Cutting Edge: Fine-Tuning of *Thpok* Gene Activation by an Enhancer in Close Proximity to Its Own Silencer

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Differentiation of MHC class II–selected thymocytes toward the CD4^{+} helper lineage depends on function of the transcription factor ThPOK, whose expression is repressed in CD8^{+} cytotoxic lineage cells by a transcriptional silencer activity within the distal regulatory element (DRE) in the *Thpok* gene. Interestingly, the DRE also functions as a transcriptional enhancer. However, how the DRE exerts such dual functionality remains obscure. In this study, we dissected the DRE and identified DNA sequences specifically responsible for enhancer activity, and designated this as the thymic enhancer. Removal of the thymic enhancer from the murine *Thpok* locus resulted in inefficient ThPOK induction, thereby inducing a redirection toward alternative CD8^{+} cytotoxic lineage in a proportion of MHC class II–selected cells, even when they express monoclonal MHC class II–restricted transgenic TCR. Thus, regulation of contiguous but separable sequences with opposite function in the DRE plays an important role in precise coupling of TCR signaling with the selection process of two opposite lineages.   *The Journal of Immunology*, 2013, 190: 1397–1401.

Two distinct T lymphocyte subsets, CD4^{+} helper and CD8^{+} cytotoxic cells, are differentiated from common precursors, the CD4^{+}CD8^{+} double-positive (DP) thymocytes. MHC class II–selected thymocytes differentiate into CD4^{+}CD8^{−} single-positive (SP) thymocytes committed to the helper lineage, whereas thymocytes expressing MHC class I–restricted TCRs differentiate into CD4^{−}CD8^{+} SP thymocytes committed to the cytotoxic lineage (1). Thus, TCR specificity for MHC molecules is matched with helper versus cytotoxic lineage choice. Transcription factor ThPOK encoded by the *Zbtb7b* gene (hereafter referred to as the *Thpok* gene) is essential for development of CD4^{+} Th cells. Previous genetic studies have shown that the presence or absence of ThPOK in postselection thymo-

cytes is a crucial factor that discriminates CD4/CD8 lineages (2, 3). Therefore, elucidation of mechanisms that control *Thpok* expression has been a major subject to understand how cell fate determination is regulated at the transcriptional level.

Kappes and colleagues (4) had identified two relevant *cis*-regulatory elements in the murine *Thpok* gene, the distal regulatory element (DRE) and the proximal regulatory element (PRE), which are located ∼3.0 kb upstream and ∼3.6 kb downstream of exon Ia, respectively (Fig. 1A). An enhancer activity within the PRE, referred to as the proximal enhancer (PE), was shown to drive a reporter transgene in the later stage of helper lineage cell development (4, 5). Alternatively, enhancer activity within the DRE was shown to drive reporter transgene expression mainly in CD4^{+}CD8^{lo} thymocytes (4). Thus, enhancer activity in the DRE has been supposed to be responsible for early *Thpok* induction. Interestingly, however, previous studies demonstrated that the DRE also possesses a transcriptional silencer activity that represses expression of reporter transgene as well as the *Thpok* gene in cytotoxic lineage cells (4, 5). Thus, the silencer activity in the DRE (referred to as the *Thpok* silencer in this study) is essential to limit *Thpok* expression to helper lineage T cells. These observations suggest that a mechanism that controls two opposite functions (silencer and enhancer) in the DRE is important to regulate *Thpok* expression. However, the molecular basis for such dual functionality of the DRE remains uncharacterized. One could speculate that functional conversion from silencer to enhancer could occur (4), as was reported for the fruit fly dorsal morphogen (6). However, it is also possible that each function is embedded in different DNA sequences. In this study, we identified core sequences responsible specifically for enhancer activity from the DRE and designated them as the "thymic enhancer" (TE). We provide concrete genetic evidence that the TE is important for efficient induction of ThPOK, and consequently to help certain MHC class II–selected thymocytes to appropriately choose the helper lineage fate.

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Materials and Methods

Mice

Thpok^{gfp} (7), *Thpok^{gfp:ΔPE}* (7), β2m^{−/−} (8), and OT-II TCR transgenic mice (9) have been described. To generate transgenic reporter mice, each of the TE fragments of different lengths obtained by PCR amplification was replaced with the *Cd4* proximal enhancer in the pE4P4-hCD2 transgene vector (10) to conjugate with the *Cd4* promoter. To generate the E4P4-Sth construct, the 562-bp *Thpok* silencer (5) was inserted into the HindIII site in the pE4P4-hCD2 vector (10). To generate mice harboring the ΔTE mutation, a targeting vector that deleted the StuI-PstI sequence was transfected into embryonic stem cells harboring the *Thpok^{+/gfp}* genotype as previously described (7) (Supplemental Fig. 2). All mice were maintained in the animal facility at RIKEN Research Center for Allergy and Immunology and all experiments were performed in accordance with institutional guidelines for animal care.

