Specialized proteasome subunits have an essential role in the thymic selection of CD8\(^+\) T cells

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The cells that stimulate positive selection express specialized proteasome \(\beta\)-subunits different from those expressed by all other cells, including those involved in negative selection. Mice that lack all four specialized proteasome \(\beta\)-subunits, and therefore express only constitutive proteasomes in all cells, had a profound defect in the generation of CD8\(^+\) T cells. While a defect in positive selection would reflect an inability to generate the appropriate positively selecting peptides, a block at negative selection would point to the potential need to switch peptides between positive selection and negative selection to avoid the two processes’ often cancelling each other out. We found that the block in T cell development occurred around the checkpoints of positive selection and, unexpectedly, negative selection as well.

During T cell development, immature thymocytes rearrange genes encoding their T cell antigen receptors (TCRs) to generate receptors of random specificities. The immune system must then positively select T cells with useful receptors, match the cells’ receptor specificities with function through the process of lineage commitment, and eliminate dangerous autoreactive clones by negative selection\(^1\). Each of these key developmental checkpoints is driven by signaling through the TCR, via interactions with proteasomes with different active-site subunit combinations and catalytic properties and allosterically influence each other\(^7\). Three different specialized proteasomes with unique \(\beta\)-subunit combinations (\(\beta\)1, \(\beta\)2, \(\beta\)5) are able to stimulate the maturation of CD8\(^+\) T cells\(^1\). Through the use of such cultures it has been found that variants of antigenic epitopes that have been modified to become antagonists or weak agonist-antagonists for mature T cells (altered peptide ligands) promote the maturation of CD8\(^+\) T cells, whereas the native peptide sequence does not\(^11\). That led to the idea that specialized epitopes might be needed to drive positive selection. Given such findings, it is logical that thymoproteasomes might help generate such special peptides, and some experiments have suggested that the developmental block in \(Psmb11\)\(^−/−\) thymi occurs before negative selection\(^12\). Accordingly, the favored model for the diminished development of CD8\(^+\) T cells in \(\beta\)5-deficient mice has been impaired positive selection due to a loss of the ‘special’ peptides needed for positive selection\(^13\). An alternate and disfavored model was that thymoproteasome-derived peptides allow cells to survive negative selection because they are displayed only by cTECs and would not be present at negative selection. Therefore, positive selection and negative selection would not cancel each other out\(^14\).

We initiated this study to investigate the role in T cell development of the specialized proteasomes that contain \(\beta\)5t (\(Psmb11\)\(^−/−\) mice) have a partial but substantial reduction in the development of mature CD8\(^+\) T cells\(^5,10\). Those findings resonate with earlier studies showing that only a minority of exogenous antigenic or splenic peptides added to fetal thymic organ cultures deficient in \(\beta\)5t-\(\beta\)2i-\(\beta\)1i-\(\beta\)1c or \(\beta\)5t-deficient mice has been investigated, thymoproteasomes generate both unique (~30%) and common (~70%) presented peptides\(^6,8,9\). Thymoproteasomes are expressed exclusively in cortical thymic epithelial cells (cTECs), and therefore some of the peptides presented on the cTECs that drive positive selection will be different from the peptides presented anywhere else in the body, including on the medullary thymic epithelial cells (mTECs) and thymic dendritic cells that drive negative selection\(^5,6\). Mice that lack the thymoproteasome subunit \(\beta\)5t (\(Psmb11\)\(^−/−\) mice) have a partial but substantial reduction in the development of mature CD8\(^+\) T cells\(^5,10\). Those findings resonate with earlier studies showing that only a minority of exogenous antigenic or splenic peptides added to fetal thymic organ cultures deficient in \(\beta\)5t-\(\beta\)2i-\(\beta\)1i-\(\beta\)1c or \(\beta\)5t-deficient mice has been investigated, thymoproteasomes generate both unique (~30%) and common (~70%) presented peptides\(^6,8,9\). Thymoproteasomes are expressed exclusively in cortical thymic epithelial cells (cTECs), and therefore some of the peptides presented on the cTECs that drive positive selection will be different from the peptides presented anywhere else in the body, including on the medullary thymic epithelial cells (mTECs) and thymic dendritic cells that drive negative selection\(^5,6\).

