in $\beta5i^{-/-}$β5t-Tg mice. Shown are representative data of the histogram 12 days after the transfer and the ratio of Bcl-6 expression in $CD44^{high}CD122^{high}$ versus $CD44^{low}CD122^{low}$ T cells. Flow cytometry data are expressed as relative values, with the mean fluorescence intensity (MFI) of Bcl-6 in $CD44^{low}CD122^{low}$ in transferred $EGFP^+CD8^+$ T cells defined as 1.

(E–H) Expression of PD-1 and Tim-3 on recipient $CD8^+$ T cells and transferred $EGFP^+CD8^+$ T cells in $\beta5i^{-/-}$β5t-Tg mice. Representative data of the histogram 12 days after the transfer (E and G) and percentages of PD-1- or Tim-3-expressing cells in recipient or transferred $CD8^+$ T cells with the indicated CD44 and CD122 profiles are shown (F and H).

Numbers adjacent to outlined areas and in histograms indicate percentages (A, C, E, and G). Horizontal lines indicate SD (B). Data are expressed as means SD (C, D, F, and H). Data were pooled from three independent experiments. Statistical significance was analyzed by Student's t test and one-way ANOVA with multiple comparisons post test: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

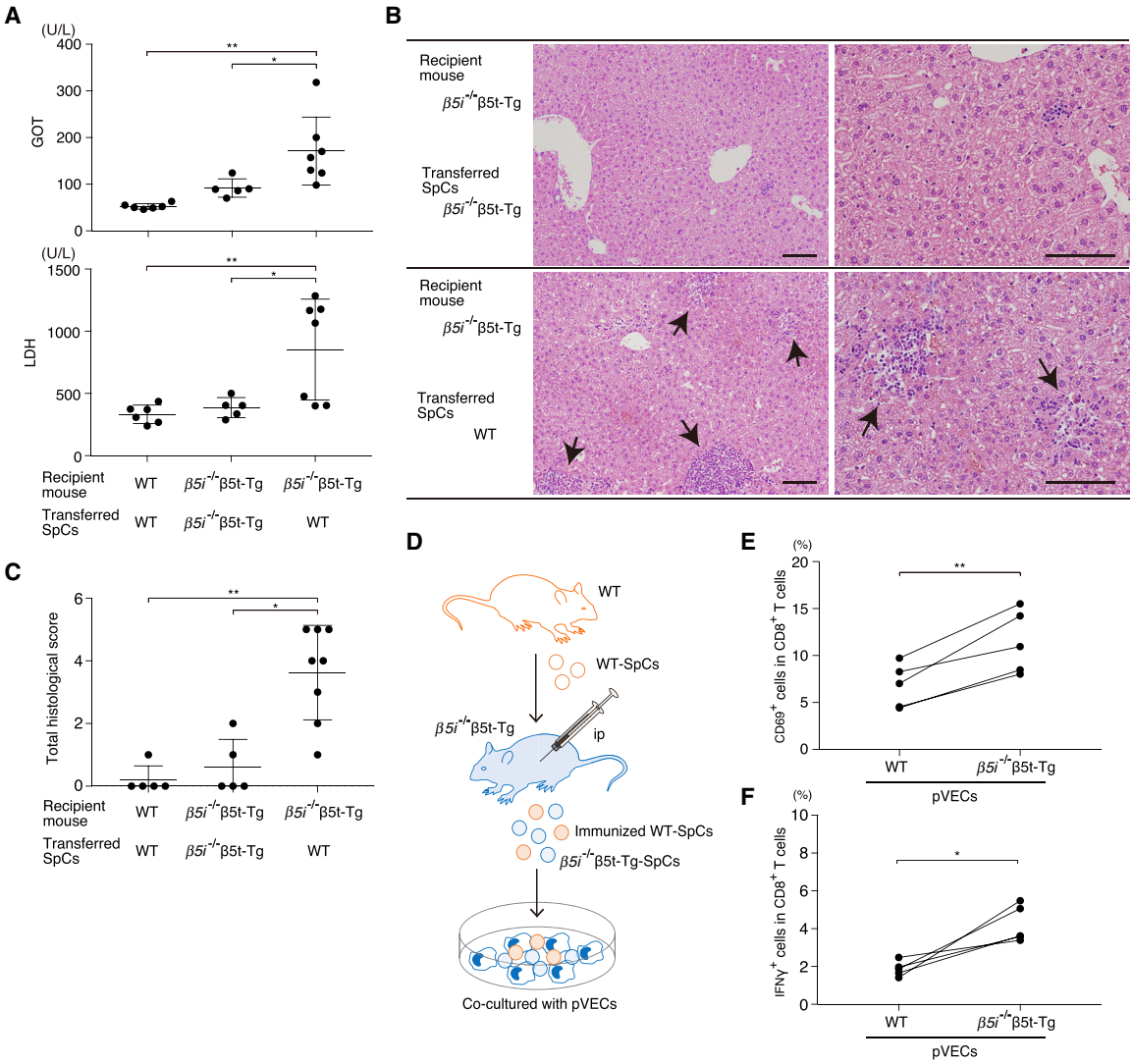

Figure 5. Aberrant Expression of β5t in the Periphery Induces a Self-Reactive T Cell Response

(A–C) Cell transfer experiments using the indicated donor SpCs and recipient mice. Plasma concentrations of GOT and LDH (A), representative histological images of liver tissues (B), and total histological scores (C) of recipient mice are shown. In the histological images, the left side represents a low-power view, the right side shows a high-power view, and arrows indicate inflammatory foci. The method used to determine the histological score is described in the STAR Methods.

(D–F) $CD8^+$ T cell responses to primary vascular endothelial cells (pVECs) from WT or $\beta5i^{-/-}$β5t-Tg mice. Shown is a schematic of *in vitro* experiments analyzing T cell responses of transferred WT-SpCs in $\beta5i^{-/-}$β5t-Tg mice (D). Also shown is expression of CD69 (E) and IFN-γ (F) in splenic $CD8^+$ T cells from WT-SpC-transferred recipient $\beta5i^{-/-}$β5t-Tg mice.