T cell isolation and flow cytometric analyses

Thymus and lymph nodes were removed from mice at 4–8 wk of age. Cells were stained with the FITC-, PE-, PerCP-, or allophycocyanin-conjugated Abs purchased from BD Biosciences and eBioscience. Multicolor flow cytometry data were collected with FACSCalibur and were analyzed with BD CellQuest (BD Biosciences).

Transfection of luciferase reporter into RM11-1 cells

Several genomic fragments, which were prepared from a 6.4-kb upstream region of exon Ia in the *Thpok* locus by digestion with appropriate restriction enzymes, were inserted into the pGL4.10 vector (Promega). Ten micrograms of each luciferase vector and an internal control pRL-CMV vector (Promega) were transfected into 5 million RM11-1 cells by electroporation. Luciferase activity was measured 48 h after transfection using the Dual-Luciferase reporter assay system (Promega).

### Chromatin immunoprecipitation assay

Preparation of chromatin DNA and primers for the PE were previously described (5). Abs used are control IgG (SC-2025) and anti-Gata3 (SC-268) from Santa Cruz Biotechnology. Primers for TE amplification were 5′-ACCTAGTGGCAGGTAGGAAGCAG-3′ and 5′-GGGTAGCACTAT-TTATAACTGCGCG-3′.

Results and Discussion

During characterization of Runx-binding sequences in the *Thpok* gene (5), we noticed that expression of a reporter transgene (Tg) driven by the 6.5-kb region upstream of exon Ia depended on a 1.5-kb StuI-Eco47III fragment encompassing the DRE (Supplemental Fig. 1). Consistent with a previous report (4), enhancer activity in this fragment drove Tg expression mainly in CD4 lineage thymocytes (Supplemental Fig. 1), prompting us to designate the enhancer activity of the DRE as the TE of the *Thpok* gene. To narrow down sequences responsible for TE activity, we next performed reporter transfection assays with CD4 SP RM11-1 thymoma cells and found that a 797-bp StuI-PstI sequence in close 5′ proximity to the silencer is necessary to enhance reporter gene expression (Fig. 1A, Supplemental Fig. 1).

To examine in vivo function as well as to identify core sequences of the TE, we generated another reporter Tg mice. Several DNA fragments derived from the StuI-PstI sequences were conjugated with the *Cd4* promoter (P4), which alone cannot drive Tg expression in the reporter construct (10). A