**ARTICLES**

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RESULTS
Specialized proteasomes required for CD8+ T cell development
To investigate the role of specialized proteasomes (i.e., thymoproteasomes, immunoproteasomes, and mixed proteasomes) in T cell development, we generated mice deficient in the proteasome subunits β1i, β2i, β5i and β5t (Psmb8−/−Psmb9−/−Psmb10−/−Psmb11−/− (4KO) mice). These mice, which have only constitutive proteasomes, were viable and healthy and bred normally (data not shown). Their thymi were of normal size (Supplementary Fig. 1a). The 4KO mice had normal numbers of CD4+CD8+ (double negative) thymocytes and CD4−CD8+ (double-positive) thymocytes relative to the abundance of these cells in C57BL6/J wild-type mice (Supplementary Fig. 1b–d). In contrast, there was a loss of >90% in mature TCRββCD4−CD8αβ+ CD8 single-positive (CD8SP) thymocytes (Fig. 1a–c and Supplementary Fig. 1f,g). Similarly, the spleens of 4KO mice had ≥90% fewer CD8+ T lymphocytes than those of C57BL6/J wild-type mice (Fig. 1e,f). This block in polyclonal T cell development in 4KO mice was substantially greater than that observed in β5t-deficient mice or immunoproteasome-deficient mice, which have reductions of ~75% and 50%, respectively, in CD8SP cells 10,5,8. Thus, our analysis of 4KO mice revealed that specialized proteasomes were required for the development of the vast majority of mature CD8+ T cells.

The CD8SP cells that developed in the 4KO thymus expressed all of the TCR β-chain variable regions we analyzed (Vβ2, Vβ3, Vβ4, Vβ5.1 or Vβ5.3, Vβ6, Vβ7, Vβ8.1 or Vβ8.2, Vβ8.3, Vβ9, Vβ10a, Vβ11, Vβ12, Vβ13 and Vβ14), although the frequency of some of these Vβ regions was modestly different in 4KO mice than in wild-type mice (Supplementary Fig. 2). Notably, CD8SP cells in 4KO mice had significantly lower expression of the negative regulator CD5 than that of CD8SP cells in wild-type mice (P = 0.0079; Supplementary Fig. 1h), which suggested that their TCRs had been selected on peptide–MHC class-I-deficient host mice Therefore, specialized proteasomes were required in thymic stromal cells for the development of most CD8+ T cells. These results indicated that the defect in 4KO mice was not simply due to a cell-intrinsic defect in T cells lacking specialized proteasomes.

Because altered expression of MHC class I can affect the development of CD8+ T cells, we next investigated MHC class I expression on TECs that lacked specialized proteasomes (Supplementary Fig. 3). We found that the expression of H-2Kb and H-2Db on 4KO CD45−CD249−CD326+ cTECs was approximately half of that of their wild-type counterparts (Supplementary Fig. 3d). We found a similar reduction in the expression of MHC class I molecules on 4KO CD45−CD326−UEA1+ mTECs (Supplementary Fig. 3e). The 50%...
reduction in MHC class I expression was similar that of dendritic cells and lymphocytes from Psmb8−/− Psmb9−/− Psmb10−/− immunoproteasome-deficient mice, which, like 4KO hematopoietic cells, have only constitutive proteasomes. To determine whether the 50% reduction in MHC class I expression could account for the block in CD8+ T cell development observed in the 4KO mice, we used the heterozygous progeny of mice with homozygous deficiency in H-2Kb and H-2Db immunoproteasome genes. As noted above, these mice suggested that there could have been defective positive selection of OT-I or P14 CD8+ T cells (Fig. 2a). Because the CD4+CD8+ phenotype indicated that the thymocytes had been positively selected, this showed that in the absence of specialized proteasomes, positive selection of the OT-I T cells was defective. These findings were consistent with the published finding that the immunoproteasome is able to generate, at least in fibroblasts, peptides known to positively select OT-I cells17. If these are actually the true peptides that positively select OT-I cells in vivo, which is unknown, then presumably constitutive proteasomes cannot generate them in sufficient amounts. We obtained similar results for the development of mature CD8SP T cells from the BM of P14 mice (with transgenic expression of a TCR specific for lymphocytic choriomeningitis virus glycoprotein epitope gp33 presented by H-2Db) deficient in the TCR α-chain (Tcra−/−), in the thymus of 4KO host mice (Fig. 2f–j).

