RESEARCH ARTICLE

IMMUNOLOGY

CD8 T cell tolerance results from eviction of immature autoreactive cells from the thymus

Mohamed Elsherif Badr*, Zhongmei Zhang, Xuguang Tai, Alfred Singer*

CD8 T cell tolerance is thought to result from clonal deletion of autoreactive thymocytes before they differentiate into mature CD8 T cells in the thymus. However, we report that, in mice, CD8 T cell tolerance instead results from premature thymic eviction of immature autoreactive CD8 thymocytes into the periphery, where they differentiate into self-tolerant mature CD8 T cells. Premature thymic eviction is triggered by T cell receptor (TCR)–driven down-regulation of the transcriptional repressor Gfi1, which induces expression of sphingosine-1–phosphate receptor-1 (S1P1) on negatively selected immature CD8 thymocytes. Thus, premature thymic eviction is the basis for CD8 T cell tolerance and is the mechanism responsible for the appearance in the periphery of mature CD8 T cells bearing autoreactive TCRs that are absent from the thymus.

Selection of a functional and self-tolerant T cell receptor (TCR) repertoire in the thymus requires that thymocytes undergo positive and negative selection on the basis of the specificity of their TCRs for self-ligands. Thymocytes that engage self-ligands with low affinity undergo positive selection into mature CD4 or CD8 T cells, whereas thymocytes with potentially autoreactive TCRs engage self-ligands with high affinity and undergo negative selection (*1*). The major consequence of negative selection is thought to be clonal deletion, which eliminates autoreactive thymocytes before their differentiation into mature T cells (*2*–*4*). However, it is now understood that TCR signaling is sustained during major histocompatibility class II (MHC-II)–specific CD4 T cell development but is disrupted during major histocompatibility class I (MHC-I)–specific CD8 T cell development (*5*–*7*). Accordingly, MHC-I–specific TCR signaling may be disrupted before deleting enough autoreactive thymocytes to induce CD8 T cell tolerance. In this work, we report that CD8 T cell tolerance does not result from clonal deletion but is induced by the premature eviction of immature autoreactive thymocytes into the periphery, where they complete their differentiation into self-tolerant mature CD8 T cells.

MHC-I–specific autoreactive thymocytes survive negative selection

Autoreactive thymocytes that avoid clonal deletion during negative selection express PD-1, an attenuator of TCR signaling, and become TCR^{hi}PD-1^{+} cells (*8*–*10*). TCR^{hi}PD-1^{+} cells were significantly higher in both frequency and PD-1 expression among MHC-I–selected compared with MHC-II–selected thymocytes from *H-2Ab1*$^{-/-}$ (MHC-II^{-}) and *b2m*$^{-/-}$ (MHC-I^{-}) mice, respectively (Fig. 1, A and B). These TCR^{hi}PD-1^{+} thymocytes lacked cleaved caspase-3, which confirmed that they were not dying cells (Fig. 1, A and B). Thus, a significantly higher frequency of MHC-I–specific compared with MHC-II–specific autoreactive thymocytes survive negative selection.

To compare developing MHC-I– and MHC-II–specific thymocytes with TCRs that recognize the same self-antigen, we examined HY^{cd4} and Marilyn mice, whose T cells both express transgenic TCRs specific for the HY male antigen and induce negative selection in male mice but positive selection in female mice (*11*, *12*). The transgenic TCRs in HY^{cd4} mice are clonotype T3.70^{+} and engage MHC-I ligands to generate CD8 T cells (*11*), whereas the transgenic TCRs in Marilyn mice are TCR-Vβ6^{+} and engage MHC-II ligands to generate CD4 T cells (*12*). Notably, in male mice, Marilyn thymocytes failed to become TCR^{hi}, whereas HY^{cd4} thymocytes became TCR^{hi} (Fig. 1, C and D). These TCR^{hi} thymocytes were generated by the transgenic TCR because their thymocyte profiles were identical in *Rag2*-sufficient and *Rag2*-deficient HY^{cd4} mice (Fig. 1, C and D). Thus, negative selection does not prevent MHC-I–selected thymocytes from differentiating into TCR^{hi} cells.

MHC-I–selected thymocytes that were TCR^{hi} in HY^{cd4} male (HY^{cd4}M) mice did not become CD8–single-positive (CD8SP) but remained CD4/8^{dull} (Fig. 1, E and F). The absence of CD8SP thymocytes in negatively selecting HY^{cd4}M mice has been attributed to clonal deletion (*11*, *13*). However, this conclusion was contradicted by the presence of HY-specific CD8αβ T cells in the periphery of male mice in greater numbers compared with HY^{cd4} female (HY^{cd4}F) mice (Fig. 1, E and F, and fig. S1A). By contrast, HY-specific CD4 T cells were not present in the periphery of Marilyn male mice (fig. S1B). Thus, unlike MHC-II–selected autoreactive CD4 T cells, MHC-I–selected autoreactive CD8 T cells survive negative selection and appear in the periphery.

To assess cell death during MHC-I–specific negative selection, we examined cleaved caspase-3 in HY^{cd4}M thymocytes (Fig. 1G). Less than 1% contained cleaved caspase-3, and these dying cells were Bcl-2^{lo}CCR7^{lo}. An even higher frequency of negatively selected thymocytes were Bcl2^{+}CCR7^{hi} and cleaved–caspase-3^{-} and were differentiating, not dying, cells (Fig. 1G). Because thymocyte clonal deletion during negative selection is induced by B7 costimulatory ligands (CD80 and CD86) (*8*, *14*, *15*), we compared CD8 T cell numbers in HY^{cd4}M mice with and without B7 deficiency. Impaired clonal deletion in B7^{DKO}HY^{cd4}M mice increased TCR^{hi} thymocyte numbers by only one-third (from 14 million to 21 million) and did not reconstitute generation of CD8SP thymocytes (Fig. 1, H and I). Thus, clonal deletion eliminated only a minority of autoreactive MHC-I–specific thymocytes and failed to explain the absence of CD8SP cells in the thymus.

Immature autoreactive CD8 thymocytes are evicted from the thymus

To understand how HY-specific CD8 T cells appeared in the periphery when they were absent from the male thymus, we characterized the TCR^{hi}CD4/8^{dull} thymocytes that populated the male thymus (Fig. 2A). Female thymi contained mature CD8SP thymocytes, whereas TCR^{hi}CD4/8^{dull} thymocytes in male thymi were PD-1^{+}Bcl-2^{+} (indicating that they had been strongly TCR signaled) and were CD24^{hi}Qa2^{-} (indicating that they were immature) (Fig. 2A). Nevertheless, immature TCR^{hi}CD4/8^{dull} male thymocytes were CD8 lineage–committed because they expressed the cytotoxic-lineage transcription factor Runx3d, albeit at lower levels compared with CD8SP thymocytes (Fig. 2B).

To determine whether immature TCR^{hi}CD4/8^{dull} thymocytes exited the male thymus, we examined thymocytes 5 min after intravenous (i.v.) injection of anti-CD5 monoclonal antibody (mAb), which only had enough time to bind to exiting thymocytes that contacted the blood circulation (*16*). Whereas CD5(i.v.)$^{+}$ cells exiting B6 thymi were single positive (SP), CD5(i.v.)$^{+}$ thymocytes exiting HY^{cd4}M thymi were T3.70^{hi}CD4/8^{dull} (Fig. 2C). Such T3.70^{hi}CD4/8^{dull} cells were also present in the periphery of male mice, and these peripheral T3.70^{hi}CD4/8^{dull} cells resembled immature thymocytes in being CD24^{hi}PD-1^{+} (Fig. 2, D and E). Thus, TCR^{hi}CD4/8^{dull} thymocytes exit the male thymus despite being immature.

Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.
*Corresponding author. Email: sherif.badr@nih.gov (M.E.B.); singera@nih.gov (A.S.)

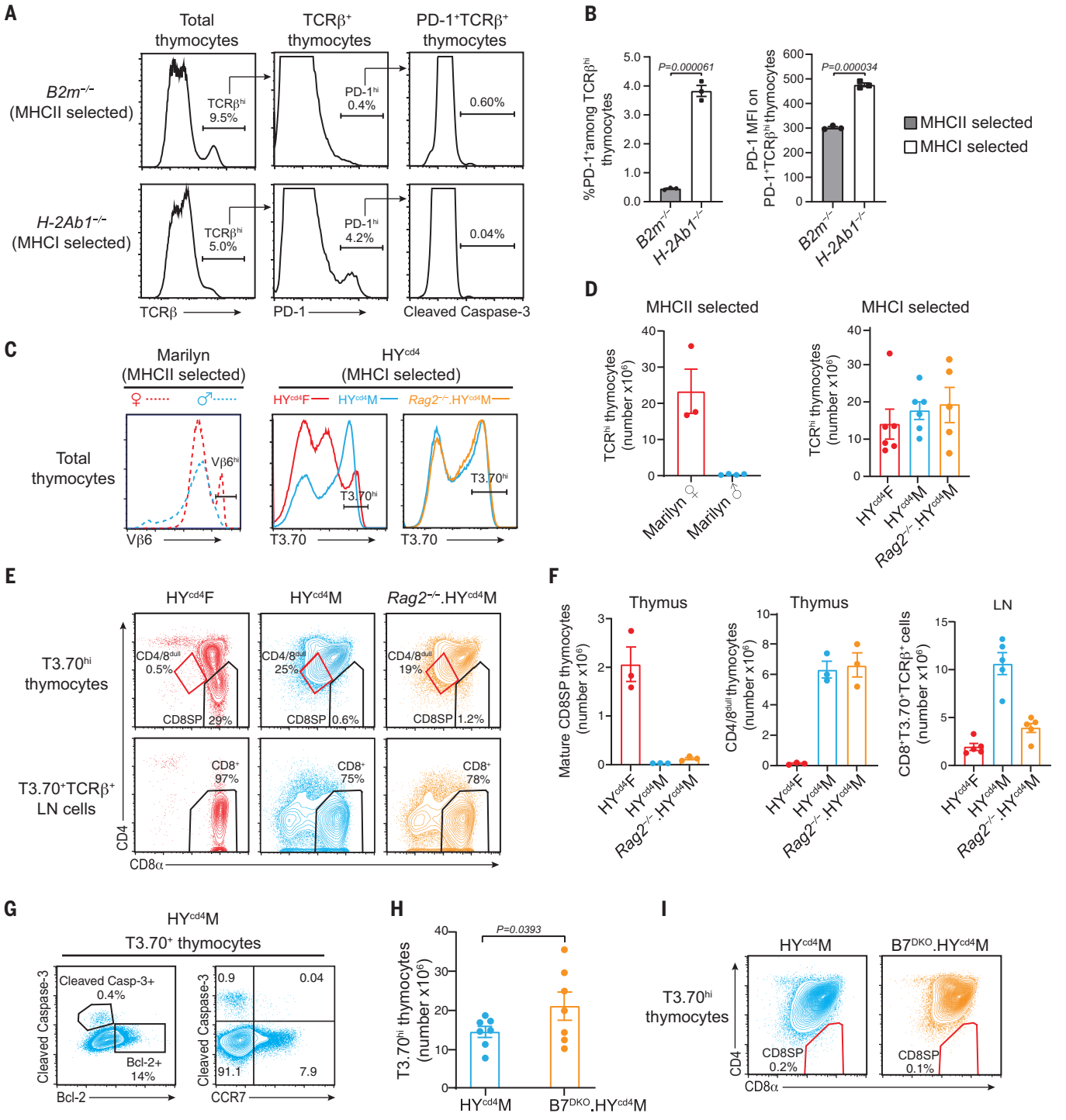

Fig. 1. Characterization of developing thymocytes and T cells that preferentially survive strong TCR signaling during negative selection in the thymus. (**A**) Thymocyte profiles from mice containing either MHC-II–selected or MHC-I–selected T cells. Shown are TCR profiles of total thymocytes (left), PD-1 profiles on TCR^{hi} (CD1d–PBS57^{-} non-iNKT) thymocytes (middle), and cleaved caspase-3 expression in PD-1^{+}TCR^{hi} (non-iNKT) thymocytes (right). (**B**) Percent PD-1^{+} cells among TCRβ^{hi} thymocytes (left) and quantification of their surface PD-1 expression as mean fluorescence intensity (MFI) (right). (**C**) TCR profiles of male versus female thymocytes from Marilyn and HY^{cd4} transgenic mice. (**D**) TCR^{hi} thymocyte numbers in Marilyn and HY^{cd4} transgenic mice. (**E**) CD4 versus CD8α expression on TCR^{hi} thymocytes (top) and TCR^{+} lymph node (LN) cells (bottom) in male and female HY^{cd4} mice. (**F**) Thymocyte and LN T cell numbers in HY^{cd4} transgenic mice. (**G**) Expression of cleaved caspase-3 versus Bcl-2 (left) or CCR7 (right) in HY-specific thymocytes from HY^{cd4} male mice. (**H**) TCR^{hi} thymocyte numbers in WT and B7-deficient [B7 double-knockout (B7^{DKO})] HY^{cd4} male mice. (**I**) CD4 versus CD8α expression on TCR^{hi} thymocytes on WT and B7-deficient HY^{cd4} male mice. [(A) to (C), (E), (G), and (I)] Representative data are from three independent experiments. [(D), (F), and (H)] Data are from two to four independent experiments. *P* values were determined using unpaired two-tailed Student's *t* tests (means ± SEMs).

