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Negative selection, not receptor editing, is a physiological response of autoreactive thymocytes

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Antigen receptor editing—a process of secondary rearrangements of antigen receptor genes in autoreactive lymphocytes—is a well-established tolerance mechanism in B cells, whereas its role in T cells remains controversial. Here, we investigated this issue using a novel Tcra knock-in locus, which ensured appropriate timing of TCRα expression and allowed secondary rearrangements. Under these conditions the only response to self-antigen that could be unambiguously identified was negative selection of CD4/CD8 double positive thymocytes. No evidence could be obtained for antigen-induced TCR editing, whereas replacement of the transgenic TCRα chain by ongoing gene rearrangement occurred in some cells irrespective of the presence or absence of self-antigen.

The diversity of antigen receptors in the adaptive immune system of vertebrates is generated by a random recombinatorial process, and thus receptors recognizing self-antigens are constantly generated, creating a risk of autoimmunity. Therefore, the immune system requires an array of mechanisms to disarm autoreactive lymphocytes. This includes negative selection, induction of anergy, diversion to lineages with regulatory properties, and antigen receptor editing.

Receptor editing is a process of secondary rearrangements of antigen receptor genes in response to recognition of self-antigen. This phenomenon is well described for B cells (Nemazee and Hogquist, 2003); however, its role in T cell tolerance remains controversial (Mostoslavsky and Alt, 2004), in part because of the lack of an appropriate mouse model. Indirect experiments using mice expressing TCR transgenes that were not contained within endogenous TCR loci suggested that editing may exist because T cells with receptors specific for peptide ligands expressed in the thymic cortex appeared to undergo rearrangements of the endogenous Tcra locus (Mgargill et al., 2000). In this setting, endogenous TCRα chains have to compete with the transgenic TCRα for pairing with TCRβ chains. The varying efficiency of such competition for different TCR transgenes may explain the fact that OT-I, but not HY or 2C TCRs, allowed for the appearance of transgene-negative, TCR-positive cells in the presence of antigen (Mayerova and Hogquist, 2004). Other studies have suggested that editing may even take place after thymic egress and peripheral antigens could induce TCRβ rearrangements in peripheral T cells specific for these antigens (McMahan and Fink, 1998). More direct experiments in which a rearranged VαJα exon of the HY TCRα chain was knocked-in into the Tcra locus in a way that mimics the product of physiological VαJα rearrangement (HY-I mouse), and thus could be deleted by secondary rearrangements, indicated, however, an absence of TCR editing.
(Buch et al., 2002). Secondary rearrangements did occur to some extent in this system in the absence of the self-antigen, but were not increased in its presence. These secondary rearrangements, also observed in another Tcra knock-in system (Wang et al., 1998), are thought to be a mechanism that enhances the generation of TCRs suitable for positive selection in WT mice. Unexpectedly, however, the knock-in Tcra allele constructed by Buch et al. (2002) was expressed prematurely, in CD4/CD8 double-negative 3 (DN3) thymocytes (Croxford et al., 2008), whereas under physiological conditions Tcra is rearranged only at the CD4/CD8 double positive (DP) stage. Thus expression of the TCR in this system could occur too early, at a point in time when editing may not yet be possible. Indeed, rearrangement of antigen receptors takes place only in nonproliferating populations, as Rag2 is unstable in dividing cells (Li et al., 1996; Lee and Desiderio, 1999), whereas expression of the TCRβ chain in DN3 cells initiates a burst of proliferation that is likely to interfere with V(D)J recombination. Although, in B cells, premature expression of an autoreactive BCR does not interfere with editing, which still occurs after a wave of proliferation (Pelanda et al., 1997), similar rules do not have to apply to T cells. Moreover, premature TCRαβ expression is known to divert cells to γδ-like lineage (Bruno et al., 1996; Terrence et al., 2000; Baldwin et al., 2005; Egawa et al., 2008) and it is unclear whether that is compatible with editing. Some in vitro experiments were performed to address this issue, and appeared to contradict this possibility (Buch et al., 2002); however, this system did not allow tackling the issue properly in the in vivo setting. Thus, a model with correct timing of TCR expression is required to address the impact of TCRα editing under physiological conditions.