Each symbol represents an individual mouse; a long horizontal line represents the mean, and short horizontal lines represent SD (A, C, E, and F). The scale bar represents 100 μm (B). Data were pooled from three (A–C) and two (E and F) independent experiments. Statistical significance was analyzed by paired Student's t test and one-way ANOVA with multiple comparisons post test: *$p < 0.05$, **$p < 0.01$.

In addition to thymic selection, we investigated whether peripheral immune regulation is affected by aberrant expression of β5t. Memory $CD8^+$ T cells accumulated in $\beta5i^{-/-}$β5t-Tg mice, and a change from a naive to memory cell phenotype was observed when naive $CD8^+$ T cells from EGFP mice were transferred into $\beta5i^{-/-}$β5t-Tg mice but not when they were transferred into WT or $\beta5t^{-/-}$ mice. Peripheral T cells react with self-peptides to maintain homeostatic proliferation during recirculation between the blood and lymphoid organs, and continuous stimulation of $CD8^+$ T cells by antigenic self-peptides can drive differentiation into a memory or exhausted phenotype (Goronzy and Weyand, 2001; Takada and Jameson, 2009). Given that the change to the memory phenotype was much more pronounced in $EGFP^+CD8^+$ T cells transferred to WT BM chimera hosts than in those transferred to $\beta5t^{-/-}$ hosts, the transferred naive $CD8^+$ T cells must have reacted with peptides they had already recognized during positive selection. In addition, $\beta5i^{-/-}$β5t-Tg mice receiving WT-SpCs exhibited a significant

increase in inflammatory foci. When the immunized WT-SpCs were co-cultured with pVECs, they reacted against pVECs derived from $\beta5i^{-/-}$β5t-Tg mice but not those derived from WT mice. These results suggest that self-peptides derived from β5t that are physiologically expressed in the thymus could serve as autoimmune antigenic peptides in the periphery. Although it remains unknown whether the aberrant expression of thymoproteasomes is associated with the pathogenesis of autoimmune disorders, some self-peptides that mimic those produced by thymoproteasomes may cause autoreactive immune responses.