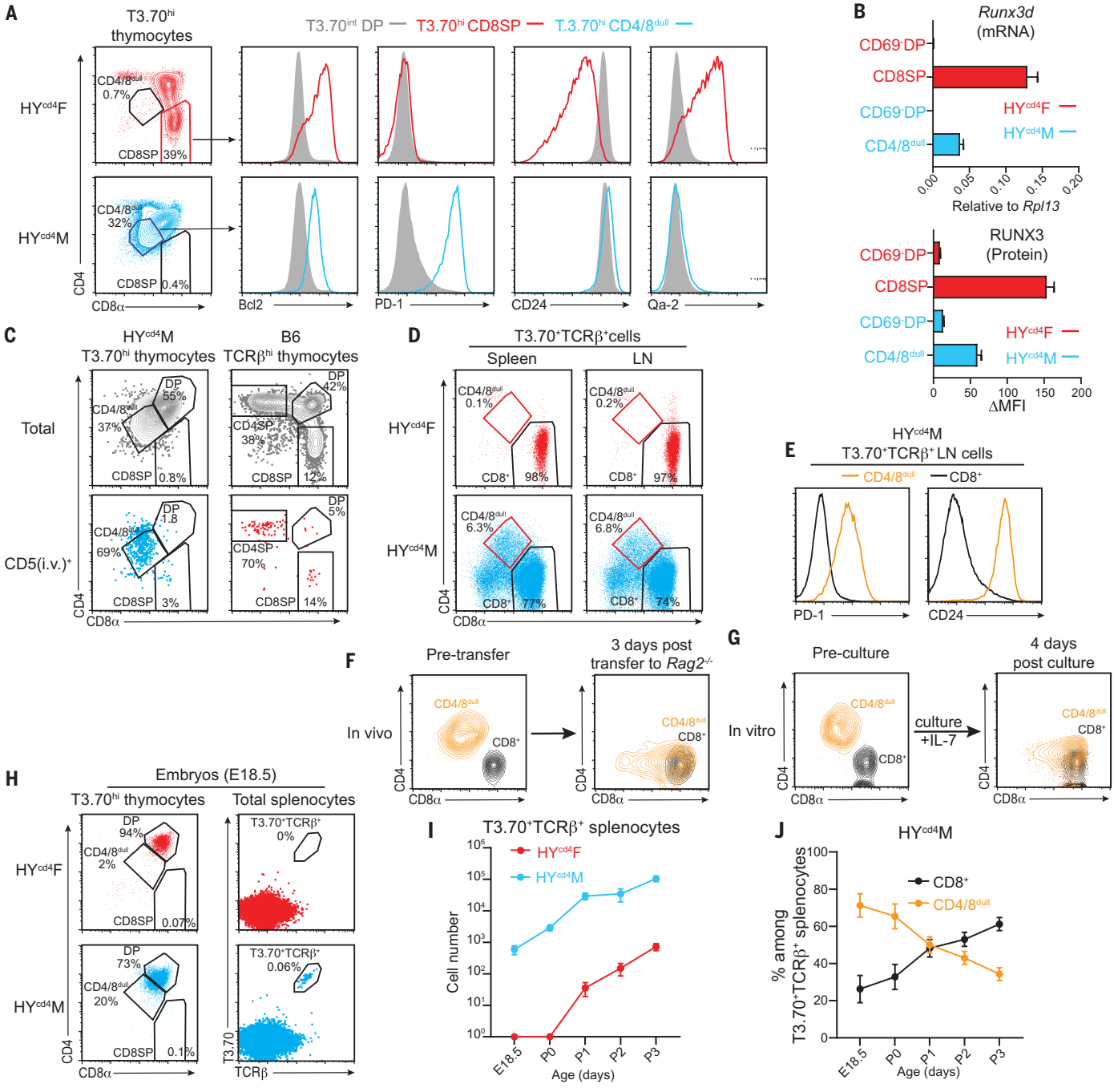

Fig. 2. Clonal eviction of autoreactive CD8 thymocytes signaled to undergo negative selection. (**A**) CD4 versus CD8α expression on TCR^{hi} thymocytes from HY^{cd4} male and female mice (left) and expression of various proteins in the indicated thymocyte subsets (single-color histograms). (**B**) Expression of *Runx3d* mRNA (top) and RUNX3 protein (bottom) in thymocyte subsets from HY^{cd4} male and female mice. (**C**) To identify cells exiting the thymus, thymocytes were harvested 5 min after in vivo i.v. injection of phycoerythrin (PE)–conjugated anti-CD5 mAb. Thymocytes that bound the injected anti-CD5 mAb were referred to as CD5(i.v.)$^+$ cells. (**D**) CD4 versus CD8 profiles of HY-specific T3.70$^+$ peripheral T cells in HY^{cd4} male and female mice. Only negatively selecting male mice contained peripheral CD4/8^{dull} HY-specific T cells. (**E**) PD-1 and CD24 expression on peripheral CD4/8^{dull} and CD4$^-$8$^+$ HY-specific T cells in HY^{cd4} male mice. (**F**) CD4/8^{dull} and CD8$^+$ HY-specific T3.70$^+$ T cells were sorted from the spleen and LN of HY^{cd4} male mice and were adoptively transferred into separate *Rag2*$^{-/-}$ hosts. CD4 versus CD8α expression on each transferred cell population was overlayed before and 3 days after transfer into host mice. (**G**) CD4/8^{dull} and CD8$^+$ HY-specific T3.70$^+$ T cells were sorted from the spleen and LN of HY^{cd4} male mice and were placed into separate in vitro cultures containing IL-7 for 4 days. Overlayed CD4 versus CD8α expression on sorted T cells before and after IL-7 culture is shown. (**H**) CD4 versus CD8α expression on HY-specific T3.70^{hi} thymocytes and splenocytes in male and female embryonic day 18.5 (E18.5) HY^{cd4} mice. (**I**) Comparison of HY-specific T3.70$^+$ T cells in HY^{cd4} male versus female neonatal mice at indicated ages. P0, postnatal day 0. (**J**) Frequencies of CD4/8^{dull} and CD8$^+$ cells among T3.70$^+$TCRβ$^+$ splenocytes in HY^{cd4}M neonatal mice. [(A) and (C) to (G)] Data are representative of three or four independent experiments. (B) Data are from three independent experiments with technical triplicates. (H) Representative data are from three or four HY^{cd4} embryos of each gender. [(I) and (J)] n = 3 to 8 from each gender at each time point. Data are expressed as means ± SEMs.