We also investigated how the loss of specialized proteasome subunits affected the development of polyclonal T cells. As noted above, the decreased expression of CD5 on polyclonal CD8SP cells in 4KO mice suggested that there could have been defective positive selection of these cells in the absence of specialized proteasomes, at least for the small subset of these cells that successfully transited all of the developmental checkpoints. We sought to investigate this issue further for the broader variety of developing cells. CD4+CD8+ T cells that have received positive selection signals upregulate their expression of the activation marker CD69 (refs. 18,19) along with expression of the TCR and downregulate expression of the co-receptor CD8. The CD69+ TCRβintCD4+CD8lo thymocyte population includes cells that will develop into both CD4SP cells and CD8SP cells. To distinguish

![Figure 2](https://example.com/figure2.png)

**Figure 2** Defect in positive selection of OT-I or P14 CD8+ T cells in 4KO thymi. (a,f) Flow cytometry of cells from Rag1−/−OT-I−/−WT or Rag1−/−OT-I−/4KO chimeras (a) or Tcra−/−P14−/−WT or Tcra−/−P14−/−4KO chimeras (f). Numbers adjacent to outlined areas indicate percent CD4+CD8+ cells (top left) or CD4+CD8− (CD8SP) cells (bottom right); numbers above bracketed lines indicate percent CD4+CD8+Vα2hi thymocytes (b) or CD4+CD8− thymocytes (c), the mean fluorescence intensity (MFI) of Vα2 on CD4+CD8+ thymocytes (d), and flow cytometry analyzing Vα2 on CD4+CD8+ thymocytes (e) in Rag1−/−OT-I−/−WT chimeras (WT; n = 9) or Rag1−/−OT-I−/−4KO chimeras (4KO; n = 16) (male and female). (g–j) Quantification of CD4+CD8−Vα2+ thymocytes (g) or CD4+CD8− thymocytes (h), geometric mean fluorescence intensity of Vα2 on CD4+CD8+ thymocytes (i), and flow cytometry analyzing Vα2 on CD4+CD8−Vα2+ thymocytes (j) in Tcra−/−P14−/−WT chimeras (WT; n = 14) or Tcra−/−P14−/−4KO chimeras (4KO; n = 15) (male and female). Each symbol (b–d,g–i) represents an individual mouse; small horizontal lines indicate the mean (± s.d.). **P < 0.0245, ***P < 0.0068, ****P < 0.0056, *****P < 0.0025 and ******P < 0.0001 (Student’s t-test). Data are representative of (a,e,f,j) or are pooled from (b–d,g–i) two experiments.

Positive selection of monoclonal and polyclonal thymocytes

We investigated how the loss of specialized proteasomes affected the positive selection of OT-I T cells (with transgenic expression of a TCR specific for ovalbumin presented by H-2Kb) through the use of chimeric wild-type or 4KO host mice given BM from OT-I mice deficient in the recombination enzyme RAG-1 (Rag1−/−). We observed much less development of mature CD8SP T cells from the OT-I BM in the thymus of 4KO host mice than in the thymus of wild-type host mice (Fig. 2a,b). This result was markedly different from results obtained for Psmb11−/− thymi, which show no substantial reduction in OT-I CD8SP cells relative to that of wild-type hosts13,16. There was also much less development of CD4+CD8+ OT-I thymocytes in the thymus of 4KO host mice than in the thymus of wild-type host mice (Fig. 2a,c). Moreover, in the 4KO host mice, the CD4+CD8lo OT-I thymocytes that did develop failed to upregulate TCR expression to wild-type amounts (Fig. 2d,e). Because the CD4+CD8lo phenotype indicated that the thymocytes had been positively selected, this showed that in the absence of specialized proteasomes, positive selection of the OT-I T cells was defective. These findings were consistent with the published finding that the immunoproteasome is able to generate, at least in fibroblasts, peptides known to positively select OT-I cells17. If these are actually the true peptides that positively select OT-I cells in vivo, which is unknown, then presumably constitutive proteasomes cannot generate them in sufficient amounts. We obtained similar results for the development of mature CD8SP T cells from the BM of P14 mice (with transgenic expression of a TCR specific for lymphocytic choriomeningitis virus glycoprotein epitope gp33 presented by H-2Db) deficient in the TCR α-chain (Tcra−/−), in the thymus of 4KO host mice (Fig. 2f–j).
between these two populations, we analyzed the cell-surface marker CD103, an integrin expressed on cells of the CD8+ lineage in the thymus. Unexpectedly, we found that the CD4+CD8αβ+CD69+CD103+ population was not smaller in 4KO mice than in wild-type mice (Fig. 3). To verify that the development of these CD103+CD8αβ cells was dependent on MHC class I, we assessed their development in MHC-class-I-deficient (B2m−/−) mice and found considerably fewer CD103+CD8αβ cells in B2m−/− mice than in wild-type or 4KO mice.