Antigen-experienced, exhausted $CD8^+$ T cells are characterized by the expression of PD-1 or Tim-3, and the abundance of PD-1^+ or Tim-3^+ $CD8^+$ T cells increases during chronic viral infections and in aging (Jin et al., 2010; Lee et al., 2016). In this study, PD-1 or Tim-3 was upregulated in transferred naive $CD8^+$ T cells after their conversion to a memory cell phenotype, suggesting that self-peptides generated by β5t in the periphery persistently stimulate $CD8^+$ T cells as potential antigenic self-peptides. Inhibitory receptors such as PD-1 and Tim-3 play a crucial role in regulating $CD8^+$ T cell function, and they have attracted attention as a target for immunomodulatory therapy in autoimmune disorders. In autoimmune disorders, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis, PD-1^+ or Tim-3^+ $CD8^+$ T cells are more abundant in peripheral blood (Liu et al., 2009; Song et al., 2015; Jiao et al., 2016; Ramwadhdoebe et al., 2016). The pathological roles of these molecules on $CD8^+$ T cells in autoimmune disorders remain controversial. Upregulation of Tim-3 is associated with disease activity in patients with SLE (Song et al., 2015; Jiao et al., 2016). A recent study showed that diabetogenic T cells are memory PD-1^+ $CD8^+$ T cells in non-obese diabetic (NOD) mice (Garyu et al., 2016). In this study, we demonstrated that naive $CD8^+$ T cells changed their phenotype to memory PD-1^+ Tim-3^+ $CD8^+$ T cells and subsequently induced autoimmune responses against self-peptides generated by β5t. Therefore, aberrant expression of β5t in the periphery may cause failure of immunological regulation and tolerance. Although further studies are required to determine the potential roles of these phenotypic alterations of $CD8^+$ T cells in immune disorders, these observations highlight the importance of the relationship between phenotypic changes of $CD8^+$ T cells and immune dysfunction.

The transcription of β5t is regulated by Foxn1, a transcription factor that is essential for thymus organogenesis and hair follicle development (Uddin et al., 2017). Aberrant expression of β5t in the periphery has not been demonstrated to date. However, alteration or degeneration of cellular proteins may change the self-peptides presented to T cells. For example, oxidative stress modifies antigenic peptides and affects T cell responses (Weiskopf et al., 2010). Oxidative modification of cellular proteins could induce alterations of self-peptides, which may mimic unique self-peptides presented in the thymic cortex for positive selection; however, this assumption needs to be proven. Inflammation causes oxidative stress and is thus an important trigger for autoimmune disorders. Although many studies of the pathogenesis of autoimmunity have focused on autoreactive $CD4^+$ T cells, future studies should investigate the pathogenic roles of $CD8^+$ T cells.

In the regulation of the immune system, processes are continuously tuned by homeostatic signals; the thymus produces T cells by thymic selection, and the interaction between the TCR and self-peptides maintains naive T cells in the periphery. Proteasomes are important for the generation of self-peptides for thymic selection and maintenance of the peripheral T cell pool. In particular, the thymoproteasome subunit β5t is selectively expressed in the thymic cortex; therefore, self-peptides for positive selection should be isolated from peripheral tissues. This containment of self-peptides generated by β5t may be important for T cell homeostasis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.12.078.