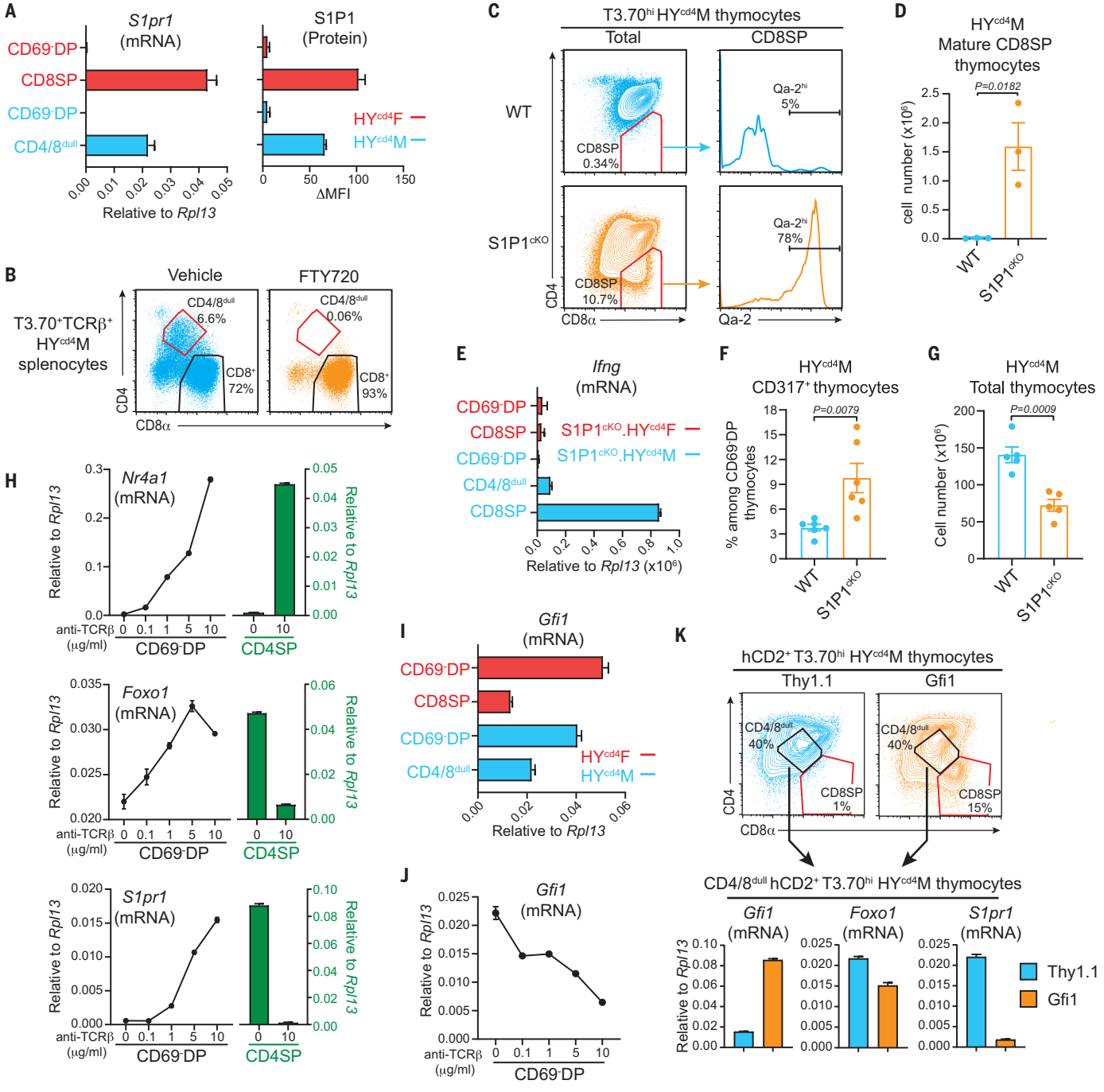

Fig. 3. Strong TCR signaling of immature thymocytes induces S1P1 expression and clonal eviction by down-regulating Gfi1. (**A**) *S1pr1* mRNA (left) and S1P1 protein (right) expression in HY^{cd4} male and female thymocytes. (**B**) CD4 versus CD8α profiles of HY-specific T3.70^{+} splenocytes in HY^{cd4} male mice after five daily injections of either FTY720 or vehicle. (**C**) CD4 versus CD8α and Qa-2 profiles of HY-specific T3.70^{+} TCR^{hi} thymocytes from S1P1-deficient [S1P1 conditional knockout (S1P1^{cKO})] and WT HY^{cd4} male mice. (**D**) Numbers of mature (CD24^{lo}) CD8SP thymocytes in S1P1^{cKO} and WT HY^{cd4} male mice. (**E**) *Ifng* mRNA in thymocyte subsets from S1P1^{cKO} and WT HY^{cd4} male and female mice. (**F**) Frequency of CD317^{+} preselection (CD69^{-}DP) thymocytes in S1P1^{cKO} and WT HY^{cd4} male mice. (**G**) Number of total thymocytes in S1P1^{cKO} and WT HY^{cd4} male mice. (**H**) *Nr4a1*, *Foxo1*, and *S1pr1* mRNA expression in immature (CD69^{-}DP) and mature (CD4SP) B6 thymocytes after overnight stimulation with immobilized plate-bound anti-TCRβ. (**I**) *Gfi1* mRNA expression in HY^{cd4} male and female thymocytes. (**J**) *Gfi1* mRNA expression in immature (CD69^{-}DP) B6 thymocytes after overnight stimulation with immobilized plate-bound anti-TCRβ. (**K**) Donor HY^{cd4}M bone marrow cells (containing CD4-cre) were infected in vitro with FLEX/Gfi1 or FLEX/Thy1.1 lentiviruses, and the lentivirus-infected bone marrow cells were then injected into lethally irradiated B6 CD45.1 host mice. Donor-origin HY^{cd4}M thymocytes were analyzed 4 to 6 weeks later, and those constitutively expressing Gfi1 or Thy1.1 lentiviral proteins were identified by surface expression of hCD2 reporter protein. [(A), (E), and (H) to (K)] Representative experiment from two to four independent experiments with technical triplicates. [(B) to (D)] Representative experiment from three or four independent experiments. [(F) and (G)] Data are from two to three independent experiments. *P* values were determined using unpaired two-tailed Student's *t* tests. Data are expressed as means ± SEMs.