We also wanted to rule out the possibility that in the absence of specialized proteasome-derived peptides in the 4KO thymus, peptides presented by MHC class II might somehow be aberrantly positively selecting CD8+ T cells. Given the close proximity of the genes encoding MHC class II to Psmb8 and Psmb9 on chromosome 17, it is impractical to breed 4KO mice with homozygous deficiency in MHC class II for such an analysis. Therefore, we blocked TCR–MHC class II interactions during development by adding antibody to MHC class II (anti–MHC class II) mice and found considerably fewer CD103+CD8αβ thymocytes in wild-type mice (anti–MHC class II) to fetal thymic organ cultures. The development of CD4+ T cells was blocked by treatment with anti–MHC class II, as expected (Fig. 4 and Supplementary Fig. 4). This served as a positive control showing that MHC class II molecules were indeed blocked by the addition of exogenous antibody. Since there were no cells of the CD4+ lineage being selected, we analyzed the development of the MHC-class-I-selected CD8+ T cells by staining cells with anti-CD69αβ and anti-TCRβ. Consistent with the results we obtained for intact 4KO mice, similar numbers of thymocytes of the CD8+ lineage were positively selected in wild-type and 4KO mice, even when MHC class II molecules were blocked with antibody. Together these data indicated that in the absence of specialized proteasomes, approximately normal numbers of polyclonal CD8+ T cells had phenotypic evidence of having undergone positive selection, whereas the development of their T cells with transgenic TCR expression was impaired at this first selection checkpoint.

**Lineage commitment of polyclonal 4KO CD8+ thymocytes**

Following positive selection, CD69αβ thymocytes upregulate their expression of CCR7, a chemokine receptor that enables the thymocytes to migrate to the thymic medulla. We found that CCR7+CD69αβCD103+ cells were present in similar numbers in wild-type thymus and 4KO thymus (Fig. 5a–d). This suggested that polyclonal CD8+ T cells in the 4KO thymus were not arresting during positive selection but had already successfully transited through this developmental checkpoint.

To further explore this issue, we investigated whether CD8+ T cells in the 4KO thymus were progressing through lineage commitment (a key event that occurs after positive selection) by analyzing the expression of Runx3d, a transcription factor that commits thymocytes to the CD8+ lineage both phenotypically and functionally. Published evidence has shown that in cells that require CD8 for signaling via the TCR, the developmental downregulation of CD8 expression interrupts TCR signaling, which in conjunction with thymic cytokines drives Runx3d expression and subsequent commitment of these cells to the CD8+ lineage. We transferred Runx3d(FP+) BM (with heterozygous expression of yellow fluorescent protein (YFP) from the distal promoter of Runx3) into wild-type, 4KO and B2m−/− hosts and quantified the YFP+ (Runx3d+) cells that developed in the three strains. Substantial numbers of Runx3d+ thymocytes developed...
in 4KO mice, significantly more than in B2m−/− mice (Fig. 5c). The total number of Runx3d+ cells was lower in 4KO mice than in wild-type mice, presumably because mature CD8SP (CD4+CD8+Runx3d+) T cells developed in wild-type thymus but not in 4KO thymus (data not shown). Consistent with that, the Runx3d expression on CD69+CD103+TCRβ+ cells from 4KO mice was not lower than that on their wild-type counterparts (Fig. 5f). Together these results suggested that polyclonal CD8+ T cells completed positive selection on MHC class I molecules in the 4KO thymus and then expressed medullary-homing chemokine receptors and successfully underwent lineage commitment.