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AUTHOR CONTRIBUTIONS

U.T. designed and performed the experiments, analyzed the data, and wrote the paper. S. Konno, S. Miyajima, R. K., M.O., and S. Kiuchi performed experiments. S. Murata generated the β5t-deficient mice and discussed the results. A.I. analyzed the data and discussed the results. M.K. supervised the experiments and discussed the conclusions.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD3-APC (145-2C11)	eBioscience	Cat# 17-0031-83, RRID:AB_469316
CD3-PerCP (145-2C11)	BD Biosciences	Cat# 553067, RRID:AB_394599
CD4-PE (GK1.5)	BD Biosciences	Cat# 553730, RRID:AB_395014
CD4-PE-Cy7 (GK1.5)	eBioscience	25-0041-82, RRID:AB_469576
CD4-BV510 (GK1.5)	Biolegend	Cat# 100449, RRID:AB_2564587
CD8-FITC (53-6.7)	BD Biosciences	Cat# 553031, RRID:AB_394569
CD8-PE-Cy7 (53-6.7)	eBioscience	Cat# 25-0081-82, RRID:AB_469584
CD8-BV510 (53-6.7)	BD Biosciences	Cat# 563068, RRID:AB_2687548
TCRβ-APC (H57-597)	eBioscience	Cat# 17-5961-83, RRID:AB_469482
CD69-PerCP/Cy5.5 (H1.2F3)	Biolegend	Cat# 104522, RRID:AB_2260065
CCR7-Alexa Fluor 488 (4B12)	Biolegend	Cat# 120110, RRID:AB_492841
CD103-APC/Cy7 (2E7)	Biolegend	Cat# 121431, RRID:AB_2566551
CD44-PE-Cy7 (IM7)	eBioscience	Cat# 25-0441-82, RRID:AB_469623
CD122-PE (TM-b1)	eBioscience	Cat# 12-1222-81, RRID:AB_465835
CD62L-PE (MEL-14)	eBioscience	Cat# 12-0621-82, RRID:AB_465721
PD-1-APC/Fire750 (29F.1A12)	Biolegend	Cat# 135239, RRID:AB_2629767
Tim-3-PerCP/Cy5.5 (RMT3-23)	Biolegend	Cat# 119717, RRID:AB_2571934
Bcl-6-APC (BCL-DWN)	eBioscience	Cat# 17-5453-80, RRID:AB_2573213
Bim-PE (C34C5)	Cell Signaling Technology	Cat# 12186
Nur77-PE (12.14)	eBioscience	Cat# 12-5965-82, RRID:AB_1257209
IFN-γ (XMG1.2)	Biolegend	Cat# 505808, RRID:AB_315402
Critical Commercial Assays		
BD Cytofix/Cytoperm Plus	BD Biosciences	Cat# 554715
Transcription factor buffer set	BD Biosciences	Cat# 562574
Experimental Models: Organisms/Strains		
C57BL/6	Japan SLC	N/A
CAG-EGFP (EGFP)	Japan SLC	N/A
β5t-Tg	Tomaru et al., 2012	N/A
β5i$^{-/-}$	RIKEN BRC	RBRC03950
β5t$^{-/-}$	RIKEN BRC	RBRC03928

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Utano Tomaru (tomaruu@med.hokudai.ac.jp).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

β5i$^{-/-}$ and *β5t*$^{-/-}$ mice were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Transgenic mice expressing β5t (β5t-Tg) were established as previously described (Tomaru et al., 2012). *β5i*$^{-/+}$β5t-Tg and *β5i*$^{-/-}$β5t-Tg mice were created by bleeding *β5i*$^{-/-}$ mice to β5t-Tg mice. C57BL/6 mice were used as wild-type (WT) controls. C57BL/6 background CAG-EGFP (EGFP) mice were purchased from Japan SLC. 8–12-week-old age and sex matched mice were used for all experiments. Both males and females were used; obvious sex difference were not noted. Mice were housed on a 12-h light–dark cycle in climate-controlled, pathogen-free barrier facilities. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals at Hokkaido University Graduate School of Medicine.

METHOD DETAILS

Flow cytometry

Cell suspensions were stained with the indicated antibodies (Abs) from eBioscience, BD Biosciences, and Biolegend. The following fluorescently-labeled Abs were used: CD3ε (145-2C11), CD4 (GK1.5), CD8α (53-6.7), CD44 (IM7), CD69 (H1.2F3), CD122 (TM-b1), CD62L (MEL-14), TCRβ (H57-597), CD103 (2E7), CCR7 (4B12), Vβ screening panel (Vβ2, Vβ3, Vβ4, Vβ5.1/5.2, Vβ6, Vβ7, Vβ8.1/8.2, Vβ8.3, Vβ9, Vβ10b, Vβ11, Vβ12, Vβ13, and Vβ14), H-2Kb (AF6-88.5.5.3), PD-1 (29F.1A12), and Tim-3 (RMT3-23). For the intracellular detection of Bim (C34C5), Nur77 (12.14), and Bcl-6 (BCL-DWN), cells were fixed and permeabilized using BD Cytofix/Cytoperm fixation/permeabilization solution or transcription factor buffer set (BD Bioscience). For detection of IFN-γ, monensin was added to the incubation medium for 6 hours before cellular fixation, and the cells were stained with Ab against IFN-γ (XMG1.2). Flow cytometric analysis was performed using a Canto II flow cytometer (BD Bioscience).