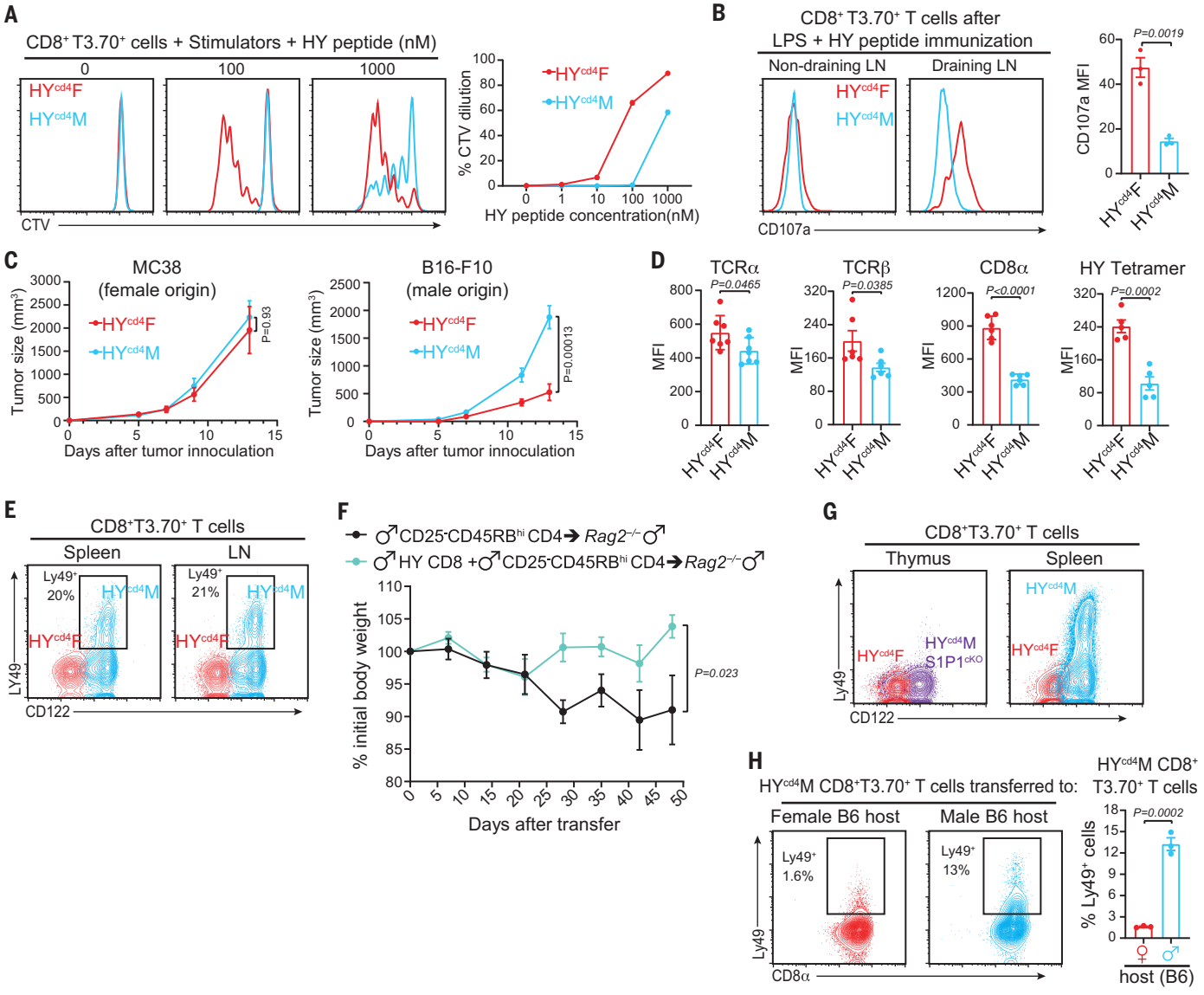

Fig. 4. Peripherally generated CD8 T cells are self-tolerant. (**A**) In vitro proliferative responses of CD8 T cells from HY^{cd4} male and female mice to 3 days of stimulation with varying doses of HY peptide presented by B6 splenocytes, as measured by the dilution of cell trace violet (CTV). (**B**) In vivo response as measured by CD107a expression of CD8 T cells from draining and nondraining popliteal LNs in HY^{cd4} male and female mice to footpad injection with HY peptide and LPS 16 hours earlier. (**C**) In vivo growth of female (MC38) or male (B16-F10) tumor cells subcutaneously injected into HY^{cd4} mice. (**D**) Surface TCR and CD8 expression and quantification of HY tetramer binding to CD8 LN T cells from HY^{cd4} male and female mice. (**E**) Ly49 versus CD122 profiles of peripheral CD8 T cells from HY^{cd4} male and female mice. (**F**) Relative body weights of male Rag2$^{-/-}$ mice after transfer of naïve CD4^{+}CD25^{-}CD45RB^{hi} B6 male T cells alone or together with male HY^{cd4} CD8 T cells. (**G**) Ly49 versus CD122 profiles of CD8SP thymocytes or CD8 spleen T cells from HY^{cd4}F mice, S1P1^{cKO}.HY^{cd4}M mice, and HY^{cd4}M mice. (**H**) Ly49 versus CD8α expression on CD8^{+}T3.70^{+} T cells from HY^{cd4}M mice analyzed 7 days after adoptive transfer into male and female B6 hosts. [(A), (B), and (E)] Representative data are from three independent experiments. (D) Data are pooled from three independent experiments. [(C) and (F)] Representative experiment from two independent experiments (n = 5 mice from each group). P values were determined using unpaired two-tailed Student's t tests except for (C) and (F), where two-way analysis of variance (ANOVA) was used instead (means ± SEMs).

To determine whether immature TCR^{hi}CD4/8^{dull} cells complete their differentiation in the periphery, we adoptively transferred them into immunodeficient Rag2$^{-/-}$ hosts and found that they became CD8 T cells (Fig. 2F). Immature CD4/8^{dull} cells also became CD8 T cells in interleukin-7 (IL-7) in vitro cultures, and their differentiation into CD8 T cells depended on Runx3d and cytokine receptor gamma chain (γc) (Fig. 2G and fig. S2), as does CD8 T cell generation in the normal thymus (*17*). Thus, during negative selection, MHC-I–selected thymocytes became immature TCR^{hi}CD4/8^{dull} cells that express PD-1 and Runx3d and that prematurely exit the thymus to complete their differentiation into mature CD8 T cells in the periphery. We refer to the exit of immature autoreactive thymocytes from the thymus into the periphery as clonal eviction.

Premature clonal eviction of autoreactive CD4/8^{dull} thymocytes also occurred during normal ontogeny, as T3.70^{+} cells appeared in the periphery of negatively selecting male mice 2 days earlier than in positively selecting