A successful approach to correct the timing of TCRα transgene expression was developed by Baldwin et al. (2005). The authors placed a floxed STOP cassette between the promoter and the HY TCRα transgene, thus preventing premature TCRα expression. The STOP cassette was then excised by CD4-Cre that is expressed from the DP stage, ensuring proper timing of TCRα expression (HYα+ mouse). However, the transgene used could not be deleted by secondary rearrangements, and thus Tcra editing could not be addressed in this system. We adopted this approach to correct the timing of TCRα expression in the HY-I system. In the resulting HY-STOP mice, the onset of HY TCRα expression took place physiologically, at the DP stage. Correction of the timing of TCR expression rescued the abnormalities in the gross thymic phenotype observed in HY-I mice. However, no evidence for TCRα editing was found in this system, and autoreactive cells instead were eliminated by negative selection.

RESULTS AND DISCUSSION

Generation of HY-STOP mice

We aimed to design a Tcra locus whose expression was only initiated at the physiologically appropriate nonproliferative DP stage, but that would allow for secondary rearrangements potentially induced by self-antigen encounter. To this end, we chose to modify the previously reported HYαΔ Tcra allele of HY-I mouse (Buch et al., 2002). This allele was designed to mimic the product of Vαβ recombination: the rearranged VαJα segment of the HY TCRα chain was inserted into the TCRα locus followed by deletion of the TCRβ locus (Buch et al., 2002). The H2Dβ-restricted HY TCR recognizes Y-chromosome-encoded antigen, and thus is self-reactive in males but not females. However, the HYαΔ allele lost its expression at the DN stages of T cell development (Buch et al., 2002; Fig. 1 B). To correct this abnormality, we generated and retargeted embryonic stem (ES) cells from HY-I mice to introduce a floxed STOP cassette with two additional polyadenylation signal sequences (Lakso et al., 1992; Buch et al., 2005) between rearranged HY VαJα and the first downstream J segment (Fig. 1 A). As polyadenylation signals in the STOP cassette lead to termination of transcription, the truncated mRNA should include a rearranged VαJα exon, but not a constant region of the TCRα, and thus the TCRα protein should not be produced unless the STOP cassette is removed. As the HYαΔ allele contains a “leftover” loxP site upstream of HY Vα (Buch et al., 2002), we used loxP2272 sites that can recombine with each other but not with conventional loxP sites (Siegel et al., 2001; Luche et al., 2007) to flank the STOP cassette. Generation of the ES cells, targeting ES cell screening and blastocyst injections, were performed as described in the Material and methods.

The resulting TcraHY-STOP chimeras were bred to Tcra−/− females containing the TCR HYB (Uematsu et al., 1988) and Rorγt-Cre (Eberl and Littman, 2004) transgenes. TcraHY-STOP−/− HYB Rorγt-Cre animals, henceforth referred to as HY-STOP mice, were used in all further analyses. The TCR HYβ transgene was required to produce a male-autoreactive HY TCR once HYα is expressed. Expression of Rorγt-Cre is initiated at the DP stage (Eberl and Littman, 2004)—the time in T cell development when rearrangement and expression of TCRα take place physiologically, and thus the STOP cassette should be removed from the TcraHY-STOP locus. In TcraHY-STOP−/− mice, the only functional copy of the Tcra locus is the one containing prerearranged HYα and the STOP cassette, and thus expression of any TCRα other than HYα is indicative of secondary rearrangements in the locus.

Thymic phenotype of HY-STOP mice

We first compared the timing of TCRαβ expression in HY-I and HY-STOP female mice. Unlike HY-I animals, where the majority of DN thymocytes were TCRβHYα−, virtually no such cells could be detected in HY-STOP animals. DP thymocytes, however, did initiate the expression of HYα (Fig. 1 B). Importantly, only a fraction of DP cells were positive for HYα, mimicking the physiological situation in WT mice where TCRα rearrangement takes place during transition through the DP stage and only a fraction of DP thymocytes expresses the αβ TCR. Thus, as expected, insertion of the STOP cassette interfered with premature expression of TCRα, and its removal by Rorγt-Cre ensured a physiological onset of TCRα expression at the DP stage.
Figure 1. Corrected timing of TCRα expression and normal gross thymic phenotype of HY-STOP mice. (A) Targeting strategy for generation of the Tcra^HY-STOP locus. Original HY-I knock-in locus (top), targeting vector (center), and targeted locus with a STOP cassette are shown. HY Vαα, recombined VαJα element of HY TCR; Neo, neomycin resistance gene; ftr, ftr sites; PA, polyadenylation signal; STOP, transcriptional STOP cassette; IoxP, leftover IoxP site in HY-I locus; IoxP2272, alternative IoxP sites; TK, thymidine kinase gene; Amp, ampicillin resistance gene. (B) Expression of surface TCRβ and HYα on CD4^−CD8^− (DN), CD4^+CD8^− (DP), and CD4^+CD8^+ (CD8SP) thymocytes from the HY-I (Tcra^HY-I/HY-I) and HY-STOP (Tcra^HY-STOP^-HYB Rorγt-Cre) female mice. Representative results of at least three independent experiments are shown. (C) Total thymocytes numbers and numbers of TCRα^−DP thymocytes in male and female HY-I and HY-STOP mice. *, P < 0.5; **, P < 0.01 (n = 3-5); student’s t test. Error bars represent the SD. Representative results of two independent experiments are shown. (D) Comparison of CD4/CD8 profiles of HY-I and HY-STOP thymocytes from male and female mice. Representative results of at least three independent experiments are shown.
As reported previously (Groettrup and von Boehm, 1993), we could detect some level of surface expression of transgenic TCRβ on DP cells even in the absence of TCRα (e.g., on a Tna−/− background; Fig. S1 A). Thus, in experiments that required strict gating on TCRαβ-negative cells, the gate was applied as shown in Fig. S1A.