FIGURE 1. Mapping of core thymic enhancer sequences in the DRE by reporter transgene expression assay. (**A**) Schematic structure of the murine *Thpok* locus is shown at the *top*. Black boxes represent exons. Circle represents the DRE and the PRE. Enlarged schematic structure of the DRE is shown at the *bottom*. Positions of core sequences for the thymic enhancer and silencer are shown as bold lines labeled as E403–E593 and S241–S362, respectively. (**B**) Schematic structures of reporter transgenes are shown at the *left*. E4, P4, Sth, and PA represent the *Cd4* proximal enhancer, the *Cd4* promoter, the 562-bp *Thpok* silencer, and SV40 polyA signal, respectively. Open boxes, black boxes, and black line represent the TE fragments, exons, and a part of the intron sequence from the murine *Cd4* gene, respectively. Histograms show the expression of the reporter hCD2 gene in the indicated T cell subsets from representative transgenic founders for each construct. The dashed line indicates nontransgenic littermate control. Numbers in the histogram indicate the percentage of cells expressing the reporter transgene. The numbers of transgenic founders expressing reporter gene among the total transgenic founders are indicated at the *right*.
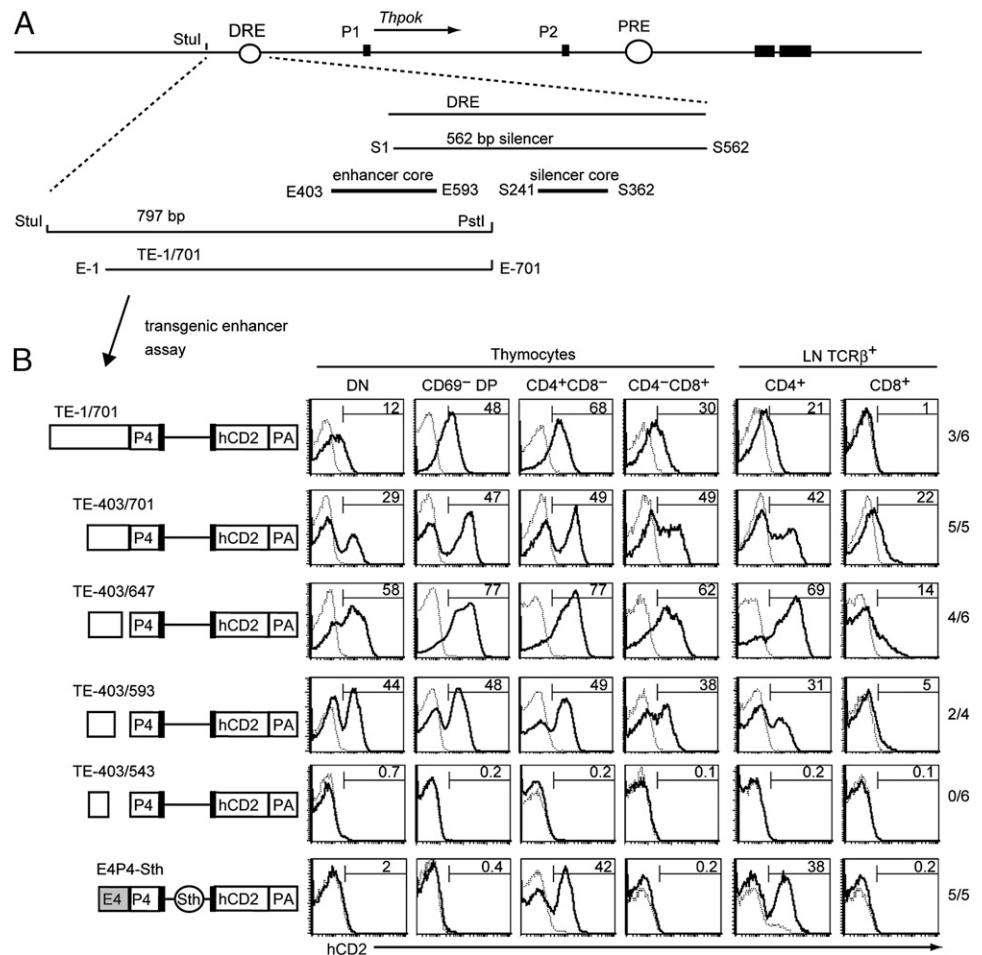
701-bp and 3' half of a 298-bp fragment (constructs TE-1/701 and TE-403/701, respectively) could drive hCD2 reporter expression in all thymocyte subsets (Fig. 1B). Further sequential deletion from the 3' side revealed that the 191-bp TE-403/593 sequences were minimal for enhancer activity in our reporter Tg assay (Fig. 1B). Of note, the levels of hCD2 expression were comparable between CD4SP thymocytes and CD4$^+$ T cells, and hCD2 expression was detected in double-negative and DP thymocytes. Thus, consistent with previous results using the *hCD2* promoter (4), the TE lost stage-specificity in conjunction with the heterologous *Cd4* promoter. Additionally, in the peripheral T cell pool, the level of hCD2 expression was significantly lower in CD8$^+$ cells than in CD4$^+$ cells with all Tg reporter constructs tested. There are two possible mechanisms that explain this helper lineage-dominant expression: the enhancer activity itself is dominant in CD4$^+$ cells, or silencer activity in the tested TE fragment represses Tg expression in CD8$^+$ cells. However, because helper lineage–dominant expression was not apparent between CD4SP and CD8SP thymocytes, silencer activity, if present, should be active at later stage of CD8 lineage cell differentiation. In contrast, when an entire 562-bp *Thpok* silencer (Sth) was inserted into the Tg driven by the *Cd4* enhancer (E4) and P4 (construct E4P4-Sth), Tg expression was repressed not only in CD8 thymocytes but also in DP thymocytes (Fig. 1B). Thus, full silencer activity in the DRE can repress Tg expression by heterologous enhancer in immature thymocytes. Furthermore, S241–S362 sequences, which do not overlap with the TE-403/593 sequences (Fig. 1A), were sufficient to repress Tg expression in DP and CD8$^+$ SP thymocytes (Supplemental Fig. 1). Even though these results do not formally exclude a possibility of residual silencer activity in the TE-403/593 sequence for repression of Tg expression in CD8$^+$ cells, they fit more

with the former possibility. We therefore propose that core sequences necessary for enhancer and silencer activity are likely to be embedded in different DNA sequences within the DRE.