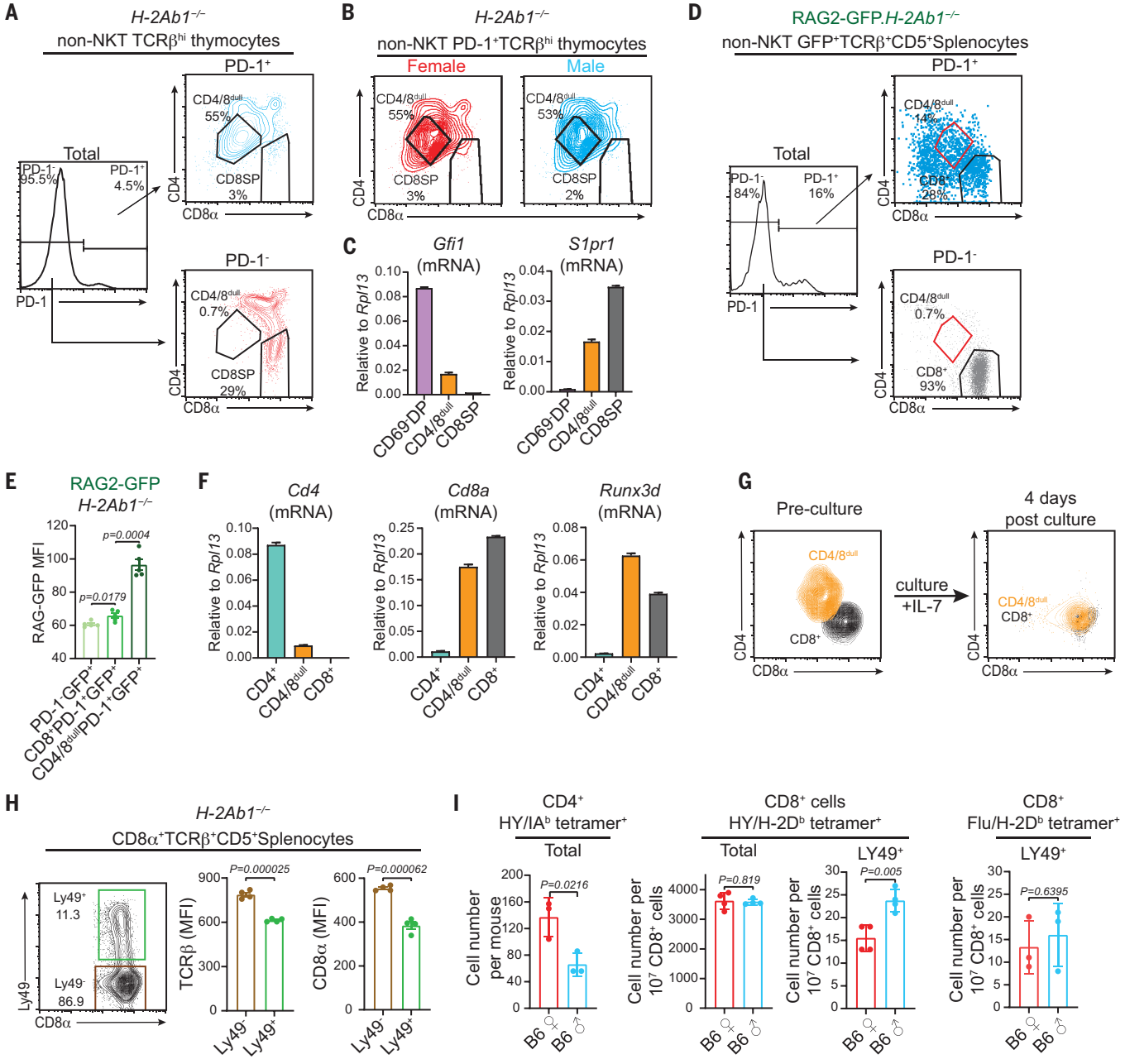

Fig. 5. Assessment of clonal eviction in normal polyclonal mice. (**A**) Analysis of MHC-I–selected thymocytes from MHC-II^{-} polyclonal mice. PD-1 expression on TCR^{hi} (non-NKT) thymocytes (left), and CD4 versus CD8α profiles on PD-1^{+} and PD-1^{-} TCR^{hi} thymocytes (right). (**B**) CD4 versus CD8α profiles on PD-1^{+}TCRβ^{hi} thymocytes from MHC-II^{-} male and female mice. (**C**) *Gfi1* and *S1pr1* mRNA expression in sorted thymocyte subsets from MHC-II^{-} mice. (**D**) Analysis of RTEs in the spleens of RAG2-GFP.MHC-II^{-} mice. PD-1 expression (left) and CD4 versus CD8α profiles on PD-1^{+} and PD-1^{-} RTEs (right). (**E**) Quantification of RAG-GFP fluorescence in spleen RTEs from RAG2-GFP.MHC-II^{-} mice. (**F**) Expression of *Cd4*, *Cd8a*, and *Runx3d* mRNA in sorted spleen and LN T cell subsets in Rag-GFP.MHC-II^{-} mice. (**G**) RTE CD4/8^{dull}PD-1^{+} and CD8^{+}PD-1^{+} were sorted from the spleens and LNs of RAG-GFP.MHC-II^{-} mice and placed into separate IL-7 in vitro cultures for 4 days. (**H**) Ly49 versus CD8α profile of total CD8 T cells from MHC-II^{-} spleens (left) and comparison of TCRβ and CD8α expression on Ly49^{-} and Ly49^{+} CD8 T cell subsets (bar graphs). (**I**) Comparison between B6 male and B6 female mice of HY–IA^{b} tetramer binding to CD4 T cells (left), HY–H-2D^{b} tetramer binding to total and Ly49^{+} CD8 T cells (middle), and Flu–H-2D^{b} tetramer binding to Ly49^{+}CD8 T cells (right). [(A) to (H)] Representative experiment from two to three independent experiments. (I) Data are from two independent experiments. *P* values were determined using unpaired two-tailed Student's *t* tests. Data are expressed as means ± SEMs.

female mice (Fig. 2, H and I). Moreover, in male mice, the earliest-appearing T3.70^{+} peripheral cells were CD4/8^{dull} cells, which increasingly became CD8 T cells over the following days (Fig. 2J).

S1P1 mediates clonal eviction, which avoids thymic stress

Because immature thymocytes cannot normally exit the thymus, we investigated how immature autoreactive thymocytes were clonally evicted during negative selection. Because the sphingosine-1–phosphate receptor-1 (S1P1) is used by mature thymocytes to exit the thymus (*18*), we assessed whether S1P1 might also mediate premature clonal eviction of immature

thymocytes. CD4/8^{dull} cells in male thymi expressed S1P1, albeit in lower amounts compared with mature CD8SP thymocytes (Fig. 3A and fig. S3, A and B). Their exit from the thymus required S1P1 because in vivo S1P1 blockade by FTY720 induced short-term thymic retention, which caused T3.70^{+}CD4/8^{dull} cells to disappear from the periphery (Fig. 3B) (*19*). Moreover, conditional deletion of S1P1 in HY^{cd4}M thymocytes, which caused prolonged thymic retention, resulted in the generation of mature CD8SP thymocytes in S1P1^{cKO}.HY^{cd4}M mice that were CD24^{lo}CCR7^{hi}Qa-2^{hi} (Fig. 3, C and D, and fig. S3C). Unexpectedly, these mature CD8SP male thymocytes were autoreactive against HY self-antigens in the thymus, as they expressed interferon-γ (IFN-γ), which induced preselection CD4^{+}CD8^{+} [double-positive (DP)] thymocytes to express CD317 (an IFN-γ–responsive protein) and caused thymic stress with reduced total thymocyte numbers (Fig. 3, E to G) (*20*–*22*). Thus, S1P1-mediated clonal eviction rids the thymus of immature autoreactive cells before they become functionally competent and cause thymic stress. Moreover, the appearance of mature CD8SP thymocytes in S1P1-deficient HY^{cd4}M mice demonstrates that their absence in wild-type (WT) HY^{cd4}M mice is not a result of clonal deletion but is instead because of clonal eviction of their immature precursors from the thymus.

Gfi1 regulates S1P1 expression and clonal eviction

Mature SP thymocytes express S1P1 only after TCR signaling is disrupted because this event up-regulates the expression of the transcription factor Foxo1, which, in turn, induces the expression of *Klf2* and *S1pr1*, the gene encoding S1P1 (*23*). By contrast, immature TCR^{hi}CD4/8^{dull} thymocytes expressed S1P1 despite being TCR signaled (Fig. 3A and fig. S3, A and B). To understand the contradictory requirements for S1P1 induction in immature and mature thymocytes, we compared TCR signaling of immature CD69^{-}DP and mature CD4SP thymocytes (Fig. 3H and fig. S4A). TCR signaling up-regulated *Nr4a1* in both thymocytes but had different effects on their expression of *Foxo1*, *Klf2*, and *S1pr1* (Fig. 3H and fig. S4, A and B). Without stimulation, mature thymocytes expressed *Foxo1*, *Klf2*, and *S1pr1*, which were then down-regulated by TCR signaling (Fig. 3H and fig. S4A), whereas immature thymocytes expressed *Foxo1*, *Klf2*, and *S1pr1* only when actively TCR signaled (Fig. 3H and fig. S4, A and B). In immature thymocytes, Foxo1 deficiency abrogated *Klf2* and *S1pr1* induction, as in mature cells (fig. S4C), which indicates that Foxo1 up-regulation initiated the same transcriptional progression to S1P1 expression in immature and mature cells (*23*). Thus, the key feature distinguishing immature and mature thymocytes is that Foxo1 up-regulation in immature thymocytes is induced by strong TCR signaling, whereas Foxo1 up-regulation in mature thymocytes is induced by TCR signaling disruption.

To induce Foxo1 up-regulation in immature thymocytes, strong TCR signaling would either up-regulate a transcriptional activator or down-regulate a transcriptional repressor. Gfi1 is a known transcriptional repressor of Foxo1 (*24*), which we found to be highly expressed in immature (CD69^{-}DP) thymocytes but whose expression was reduced in more differentiated CD4/8^{dull} and CD8SP thymocytes (Fig. 3I). Gfi1 expression in immature thymocytes was down-regulated by strong TCR signaling (Fig. 3J), which indicates that strong TCR signaling of immature thymocytes induces Foxo1 up-regulation by down-regulating Gfi1 expression.

To assess the role of Gfi1 in S1P1 induction and clonal eviction, we used the lentivirus "flex-switch" system and the CD4-Cre recombinase (present in HY^{cd4} thymocytes) to induce constitutive expression of lentiviral Gfi1 or lentiviral control Thy1.1 proteins in HY^{cd4} thymocytes (fig. S4D) (*25*). Thymocytes that constitutively expressed lentiviral Gfi1 or Thy1.1 proteins also constitutively expressed hCD2 reporter proteins (fig. S4, D and E). Examination of hCD2^{+}CD4/8^{dull} thymocytes constitutively expressing Gfi1 or Thy1.1 proteins revealed that constitutive Gfi1 expression reduced *Foxo1*, *Klf2*, and *S1pr1* but did not affect PD-1 or Qa-2 (Fig. 3K and fig. S4, F and G). Gfi1-induced down-regulation of *S1pr1* resulted in thymic retention and the appearance of mature Qa-2^{+} CD8SP thymocytes (Fig. 3K and fig. S4H). Thus, it is by down-regulating Gfi1 that strong TCR signaling induces immature thymocytes to express S1P1 and undergo premature clonal eviction (fig. S5).