Unlike the original HY-I mice, which had a strong decrease in the DP compartment and a relative increase in DN cells even in female mice because of premature TCR expression, both female and male HY-STOP mice had a normal frequency of DP and DN thymocytes (Fig. 1 D). Thymic cellularity, which was severely reduced in male HY-I mice due to premature deletion of DP cells, was consistently increased in male HY-STOP mice. However, male HY-STOP mice still had an approximately twofold decrease in total thymocyte numbers when compared with HY-STOP females (Fig. 1 C). This yet to be explained phenomenon is not caused by premature deletion of H-Y–specific cells, as TCR− DP cells are also affected (Fig. 1 C), and thus seems to represent a bystander effect. We conclude that corrected timing of TCR expression largely rescued the severe abnormalities found in the gross thymic phenotype of HY–I mice. This conclusion concurs with earlier studies in the HY−/− model (Baldwin et al., 2005).

We next tested whether the HY-STOP locus is functional in terms of Vαβ recombination. It was reported for HY-I mice that even in the absence of self-antigen, their TCRα locus underwent some level of Vα−β rearrangement resulting in deletion of the HYα Vαβ in a fraction of thymocytes, a process that in normal mice is believed to enhance the formation of receptors suitable for positive selection. Consistently, in HY-STOP female mice, some thymocytes were HY TCR− but expressed high levels of surface TCRβ. These cells expressed Vαβ joins other than that of the HY TCR, as staining was detectable withVa2 and Va3.2 antibodies (Fig. 2 A). These non-HYα chains can only be a product of rearrangements in the TnaH/STOP allele, as TnaH/STOP−/− animals were used. Therefore, insertion of the STOP cassette did not disrupt the Tna locus architecture and did not interfere with its ability to undergo secondary rearrangements. However, these rearrangements were not induced by antigen, as they occurred in female mice. These TCRβHYα− cells differentiated toward both CD4 and CD8 lineages and, as expected, were uniformly HYβ− as judged by VB8.1/8.2 antibody staining. This demonstrates that, as reported previously (Uematsu et al., 1988), the transgenic TCRβ chain was able to support positive selection in the context of TCRα chains other than HYα (Fig. 2 B).

In summary, HY-STOP mice had a grossly normal thymic phenotype, their TCRα expression timing was physiological, and their Tna locus was capable of secondary rearrangements. These properties, not found together in any other model system, make HY-STOP mice suitable to definitively address the question of TCR editing.

Lack of evidence for TCR editing in HY-STOP animals
We next sought to find evidence for or against TCR editing in this system. To this end, we compared the frequencies and numbers of clonotype-positive and negative TCRαβ-expressing cells in male and female HY-STOP mice. BM chimeras with sex-matched donors and recipients were used in these experiments to increase the numbers of animals analyzed in parallel.