To address the physiological function of the TE in regulating *Thpok* gene expression and T cell development, we have deleted a 797-bp StuI-PstI region from the *Thpok* or *Thpok^{gfp}* reporter allele (7), generating a *Thpok$^{\Delta TE}$* or *Thpok$^{gfp:\Delta TE}$* allele, respectively (Supplemental Fig. 2). In *Thpok$^{+/gfp:\Delta TE}$* mice, in which T cell development was supported normally by half dosage of ThPOK protein, GFP expression in the CD4$^+$CD69$^+$TCR^{hi} population decreased in terms of both frequency of GFP$^+$ cells and the amount of GFP per cell compared with those in control cells of *Thpok$^{+/gfp}$* mice (Fig. 2). However, the amount of GFP expressed from the *Thpok$^{gfp:\Delta TE}$* allele increased during maturation to the helper lineage thymocytes and reached a level comparable to that from the control *Thpok^{gfp}* allele in peripheral CD4$^+$ T cells, whereas deletion of the PE led to a decreased amount of GFP in those cells (Fig. 2A). This result indicates not only an essential role for the TE in efficient induction of the *Thpok* gene but also distinct stage-specific functions for the TE and PE. The Gata3 transcription factor was shown to be essential for activation of the *Thpok* gene via binding to a region upstream of exon II and the PE (11). We therefore tested whether Gata3 also binds to the TE by chromatin immunoprecipitation (ChIP) assay. Consistent with recent ChIP-Seq results (12), Gata3 also bound to the TE (Fig. 2B), suggesting that Gata3 may activate the *Thpok* gene through regulating both the TE and PE activity. Of note, there was low, but significant, GFP expression in some CD8$^+$ T cells in the *Thpok$^{+/gfp:\Delta TE}$* mice (Fig. 2A), suggesting that sequences additionally deleted with the TE core by the ΔTE mutation are necessary for full silencer activity.