Block in CD8+ thymocyte development at negative selection
As positively selected cells of the CD8+ lineage (CD4+CD8α-CD69+CD103+TCRβ+) progress in development, they upregulate their expression of CD8 and TCR to become CD4+CD8α-CD69+CD103+TCRβ+CCR7hi cells. This population of cells is committed to the CD8+ lineage20,26 and is subjected to negative selection37,38. Interestingly, we found that the number of CD4+CD8α-CD69+CD103+TCRβ+CCR7hi cells was significantly decreased (by 68%) in 4KO mice relative to their number in wild-type mice (Fig. 6a,b). The number of all TCRβ+CCR7hiCD8SP cells (normally found in the medulla)29, including both immature CD69+ cells and mature CD69− cells, was decreased by >90% in 4KO mice relative to their number in wild-type mice (Fig. 6a,c,d and Supplementary Fig. 5). Therefore, a block in the development of CD8+ T cells in the absence of specialized proteasomes subunits appeared to be occurring after positive selection and coincident with the final TCR–MHC class I checkpoint: negative selection.

We sought to further test that idea by analyzing whether impairing negative selection in 4KO mice would restore the development of CD8+ T cells. Therefore, we reconstituted 4KO mice with BM from mice deficient in the proapoptotic protein Bim (Bcl2l11−/−), a strain with a substantial defect in negative selection (although cells that fail to undergo positive selection still die normally)27,28. We found that the development of mature CD8+ T cells in the thymus of 4KO host mice given Bcl2l11−/− → WT mice (WT→WT) (Fig. 7a,b). The loss of Bim in Bcl2l11−/− → 4KO chimeras did not increase the number of CD8+ T cells to that observed in wild-type host mice given Bcl2l11−/− → B2m−/− (B2m−/− → WT) (instead, it was ~40% less) (Fig. 7a,b), but this was not unexpected, because some T cells might have been negatively selected by a Bim-independent pathway20 perhaps involving other pro-apoptotic factors, such as Puma, that contribute to negative selection39 and also disproporionately eliminate more cells in the 4KO thymus than in the wild-type thymus. Notably, however, there was a 22.7-fold ± 8.8-fold greater abundance of mature CD8SP thymocytes in Bcl2l11−/− → 4KO chimeras than in 4KO host mice given wild-type BM (WT→4KO), compared with a greater abundance of only 5.0-fold ± 2.3-fold in Bcl2l11−/− → WT chimeras relative to that in WT→WT chimeras (mean ± s.d.; Fig. 7c). In contrast, the changes in the number of CD4+CD8α- or CD4SP cells were much more modest; the increase in CD4SP cells in Bcl2l11−/− → 4KO chimeras (relative to that in WT→4KO chimeras) was actually less than that in the Bcl2l11−/− → WT chimeras (relative to that in WT→WT chimeras) (Supplementary Fig. 6).

The findings reported above were unexpected, because published studies of less-complete deficiency in specialized proteasomes (mice deficient in β5i only) have concluded that the block in CD8+ T cell development is at an earlier stage than negative selection5,10; however, in our opinion, those earlier findings did not definitively demonstrate that this is indeed the case. One study found that transplantation of MHC-class-I-deficient (B2m−/−) BM into irradiated Psmb11−/− mice did not restore the development of CD8+ T cells13. It was suggested that if the problem in these mice was due to excessive negative selection, then the loss of MHC class I on cells of hematopoietic origin should have ‘rescued’ thymocytes from negative selection; the finding that this did not occur led to the suggestion that the defect in these mice must be at an earlier stage. However, that was not a definitive conclusion, because B2m−/− hematopoietic cells also do not ‘rescue’ CD8SP cells (i.e., increase their numbers) in the wild-type thymus. This might be because B2m−/− hematopoietic cells can also present some peptides32 or because non-BM-derived cells express MHC class I and can either drive negative selection directly or transfer MHC class I complexes to hematopoietic cells (‘cross dressing’)33. Therefore,
DISCUSSION