In female HY-STOP mice, the majority of TCR+ cells express the clonotypic HY TCR, whereas a minority undergoes secondary rearrangement and are TCRβ+HYα− (Fig. 1 B). Two scenarios are conceivable in male mice. If self-reactive thymocytes would undergo TCR-editing, cells that were HYα−positive in females would convert to an HYα−negative state in males. Alternatively, if negative selection is the main tolerogenic mechanism, these cells would be eliminated by apoptosis. Both scenarios would predict a proportional decrease in HYα− cells among TCR+ cells (Fig. 3 A). However, in the case of TCR editing, this would be accompanied by a corresponding increase in absolute numbers of TCRβ+HYα− thymocytes (converted from autoreactive HYα+ cells), whereas if the predominant mechanism of tolerance is negative selection of TCRβ+HYα− cells, no increase in TCRβ+HYα−
Brief Definitive Report

Potential causes of thymocyte death in HY-STOP mice were investigated by monitoring Rag1 and Rag2 expression in thymocytes sorted from HY-STOP male and female mice. As expected, expression of HY TCR led to a 5–10 fold decrease in Rag gene expression in female DP thymocytes. However, even a more prominent 55–170 fold decrease was observed in male HYα+ DP cells, suggesting that the stronger TCR signal from an autoreactive TCR was repressing Rag expression more efficiently rather than prolonging it. We conclude that no evidence for antigen-induced TCR editing can be found in the HY-STOP system.

Autoreactive T cells in HY-STOP mice undergo negative selection

We next compared the phenotype of clonotype-positive cells in male and female HY-STOP mice. As expected, no CD4−CD8− cells were found among HYα+ cells (Fig. 4A), confirming appropriate timing of TCRα expression. CD8SP HYα+ cells were detected in female but not male mice, and HYα+ DP cells were present both in males and females. However, the levels of co-receptor expression were different from male and female HY-STOP mice by qPCR (Fig. 3D). As expected, expression of HY TCR led to a 5–10 fold decrease in Rag gene expression in female DP thymocytes. However, even a more prominent 55–170 fold decrease was observed in male HYα+ DP cells, suggesting that the stronger TCR signal from an autoreactive TCR was repressing Rag expression more efficiently rather than prolonging it. We conclude that no evidence for antigen-induced TCR editing can be found in the HY-STOP system.

**Figure 3.** Presence of self-antigen does not enhance secondary rearrangements. (A) Schematic representation of the predicted effects of deletion and TCR-editing scenarios on numbers of clonotype-positive and -negative thymocytes from HY-STOP male and female BM chimeras. Representative results of at least five independent experiments are shown. (B) Expression of surface TCRβ and HYα on DP and CD8SP thymocytes from HY-STOP male and female BM chimeras. **, P < 0.01; NS, not significant (P > 0.05); student’s t test; n = 5. Error bars represent the SD. Representative results of two independent experiments are shown. (C) Absolute numbers (left) and percentage from total thymocytes (right) of TCRβ−HYα+ and TCRβ+HYα− DP cells in male and female HY-STOP BM chimeras. ***, P < 0.001; **, P < 0.01; student’s t test; NS, not significant (P > 0.05).
between males and females. Whereas all DP cells in females expressed high levels of CD4 and CD8, a large fraction of male HYα+ DP cells had a CD4hiCD8lo phenotype (Fig. 4 A). This DPhi phenotype was previously reported to be associated with negative selection (Page et al., 1993). Likewise, male but not female cells expressed high levels of the co-inhibitory receptor PD-1 and the transcription factor Helios (both known to be up-regulated in cells undergoing negative selection (Baldwin and Hogquist, 2007; Daley et al., 2013; Mingueu

e et al., 2013), with the male DP hi population expressing the highest levels of both markers (Fig. 4 B). To assess directly whether clonotype-positive cells in males undergo apoptosis, we performed staining for the active form of the “executioner” caspase-3 (act-Casp3). Male, but not female, HYα+ DP cells had a detectable population of act-Casp3+ cells (Fig. 4 C) at a frequency close to that reported previously for the HY41 model, where TCR editing is not possible (McCaughtry et al., 2008). We conclude that, under conditions permissive for TCR editing, encounter of self-antigen in the thymus results in apoptosis rather than secondary rearrangement of the TCR.

Interestingly, in male HY-I mice (i.e., the experimental system previously used by Buch et al. [2002]) the frequency of act-Casp3+ HYα+ thymocytes was approximately fivefold lower than in HY-STOP males (Fig. 4 C). Thus, premature TCR expression partially interferes with negative selection—possibly due to rescue of some of the autoreactive thymocytes by their divergence to γδ-like lineage. This result emphasizes the importance of models with physiological timing of TCR expression for studies of T cell tolerance.

In spite of the virtual nonexistence of HYα+ CD8SP thymocytes in male mice, few such cells escaped to the periphery and acquired a CD8αβ*CD122*CD44* phenotype. These cells expressed lower levels of TCR and CD8 than clonotype-positive cells in females, and many of them up-regulated Ly49, a phenotype previously reported for CD8 T cells with regulatory properties (Kim et al., 2010, 2011; not depicted).