FIGURE 2. Thymic enhancer is essential for efficient *Thpok* induction. (**A**) Histograms showing GFP expression from the *Thpok^{gfp}*, *Thpok$^{gfp:\Delta TE}$*, and *Thpok$^{gfp:\Delta PE}$* alleles in the indicated cell subsets from thymus and lymph node (LN). Numbers in the histogram indicate the percentage of GFP$^+$ cells, and numbers in parentheses indicate mean fluorescence intensity (MFI) of GFP in GFP$^+$ cells. Data are representative of five experiments. (**B**) Graphs showing statistical analyses of percentage of GFP$^+$ cells (*left*) and relative MFI to that from the *Thpok^{gfp}* allele (*right*) in the indicated cell subsets. Lanes 1, 2, and 3 in the *left* graph are *Thpok$^{+/gfp}$*, *Thpok$^{+/gfp:\Delta TE}$*, and *Thpok$^{+/gfp:\Delta PE}$* mice, respectively. (**C**) Analytical ChIP assay for Gata3 bindings to the TE and PE in the *Thpok* locus in CD4$^+$ SP thymocytes. The E8I enhancer in the *Cd8* gene was used as a negative control (N.C.). One representative result from three independent experiments is shown.
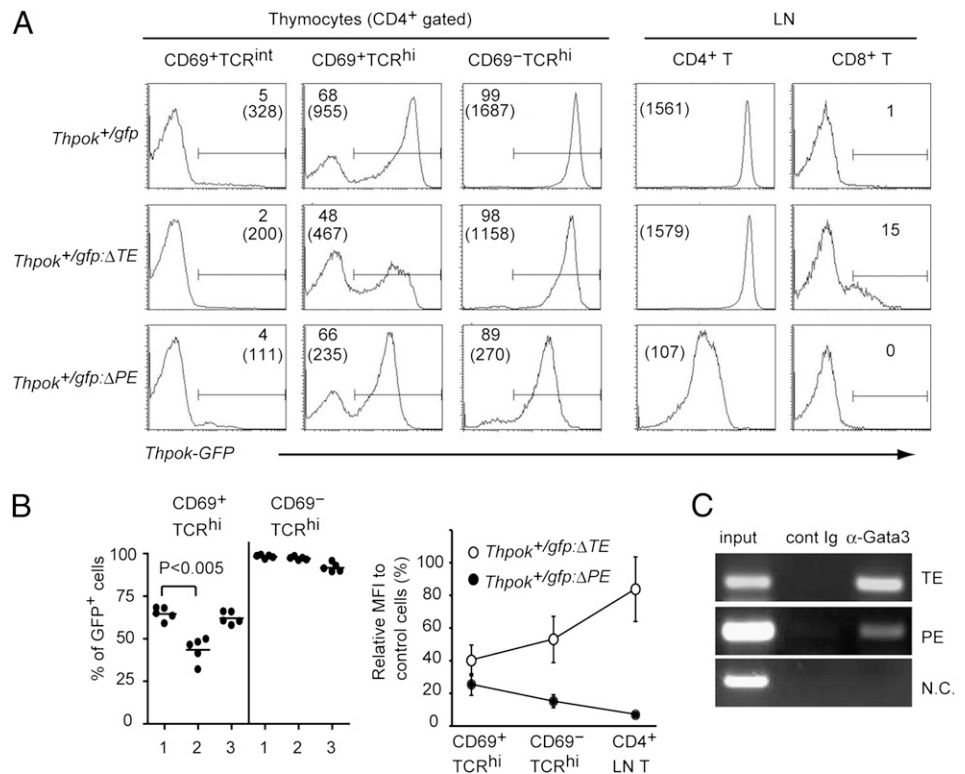
We next examined whether inefficient ThPOK induction due to lack of TE affects the development of MHC class II–selected cells. In $Thpok^{\Delta TE/\Delta TE}$ mice, there was a slight decrease and increase of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ subsets, respectively, among the TCRβ^{hi} thymocyte population (Fig. 3A). Furthermore, in $Thpok^{\Delta TE/gfp}$ mice in which MHC class II–selected T cells are marked by GFP expression from the $Thpok^{gfp}$ allele that harbors normal regulatory regions (7), the frequency of GFP$^+$ cells was increased in CD8$^+$ SP thymocytes (Fig. 3A). This result suggests a redirected differentiation of some MHC class II–selected cells into the CD8 lineage. Indeed, an emergence of mature CD4$^-$CD8$^+$ T cells was observed in the mature thymocyte population of $Thpok^{\Delta TE/\Delta TE}$:$\beta 2m^{-/-}$ mice, whereas those cells are absent in control mice (Fig. 3B). These results demonstrated that a small, but significant, proportion of MHC class II–selected cells undergo redirection to the CD8$^+$ cytotoxic lineage by loss of the TE from the *Thpok* locus.

Because strong TCR signals have been thought to be involved in helper lineage choice (13) and ThPOK expression (14), differences in TCR affinity to self-peptide would affect the initial amount of ThPOK protein and thereby be involved in cell fate discrimination in $Thpok^{\Delta TE/\Delta TE}$ mice. It was therefore important to test whether redirected differentiation occurs when all thymocytes expressed the identical TCR. To this end, we crossed transgenic mouse strain expressing MHC class II–restricted OT-II TCR (9) with $Thpok^{\Delta TE/\Delta TE}$ mice. To inhibit VDJ recombination of the endogenous *Tcr* genes, a null mutation of the *Rag1* gene was further introduced. Whereas no CD8$^+$ cells expressing OT-II TCR were detected in mature thymocytes from control $Thpok^{+/+}$ mice, a few CD8$^+$ mature thymocytes emerged in $Thpok^{\Delta TE/\Delta TE}$ mice (Fig. 3B). Thus, partial redirection to CD8 lineage still occurs even when all thymocytes express a monogenic TCR. These results indicate that TCR specificity to self-Ags is not the sole

determinant to discriminate CD4/CD8 lineage choice in the $Thpok^{\Delta TE/\Delta TE}$ setting. Supposing that the number of thymic epithelial cells presenting natural selective ligands to transgenic TCR is limited, it is possible that intraclonal competition within the postselection thymocyte population takes place when they all express identical TCR, as was reported during differentiation of regulatory T cells (15). A small proportion of postselection thymocytes that are losers in this competition might receive shorter TCR signals than do the winners. Although such shortened TCR signals would still be sufficient to induce the minimum amount of ThPOK required for helper lineage development in the presence of the TE, they would fail to induce a sufficient amount of ThPOK in the absence of the TE. Our results thus revealed an important requirement for the TE in guiding a small, but significant, proportion of MHC class II–selected thymocytes to appropriately differentiate into the helper lineage via fine-tuning of *Thpok* gene expression.