Here we have shown that specialized proteasome subunits were more essential for the development of CD8⁺ T cells than previously appreciated from studies of the β5t-subunit-deficient mice. Moreover, impaired development of CD8⁺ T cells in Psmb11−/− mice was attributed solely to a defect in positive selection, analysis of 4KO mice revealed an additional major block around negative selection. A block at negative selection would be consistent with a peptide-switching model wherein most developing CD8⁺ T cells need to be selected on different peptides at positive selection and negative selection.\(^{14,15}\) In this model, the uniquely presented peptides (~30%) generated by thymoproteasomes allow the system to select host MHC-class-I-restricted TCRs on cTECs. Negative selection then occurs on peptides produced by constitutive proteasomes, immunoproteasomes and/or mixed proteasomes. These are the peptides that are presented in the

![Figure 6](image_url)

**Figure 6** 4KO thymocytes are lost during negative selection. (a) Flow cytometry of thymocytes from wild-type and 4KO mice (n = 9 mice (male and female) per genotype). Numbers in quadrants (far left) indicate percent CD4⁺CD8α⁺ cells (top right) or CD4⁺CD8α⁺ cells (bottom left); numbers above bracketed lines (middle left) indicate percent CD4⁺CD8α⁺ CD69⁺ cells; number adjacent to outlined areas (middle right) indicate percent CD4⁺CD8α⁺CD69⁺ TCRβ⁺CCR7⁺ thymocytes (left) or CD4⁺CD8α⁺ TCRβ⁺CCR7⁺ thymocytes (right); and numbers above bracketed lines (far right) indicate percent CD4⁺CD8α⁺ TCRβ⁺CCR7⁺ CD69⁺ cells (left) or CD4⁺CD8α⁺ TCRβ⁺CCR7⁺ CD69⁺ cells (right), all among total thymocytes. (b–d) Quantification of CD4⁺CD8α⁺CD69⁺ TCRβ⁺CCR7⁺ thymocytes (b), CD4⁺CD8α⁺CCR7hiCD69⁻ TCRβ⁺ thymocytes (c) and CD4⁺CD8α⁺CCR7⁺CD69⁻ TCRβ⁺ thymocytes (d) as in a. Each symbol (b–d) represents an individual mouse; small horizontal lines indicate the mean (± s.d.). *P = 0.0002 and **P < 0.0001 (Student's t-test). Data are representative of (a) or are pooled from (b–d) three experiments.

this model still had MHC class I molecules that could participate in negative selection. Other experiments have found that CCR7 deficiency (which prevents thymocytes from migrating to the medulla, where negative selection on mTECs occurs) does not ‘rescue’ the development of CD8⁺ T cells.\(^{13}\) However, negative selection also occurs in the thymic cortex\(^ {27,34}\) and therefore the block in development could still be caused by negative selection. Yet another study found that Bcl2l11−/− BM ‘rescued’ the development of some CD8⁺ T cells, but it was concluded that the magnitude of the ‘rescue’ was smaller than the authors expected for a defect at negative selection.\(^ {10}\) However, in that study, Bcl2l11−/− BM caused a proportionally greater increase in CD8SP cells in the Psmb11−/− thymus than in the wild-type thymus,\(^ {10}\) and in our current study, the increase in the 4KO thymus was even greater. Collectively, our findings that the block in CD8⁺ thymocyte development mapped to negative selection and that the development of CD8⁺ T cells could be ‘rescued’ by Bim-deficient cells suggested that the majority of CD8⁺ T cells that were positively selected in 4KO mice were failing to pass the negative-selection checkpoint (Supplementary Fig. 7).