Cell sorting

Thymic stromal cells were prepared by enzymatic digestion of mice thymi (Williams et al., 2009). After thymocyte depletion, cells were stained using UEA-I and Abs against CD45 (30-F11), MHC class II (I-A/I-E, M5/114.15.2), EpCAM (G8.8), and Ly-51 (6C3). UEA-I was obtained from Vector Laboratories, and Abs were purchased from BD Biosciences. Splenic cells (SpCs) were stained with Ab against CD11c (HL3). $CD45^{-}MHCII^{+}EpCAM^{+}Ly51^{+}UEA\text{-}I^{-}$ cTECs, $CD45^{-}MHCII^{+}EpCAM^{+}Ly51^{-}UEA\text{-}I^{+}$ mTECs, and $CD11c^{+}$ DCs were sorted using an Aria III flow cytometer (BD Bioscience). $CD44^{low}CD122^{low}$ $EGFP^{+}CD8^{+}$ cells or $CD44^{low}CD62L^{high}$ $EGFP^{+}CD4^{+}$ cells were sorted from EGFP mice or BM chimeras for cell transfer experiments.

Western blotting

Tissues or sorted TECs and DCs were lysed in a buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.2% NP-40, and 1 mM DTT, and then centrifuged at 15,000 *g* for 10 min. The supernatants (10 μg aliquot of total proteins) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. The blots were probed with Abs and reacted with horseradish peroxidase–conjugated anti-rabbit or goat IgG (Jackson ImmunoResearch, West Grove, PA, USA) for immunodetection. The immune complexes were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) and analyzed by Image Gauge software (Fujifilm, Tokyo, Japan). For immunoblotting using purified proteasomes, proteasomes were enriched using the CycLex Proteasome Enrichment & Activity Assay Kit (MBL). Tissue lysates (2 mg of protein) were incubated with control-resin or ubiquitin-like domain (UbL)-resin, and then subjected for immunodetection according to the manufacturer's instructions. Abs for β5t, β5i, β5, and β6 were purchased from MBL, Enzo Life Sciences, Santa Cruz Biotechnology, and Abcam, respectively. Abs for β1, β2, β1i, β2i were provided by Prof. Shigeo Murata.

Bone marrow chimera and cell transfer experiments

Bone marrow cells (5×10^6) were injected into irradiated (7 Gy) host mice. For analysis of phenotypic alterations, 5×10^5 sorted $CD44^{low}CD122^{low}$ $EGFP^{+}CD8^{+}$ or $CD44^{low}CD62L^{high}$ $EGFP^{+}CD4^{+}$ cells were intravenously transferred into WT, $\beta5t^{-/-}$, or $\beta5i^{-/-}$β5t-Tg mice. At 5 and 12 days after the transfer, $EGFP^{+}CD8^{+}$ T cells were examined by flow cytometry. At 12 days after the transfer, $EGFP^{+}CD4^{+}$ T cells were examined by flow cytometry. For cell transfer experiments using sorted $CD44^{low}CD122^{low}$ $EGFP^{+}CD8^{+}$ cells from BM chimera hosts, 1×10^5 cells were used, and flow cytometry analysis was performed 10 days after the transfer. For cell transfer experiments to analyze T cell responses, SpCs from WT (SpCs-WT) were injected into WT or $\beta5i^{-/-}$β5t-Tg mice, and SpCs-$\beta5i^{-/-}$β5t-Tg were injected into $\beta5i^{-/-}$β5t-Tg mice; 1.5×10^7 cells were injected four times into each mouse at 3-day intervals. The recipient mice underwent laboratory analysis and histological examination 28 days after the first transfer.

Histological analysis

Formalin-fixed tissue sections were stained with hematoxylin and eosin (H&E). The results of histological examination were scored according to the number of inflammatory foci and infiltrating cells as follows: score 0, normal; score 1, mild; score 2, moderate; and score 3, severe. The total histological score was the sum of histological scores for the liver, kidney, and lung.

T cell stimulation

SpCs from WT-SpC–transferred recipient $\beta5i^{-/-}$β5t-Tg mice were incubated with primary vascular endothelial cells (pVECs) from WT or $\beta5i^{-/-}$β5t-Tg mice for 8 days in culture medium containing 40 U/ml of interleukin 2, and then examined by flow cytometry. pVECs were isolated from lung and liver tissues of WT or $\beta5i^{-/-}$β5t-Tg mice using collagenase digestion.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses of two unmatched or matched groups were performed using the unpaired or paired two-tailed Student's t test, respectively. For the analysis of three or more unmatched groups, one-way ANOVA with multiple comparisons / post hoc tests was performed. *P values* < 0.05 were considered significant.