In this study, we characterized core sequences responsible for enhancer activity within the DRE and showed that two individual sequences, rather than functional conversion of one element, endow the DRE with dual functionality. Whereas the silencer is dominant and assures helper lineage specificity by inactivation of the *Thpok* gene, the TE located very near to the silencer is necessary for efficient activation of the *Thpok* gene, presumably with cooperative help of the PE. Inactivation of the silencer is also involved in efficient *Thpok* activation. However, given the helper lineage–specific expression of the E4P4-Sth reporter transgene, inactivation of the silencer could occur independently of the TE or PE function. It is important to further unravel molecular mechanisms regulating not only activity of each regulatory region in the DRE but also how multiple regulatory elements work cooperatively to finely tune *Thpok* expression under TCR signals.
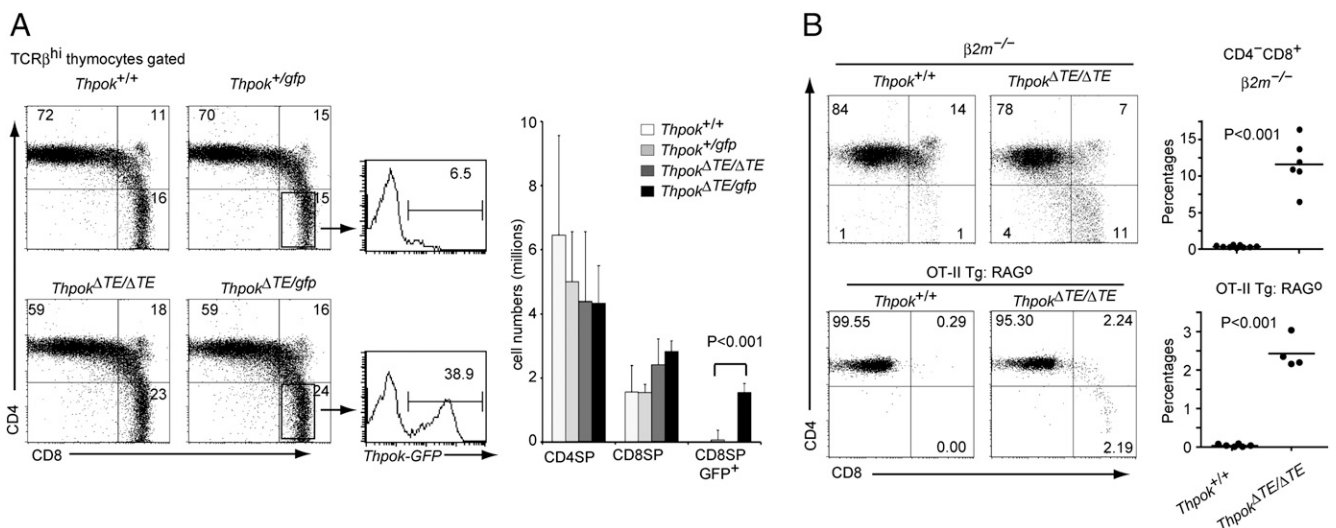
FIGURE 3. Thymic enhancer is essential for appropriate helper lineage choice by MHC class II–selected cells. (**A**) Expression of CD4 and CD8 in TCRβ^{hi} mature thymocytes from mice of the indicated genotypes. Numbers in quadrants indicate the percentage of cells in each quadrant. Histograms show GFP expression in the CD4$^-$CD8$^+$ SP thymocytes of $Thpok^{+/gfp}$ and $Thpok^{\Delta TE/gfp}$ mice. Numbers indicate percentage of GFP$^+$ cells. Data are representative of at least three individual mice of each genotype. Graph shows cell numbers of indicated cell subsets of indicated genotype as mean ± SD. (**B**) Expression of CD4 and CD8 in mature thymocytes from $Thpok^{+/+}$:$\beta 2m^{-/-}$ and $Thpok^{\Delta TE/\Delta TE}$:$\beta 2m^{-/-}$ mice (*upper plots*) and $Thpok^{+/+}$ and $Thpok^{\Delta TE/\Delta TE}$ mice harboring OT-II transgene on a Rag1-deficient background (*lower plots*). Numbers in quadrants indicate the percentage of cells in each quadrant. Percentages of CD4$^-$CD8$^+$ cells in mature thymocyte subsets from each mouse analyzed are shown as a dot in the *right panel*. Black bar indicates the mean.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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