Peripheral CD8 T cells are self-tolerant despite expressing autoreactive TCRs

Although HY^{cd4}M mice contain peripheral CD8 T cells bearing autoreactive TCRs, these animals remain healthy throughout their lives, which suggests that their peripheral CD8 T cells are self-tolerant. Compared with female peripheral CD8 T cells, male peripheral CD8 T cells were 10-fold less reactive against antigenic HY peptide in vitro and were also less reactive to HY peptide injected in vivo as revealed by CD107a expression—a marker of CD8 T cell degranulation (*26*) (Fig. 4, A and B). Additionally, the growth of male-origin tumor cells (B16-F10) was significantly less constrained in male compared with female HY^{cd4} mice, whereas female-origin tumor cells (MC38) grew equally in both (Fig. 4C and fig. S6A). Thus, despite bearing HY-specific TCRs, peripheral CD8 T cells in male mice are hyporesponsive to HY male antigen. To determine the basis of this hyporesponsiveness, we examined surface expression of TCRs and CD8 co-receptor proteins on peripheral male CD8 T cells (Fig. 4D). Surface TCR and CD8 were both significantly reduced on male CD8 T cells, which bound only one-third the number of HY–MHC-I tetramer complexes compared with female CD8 T cells (Fig. 4D) (*27*). Thus, reduced surface TCR and co-receptor expression causes reduced binding of HY self-ligands (Fig. 4D).

We considered that male CD8 T cells may also be hyporesponsive because they have regulatory function, even though they did not express Foxp3, the master gene regulator of CD4 regulatory T cells (fig. S6B) (*28*). CD8 regulatory T cells are instead thought to be CD122^{+}Ly49^{+} and to express Helios (*29*–*31*). Peripheral CD8 T cells in HY^{cd4}M mice included a substantial number of CD122^{+}Ly49^{+} cells that expressed Helios (Fig. 4E and fig. S6C) and prevented the induction of inflammatory bowel disease in male *Rag*-deficient mice by transferred naïve CD4 T cells. Thus, clonally evicted peripheral CD8 male T cells have regulatory function (Fig. 4F).

Because peripheral CD8 male T cells contained an Ly49^{+} subset and were hyporesponsive to HY antigens, we assessed Ly49 expression on CD8SP thymocytes that arose during thymic retention and were reactive against intrathymic HY antigens (Fig. 3, E to G). Retained thymic CD8 male T cells were exclusively CD122^{+}Ly49^{-}, unlike peripheral CD8 male T cells (Fig. 4G). We therefore wondered whether their Ly49 expression was induced by encountering HY self-antigens in the periphery. Ly49 expression persisted and remained significantly more frequent after transfer of CD8 T cells into male compared with female host mice (Fig. 4H), and a substantial fraction of Ly49^{-} male cells converted into Ly49^{+} cells after transfer into male hosts, which indicates that these were not distinct subsets of autoreactive CD8 T cells (fig. S6D). Thus, immature autoreactive thymocytes evicted from the thymus encounter peripheral self-ligands to become self-tolerant CD8 T cells, some of which express Ly49.

To verify that CD8 T cell tolerance depends on clonal eviction, we compared the reactivity of CD8SP thymocytes retained in the thymus because of S1P1 deficiency versus CD8 T cells evicted into the periphery (fig. S6E). Retained CD8SP thymocytes were CD69^{hi} in vivo and further up-regulated CD69 upon exogenous in vitro stimulation with HY peptide presented by irradiated splenocytes (fig. S6E). By contrast, peripheral CD8 T cells were CD69^{lo} in vivo and hardly responded to exogenous in vitro HY peptide stimulation (fig. S6E). Thus, CD8 T cell tolerance to self-antigen depends on clonal eviction of immature autoreactive thymocytes into the

periphery before they have acquired functional competence.

Clonal eviction of normal polyclonal CD8 T cells

Finally, we wondered whether our understanding of clonal eviction in HY-specific TCR transgenic mice (schematized in fig. S7) also applied to CD8 T cells in normal polyclonal mice. We found that ~5% of MHC-I–selected TCR^{hi} thymocytes in MHC-II^- polyclonal mice were PD-1^+ cells (Fig. 5A). These TCR^{hi}PD-1^+ thymocytes were CD4/8^{dull} and resembled HY^{cd4}M thymocytes that had survived negative selection, whereas their TCR^{hi}PD-1^- polyclonal thymocytes contained CD8SP cells and resembled HY^{cd4}F thymocytes that had undergone positive selection (Fig. 5A). MHC-I–selected TCR^{hi}PD-1^+CD4/8^{dull} thymocytes were present in both male and female polyclonal mice (Fig. 5B), which indicates that they had been signaled to undergo negative selection by sex-independent self-ligands. These negatively selected PD-1^+TCR^{hi}CD4/8^{dull} thymocytes had down-regulated *Gfi1* and up-regulated *S1pr1* expression compared with preselection CD69$^-$DP thymocytes (Fig. 5C), which indicates that they had been strongly TCR signaled in the thymus. To determine whether these cells underwent clonal eviction into the periphery, we identified PD-1^+ cells among recent thymic emigrants (RTEs) by their expression of the reporter protein Rag-GFP [green fluorescent protein (GFP)] (Fig. 5D) (*32*). PD-1^+ RTEs were CD4/8^{dull} and had the highest content of Rag-GFP, which revealed that they were the earliest RTEs in the periphery (Fig. 5E). In addition, CD4/8^{dull} RTEs differentiated into mature CD8 T cells, as their mRNA expression profile was *Cd4*$^-$*Cd8*$^+$*Runx3d*$^+$, and they became CD8 T cells when cultured with IL-7 (Fig. 5, F and G).

MHC-I–selected peripheral CD8 T cells in polyclonal mice contained an Ly49$^+$ subset that resembled self-tolerant CD8 T cells in HY^{cd4}M mice in that peripheral Ly49$^+$CD8 T cells expressed lower levels of both surface TCR and CD8 co-receptors (Fig. 5H). This resemblance suggests that peripheral Ly49$^+$ CD8 T cells may have been clonally evicted during negative selection and encountered their self-ligand during peripheral differentiation into mature CD8 T cells.

Finally, we quantified the number of CD4 and CD8 T cells bearing HY-specific autoreactive TCRs in male and female B6 mice (Fig. 5I). HY/I-A^b tetramer–binding CD4 T cells were significantly reduced in male versus female B6 mice, whereas HY/H-$2D^b$ tetramer–binding CD8 T cells were equally represented in both males and females, which indicates significant clonal deletion of HY-specific CD4 T cells but not HY-specific CD8 T cells in WT B6 mice (Fig. 5I). Additionally, HY–H-$2D^b$ tetramer–binding cells were significantly more frequent among Ly49$^+$CD8$^+$ T cells in male versus female mice, but this was specific for negatively selected HY–H-$2D^b$ tetramer–binding cells because it was not the case for unrelated Flu–H-$2D^b$ tetramer–binding cells, which bear TCRs specific for the influenza foreign antigen (Fig. 5I). Thus, these results further support the conclusion that Ly49$^+$ CD8 T cells are clonally evicted during negative selection and differentiated in the periphery into self-tolerant CD8 T cells.