**Figure 7** ‘Rescue’ of the development of CD8⁺ T cells in 4KO thymus by Bcl2l11−/− thymocytes. (a) Flow cytometry of thymocytes in various chimeras (above plots). Numbers in quadrants indicate percent cells in each. (b) Quantification CD4⁺CD8⁺ TCRβ⁺ thymocytes in WT→WT chimeras (n = 22), Bcl2l11−/−→WT chimeras (n = 16), WT→4KO chimeras (n = 16) and Bcl2l11−/−→4KO chimeras (n = 16) (male and female). (c) Quantification of CD4⁺CD8⁺ TCRβ⁺ thymocytes in Bcl2l11−/−→WT chimeras relative to that in WT→WT chimeras (WT), and in Bcl2l11−/−→4KO chimeras relative to that in WT→4KO chimeras (4KO). Each symbol (b,c) represents an individual mouse; small horizontal lines indicate the mean (± s.d.). *P < 0.05 and **P < 0.0001 (one-way ANOVA with Dunnett's multiple-comparison post-test (b) or Student's t-test (c)). Data are representative of (a) or are pooled from (b,c) five experiments.
periphery, and therefore it is essential to eliminate autoreactive T cells that recognize them. In the 4KO thymus, such peptide switching cannot occur and, consequently, both selection steps take place on peptides generated by constitutive proteasomes. While some unique peptides might come from proteins that are expressed only by cTECs, these unique proteasomes are rare and, without proteasome switching, they seem to be at most to select only the small number of CD8+ T cells found in 4KO mice. An analogous peptide-switching mechanism might also operate in the selection of CD4+ T cells in 4KO mice. A published study has found that a switch between B1i, B2i and B5i proteasomes in cTECs and B1i, B2i and B5c proteasomes elsewhere in the thymus results in a reduction of ~75% in the abundance of CD8SP cells (similar to the reduction in Psmb11−/− mice)10. However, there might be little peptide switching in this system, because the difference in antigen presentation by hematopoietic antigen-presenting cells that contain B1i, B2i and B5c proteasomes relative to that in their counterparts with B1i, B2i and B5i proteasomes is relatively minor. The uncertainty about how much peptide switching occurs in mice with partial deficiency in β-subunits is a limitation of these models.

The selection of thymocytes has been thought to be via a differential-affinity mechanism wherein positive selection is triggered by a TCR of lower affinity than that of the TCR that triggers negative selection. Such a mechanism is not mutually exclusive with the peptide-switching model, but peptide switching offers the advantage that it could in theory generate a larger TCR repertoire than that generated by differential affinity. This is because the window of permissible affinities could be much larger for the peptide-switching mechanism; TCRs of higher affinity that are positively selected on unique thymoproteasome-derived peptides would not subsequently encounter these same peptides at negative selection. The survival of TCRs of higher affinity would also increase the functionality of the T cell repertoire, because such TCRs are the most effective for immunity to pathogens40. On the other hand, in fetal thymic organ cultures, positive selection on some high-affinity agonist peptides results in T cells with abnormal phenotype and impaired function41. However, it is not clear whether this effect is seen with all high-affinity peptides and/or when such presentation is restricted to cTECs. In fact, functional high-affinity T cells are clearly positively selected in vivo, because they cause autoimmunity when negative selection is blocked42,43. It might be argued that the selection of high-affinity TCRs for unique thymoproteasome-generated peptides would put cTECs at risk of attack from mature T cells. However, cTECs lack the costimulatory molecules needed to activate T cells44,45 and, even more importantly, mature T cells do not traffic into the thymic cortex46.

The peptide-switching model might also explain why the development of OT-I and P14 cells in the 4KO thymus was blocked at positive selection. Such transgenically expressed TCRs that were originally positively selected on unique thymoproteasome peptides (in a wild-type thymus) would not encounter their positively-selecting peptides on 4KO cTECs. Polyclonal T cells do not suffer the same fate because this population begins with a random TCR repertoire.

The peptide-switching model raises a number of unanswered questions. Why do the peptides of higher affinity on cTECs not delete CD8+ T cells? This might be because negative selection in the thymic cortex occurs on dendritic cells and not cTECs44. Another question is how T cells that have been selected by unique thymoproteasome-generated peptides are maintained in the periphery. It has been suggested that the peptides that are needed to sustain CD8+ T cells in the periphery are the same ones on which the thymocytes were positively selected47,48. However, an essential role for positively selecting peptides has not been established for CD8+ T cell homeostasis, and if this were required, then presumably T cells selected by unique thymoproteasome-peptides would be sustained in the periphery on low-affinity, cross-reactive peptides.