Although antigen receptor editing is a well-established tolerance mechanism in B cells, studies in T cells provided conflicting results. In the two systems where TCR editing was suggested to occur, it would have to take place under non-physiological circumstances. In the OT-I system, endogenous TCRα chains had to compete with the transgenic TCRα expressed by all thymocytes (McGargill et al., 2000). Another study used a knock-in into the Tcrd locus that contained a PGK promoter-driven neomycin resistance gene and an Ig heavy chain enhancer in addition to the rearranged Vα-Jα locus. This result emphasizes the accessibility of the locus to the recombination machinery. Moreover, this locus did not mimic a normal Vα-Jα recombination product in that it contained intact Tind gene segments. Experiments in a more physiological Tind knock-in allele that closely mimicked a product of Vα-Jα recombination failed to find evidence for TCR editing (Buch et al., 2002). However, all these models suffered from premature expression of TCRα that is known to alter T cell development. Here, we revisited the problem of TCR editing with a new Tind knock-in allele that ensured physiological timing of TCRα expression and was capable of secondary rearrangements. No evidence for TCR editing was found in this system and autoreactive thymocytes were instead eliminated by negative selection.

It remains theoretically possible that TCR editing can still contribute to tolerance under certain circumstances—for example for MHC class II–restricted TCRs, when antigen is expressed in a particular subcompartment of the thymus (i.e., the medulla rather than the cortex) or by a particular type of
antigen–presenting cell. However, both TCRs for which editing was suggested to take place were MHC class I restricted (Wang et al., 1998; McGargill et al., 2000). Moreover, initial evidence for TCR editing in the OT-I model was provided in a system where antigen expression was restricted to thymic cortex (McGargill et al., 2000) and later extended to a situation when it was expressed ubiquitously (Mayerova and Hogquist, 2004). This pattern of antigen expression is thus very similar to the situation in male HY-STOP mice, where ubiquitously expressed antigen is first encountered by autoreactive thymocytes in the cortex, and therefore cannot explain the observed differences.

It was suggested that TCRs have certain intrinsic differences determining their ability or inability to induce editing (Mayerova and Hogquist, 2004). An obvious candidate for such a difference is the affinity of the TCR for self-ligand. Both TCRs that were suggested to induce TCR editing were relatively inefficient in induction of negative selection, as judged by the high frequency of TCR-transgenic DP thymocytes in the presence of antigen (Wang et al., 1998; McGargill et al., 2000), whereas negative selection by the HY TCR is highly efficient. Thus, it is conceivable that only more weakly autoreactive TCRs mediate TCR editing. Editing, however, would require prolongation or reinduction of Rag expression in autoreactive thymocytes. We demonstrate here that both weak (female HY mice) and strong (male HY mice) TCR signals repress Rag expression. It does not seem probable, although remains formally possible, that a TCR signal of intermediate strength would have an exclusive ability to induce Rag. Along these lines, McGargill et al. (2000) reported less efficient Rag down-regulation in unfraccionated OT-I DP thymocytes when antigen was present. However, as the DP compartment includes cells both before and after TCR signaling, this result can be explained by selective survival of Rag-high preselection DP thymocytes.

Antigen-independent secondary rearrangements reported previously in other models (Wang et al., 1998; Buch et al., 2002) also took place in the HY-STOP system. Such secondary rearrangements together with the fact that TCR editing may be simply explained by selective survival of the cells that rearranged their Tcrα loci before antigen exposure and therefore escaped negative selection.

In conclusion, the experiments in this novel physiological knock-in model do not provide any evidence for self-antigen–induced TCR editing, although unequivocally showing negative selection by deletion of autoreactive thymocytes.

MATERIALS AND METHODS

Generation of HY-1 (Tcrα/Tcrβ<sup>−/−</sup>/C-terminal knockout) ES cells. All ES cell work was performed in ES cell medium/DMEM containing 15% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM-non essential amino acids, 2,000 U/ml LIF, and 0.1 mM β-mercaptoethanol. C57BL/6 females were superovulated and mated with HY-1 males. 3.5 d after coitus, blastocysts were isolated and cultured on embryonic fibroblast feeders in ES cell medium supplemented with PD98059 MEK Inhibitor (final concentration 13.5 μg/ml). On day 4 or 5 after plating, the blastocysts, outgrowing inner cell mass–derived clumps, were trypsinized and plated on fresh feeder cells. Presence of HY-1 allele in the resulting ES cell lines was confirmed by PCR.