Discussion

This study identifies premature clonal eviction from the thymus into the periphery as an important consequence of negative selection that results in extrathymic T cell differentiation of self-tolerant CD8 T cells. Premature clonal eviction is triggered by TCR-signaled down-regulation of the transcriptional repressor Gfi1 and induction of S1P1 in immature CD8 thymocytes that survive negative selection. The mechanism establishing CD8 T tolerance has remained enigmatic because functionally mature CD8 T cells bearing autoreactive TCRs are generally absent from the thymus but nevertheless appear in the periphery of both humans and mice (*33*–*40*). It has been thought that CD8 T cells with autoreactive TCRs appear in the periphery because clonal deletion in the thymus is imperfect (*36*, *40*), but this perspective does not explain how autoreactive CD8 T cells that are absent from the thymus are nevertheless undiminished in number in the periphery.

This study provides clonal eviction of immature autoreactive CD8 T cells as a solution to this enigma. Immature CD8 T cells with autoreactive TCRs are strongly TCR signaled during negative selection in the thymus to down-regulate Gfi1 and up-regulate S1P1 expression, which results in the premature eviction of immature CD4/8^{dull} thymocytes from the thymus into the periphery, where they complete their differentiation into functionally mature but self-tolerant CD8 T cells, some of which become Ly49$^+$ regulatory CD8 T cells. We expect that prematurely evicted immature cells from the thymus differentiate into self-tolerant CD8 T cells in part because they are functionally immature when they first encounter peripheral self-ligands, which reduces surface expression of both TCR and CD8 co-receptor molecules, and in part because they acquire regulatory function. Thus, clonal eviction is the primary mechanism underlying CD8 T cell tolerance to self-ligands.

REFERENCES AND NOTES

1. K. M. Ashby, K. A. Hogquist, *Nat. Rev. Immunol.* 10.1038/s41577-023-00911-8 (2023).
2. T. M. McCaughtry, K. A. Hogquist, *Semin. Immunopathol.* **30**, 399–409 (2008).
3. J. W. Kappler, N. Roehm, P. Marrack, *Cell* **49**, 273–280 (1987).
4. E. Palmer, *Nat. Rev. Immunol.* **3**, 383–391 (2003).
5. E. Brugnera *et al.*, *Immunity* **13**, 59–71 (2000).
6. A. Singer, S. Adoro, J. H. Park, *Nat. Rev. Immunol.* **8**, 788–801 (2008).
7. M. Shinzawa *et al.*, *Nat. Immunol.* **23**, 731–742 (2022).
8. L. A. Pobezinsky *et al.*, *Nat. Immunol.* **13**, 569–578 (2012).
9. Y. Ishida, Y. Agata, K. Shibahara, T. Honjo, *EMBO J.* **11**, 3887–3895 (1992).
10. J. L. Riley, *Immunol. Rev.* **229**, 114–125 (2009).
11. T. A. Baldwin, M. M. Sandau, S. C. Jameson, K. A. Hogquist, *J. Exp. Med.* **202**, 111–121 (2005).
12. O. Lantz, I. Grandjean, P. Matzinger, J. P. Di Santo, *Nat. Immunol.* **1**, 54–58 (2000).
13. T. M. McCaughtry, T. A. Baldwin, M. S. Wilken, K. A. Hogquist, *J. Exp. Med.* **205**, 2575–2584 (2008).
14. J. A. Punt, B. A. Osborne, Y. Takahama, S. O. Sharrow, A. Singer, *J. Exp. Med.* **179**, 709–713 (1994).
15. L. Klein, B. Kyewski, P. M. Allen, K. A. Hogquist, *Nat. Rev. Immunol.* **14**, 377–391 (2014).
16. M. A. Zachariah, J. G. Cyster, *Science* **328**, 1129–1135 (2010).
17. R. Etzensperger *et al.*, *Nat. Immunol.* **18**, 1218–1227 (2017).
18. M. L. Allende, J. L. Dreier, S. Mandala, R. L. Proia, *J. Biol. Chem.* **279**, 15396–15401 (2004).
19. S. Mandala *et al.*, *Science* **296**, 346–349 (2002).
20. B. W. Dulken *et al.*, *Nature* **571**, 205–210 (2019).
21. Y. Kato *et al.*, *Cell. Immunol.* **177**, 103–108 (1997).
22. R. Fayad *et al.*, *Eur. J. Immunol.* **35**, 2304–2312 (2005).
23. S. M. Hedrick, R. H. Michelini, A. L. Doedens, A. W. Goldrath, E. L. Stone, *Nat. Rev. Immunol.* **12**, 649–661 (2012).
24. L. Z. Shi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **114**, E67–E74 (2017).
25. F. Schnütgen *et al.*, *Nat. Biotechnol.* **21**, 562–565 (2003).
26. M. R. Betts *et al.*, *J. Immunol. Methods* **281**, 65–78 (2003).
27. J. H. Park *et al.*, *Nat. Immunol.* **8**, 1049–1059 (2007).
28. S. Hori, T. Nomura, S. Sakaguchi, *Science* **299**, 1057–1061 (2003).
29. N. Saligrama *et al.*, *Nature* **572**, 481–487 (2019).
30. J. Li *et al.*, *Science* **376**, eabi9591 (2022).
31. H. J. Kim, B. Verbinnen, X. Tang, L. Lu, H. Cantor, *Nature* **467**, 328–332 (2010).
32. T. E. Boursalian, J. Golob, D. M. Soper, C. J. Cooper, P. J. Fink, *Nat. Immunol.* **5**, 418–425 (2004).
33. H. S. Teh, H. Kishi, B. Scott, H. Von Boehmer, *J. Exp. Med.* **169**, 795–806 (1989).
34. H. von Boehmer, J. Kirberg, B. Rocha, *J. Exp. Med.* **174**, 1001–1008 (1991).
35. S. Dhanji, S. J. Teh, D. Oble, J. J. Priatel, H. S. Teh, *Blood* **104**, 2116–2123 (2004).
36. C. Bouneaud, P. Kourilsky, P. Bousso, *Immunity* **13**, 829–840 (2000).
37. J. H. Velthuis *et al.*, *Diabetes* **59**, 1721–1730 (2010).
38. D. Kronenberg *et al.*, *Diabetes* **61**, 1752–1759 (2012).
39. Y. Maeda *et al.*, *Science* **346**, 1536–1540 (2014).
40. W. Yu *et al.*, *Immunity* **42**, 929–941 (2015).

ACKNOWLEDGMENTS

We thank J. DiCarlo, M. Y. Kimura, C. T. Mayer, J.-H. Park, D. Singer, and Y. Takahama for critically reading the manuscript; T. Baldwin and K. Hogquist for generously providing HY^{cd4} mice; M. Nussenzweig for Rag-GFP transgenic mice; A. Sharpe for B7-deficient mice; and D. Littman for *Runx3d*$^{yfp/yfp}$ mice. We also thank S. Schwab for providing the S1P1 staining protocol; T. Adams, K. Bisht, A. Crossman, L. Granger, W. Hajjar, and S. Sharrow for flow cytometry support; and the NIH Tetramer Core Facility for providing peptide-MHC tetramer complexes. **Funding:** This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. **Author contributions:** M.E.B. conceptualized the study, designed and performed experiments, analyzed data, and wrote the manuscript. Z.Z. and X.T. provided resources and performed experiments. A.S. conceptualized and supervised the study and wrote the manuscript. **Competing interests:** All authors declare that they have no competing interests. **Data and materials availability:** All data are available in the manuscript or the supplementary materials.

SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.adh4124
Materials and Methods
Figs. S1 to S7
Table S1
References (*41*–*44*)
MDAR Reproducibility Checklist

Submitted 1 March 2023; resubmitted 21 June 2023
Accepted 22 September 2023
10.1126/science.adh4124