Generation of HY-STOP mice. Targeting pC3T1STOP construct was derived from pC3T1 vector used for generation of the original HY-1 mouse (Buch et al., 2002) by removal of elements upstream of HY V<sub>α2</sub>seq and insertion of floxed STOP cassette (Buch et al., 2005) downstream of it. Conventional loxP sites flanking the STOP cassette were replaced with loxP227 sites that can only recombine with each other (Siegel et al., 2001; Luche et al., 2007). Linearized pC3T1STOP vector was electroporated into HY-1 ES cells. ES cells were selected with G418 and ganciclovir. ES cell clones were screened by PCR with primers upstream of short arm of homology (5′-AAGGCTGTTGTGATAAATGTGC-3′ and in the STOP cassette (5′-CGGAATAGGAACCTCGAG-3′; Fig. 1 A). Integration site was confirmed by sequencing the PCR product. The fact that HY-1 and not WT allele was targeted was confirmed by PCR that detects disruption of the original HY-I locus by STOP cassette insertion. Two clones satisfying these criteria were injected into C57BL/6 albino blastocysts to generate chimeric males that were directly bred to Tcrα<sup>−/−</sup> females containing TCR HYβ and Royrt-Cre transgenes to generate mice with Tcrα<sup>−/−</sup> HYβ Royrt-Cre genotype referred as HY-STOP mice. All mice were bred and maintained in the specific pathogen–free animal facilities of the Dana-Farber Cancer Institute (DFCI). All animal procedures were done in compliance with the guidelines of the DFCI Animal Resources Facility, which operates under regulatory requirements of the U.S. Department of Agriculture and the Association for Assessment and Accreditation of Laboratory Animal Care.

BM chimeras. In some experiments BM chimeras were used to amplify mice with Tcrα<sup>−/−</sup> HYβ Royrt-Cre genotype. To this end, C57BL/6 males and females were lethally irradiated (1,000 rad) with a γ-cell 40 irradiator (with a cesium source) and injected with 2–8 × 10<sup>6</sup> T cell-depleted, sex-matched BM cells from Tcrα<sup>−/−</sup> HYβ Royrt-Cre animals. Mice were analyzed 4–10 wk after the transfer.

Flow cytometry. mAbs specific for CD4 (RM4-5), CD8α (53–6.7), CD69, TCRβ (H57-597), TCR V<sub>Bβ</sub>1–8/2 (J16–133.18), HYα (T3.70), Vα2 (B20.1), Vα3.2 (RR3–16), PO-1 (29F.1A12), and Helios (22.F6) were purchased from BD, eBioscience, or BioLegend and were used as biotin, FITC, phycoerythrin (PE), peridinin chlorophyll protein (PerCP), PerC-P6.5, PE-Cy7, allophycocyanin (APC), APC-Cy7, or Pacific blue conjugates. Fluorochrome-conjugated streptavidin was used to reveal staining with biotinylated mAb. Intracellular staining for Helios was performed using the Foxp3 staining buffer set (eBioscience). Intracellular staining for cleaved caspase-3 was performed using 5A1E rabbit monoclonal antibody, Alexa Fluor 647–labeled anti–rabbit F(ab′)2 fragment (both from Cell Signaling Technology) and the Cytofix/Cytoperm kit (BD) according to the manufacturer’s instructions. Flow cytometry was performed on FACSaria (BD). Data were analyzed with Flowjo software (Tree Star).

Real-time PCR. Indicated populations were sorted, total RNA was prepared with RNeasy Plus kit (QIAGEN) with gDNA Eliminator columns. cDNA was synthesized using Superscript II reverse transcription (Invitrogen) according to the manufacturer’s recommendations. Real-time RT-PCR was performed on CFX Connect cycler (Bio-Rad Laboratories) using TaqMan PCR master mix (Bioline). Mm01270936_m1 (Rag1), Mm00501300_m1 (Rag2), and Mm99999915_g1 (Gapdh) TaqMan Gene Expression Assays (Applied Biosystems) were used. No Rag1 or Rag2 signals were detected in the absence of reverse transcription. Standard curve method was used for data analysis.

Online supplemental material. Fig. S1 shows TCRβ and HYα staining of WT, Tcrα<sup>−/−</sup>, Tcrβ<sup>−/−</sup> HYβ, and HY-STOP DP thymocytes, isotype controls for
REFERENCES