Our observations would suggest that evolution of the thymoproteasome and immunoproteasome subunits might have been essential for the development of the CD8+ arm of adaptive immunity. This might explain why these subunits arose in phylogeny at the same point as the RAG recombination enzymes and the genes encoding TCRs that needed to be selected49. Our analysis of 4KO mice has revealed that the absence of specialized proteasome subunits in non-hematopoietic cells disrupted both positive selection and negative selection. These findings support the idea that peptide switching between positive selection and negative selection is important for the establishment of a broad TCR repertoire.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. All mouse strains were bred and maintained in specific pathogen–free conditions in the animal facilities at the University of Massachusetts Medical School. All experiments involving live animals were approved by and performed in accordance with guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institutional Animal Care and Use Committee. 

Psmb8−/− Psmb9−/− Psmb10−/− Psmb11−/− immunoproteasome- or thymoproteasome-deficient mice were created by breeding Psmb8−/− Psmb9−/− Psmb10−/− mice8 to Psmb11−/− mice5. All wild-type animals were C57BL/6J (Jackson Labs). B6;129P2-Runx3tm1Litt (Runx3dYFP/YFP (ref. 20)) mice were from NYU SOM, New York, NY. B6.SJL-PtprcaPep3b/BoyJ (CD45.1, Jackson Labs) C57BL/6-, B6.129P2-H2-Kbtm1H2-Dbtm1 (H-2Kb−/− H-2Db−/−, Taconic), B6.129S7-Rag1tm1Mom Tg(TcraTcrb)1100Mjb (Rag1−/− OT-I, Taconic), B6.Cg-Tcratm1Mom Tg(TcrLCMV)327Sdz (Tcra−/− P14, Taconic) were purchased from the indicated vendor and/or bred in the UMMS animal facility. Male and female mice were used in approximately equal numbers. All mice were between 6 and 12 weeks old, except BM chimeras, as described below.

BM chimeras. BM recipients (at least 8 weeks old) were irradiated with Cesium 137, in two rounds of 550 rad each, 5 h apart, using a Gammacell 40 irradiator (Atomic Energy of Canada). One day later, BM cells (isolated as previously described8) were injected retroorbitally. All mice received Sulfamethoxazole and Trimethoprim, Oral Suspension, USP 200 mg/40 mg per 5 mL in their drinking water for 6 weeks while engraftment took place.

Cell preparation and flow cytometry. Mice were sacrificed and spleen, inguinal lymph nodes and/or thymus were harvested. In the case of spleens, single-cell suspensions were isolated, erythrocytes were lysed using ACK buffer. In the case of thymus, the tissue was disrupted and passed through a 70-µm cell strainer (Costar). In thymic dissection, care was taken to exclude lymph nodes; the normal numbers of thymic CD4SP cells and absence of increased numbers of B cells (Supplementary Fig. 1) in the 4KO thymi verified that thymus preparations were not contaminated with lymph nodes. To analyze TECs, the tissue that remained in the strainer was digested using the Miltenyi Splenocyte isolation kit and the GentleMACS dissociator (using program m_spleen_02 before incubation at 37 °C and m_spleen_01 after). For TEC staining, 1:400 biotin-anti-CD249 (eBioscience) or 1:100 biotin-UEA-1 (Vector Labs) was added to the staining panel, followed by 1:400 V450 Streptavidin (BD Bioscience). Cells were stained for 20 min at 4 degrees using the antibodies and dilutions in Supplementary Table 1. For CCR7 staining, cells were first stained as described above, then incubated at 37deg for 30 min, with 1 µg biotin-anti-CD197 per 2,500,000 cells, followed by 1:200 Brilliant Violet 421 Streptavidin (BD Bioscience). Flow Cytometry was performed on a FACS Caliber or LSR II (Becton Dickenson), and data were compensated and analyzed using FlowJo (TreeStar). We used biexponential transformations (which affects only the visualization of the data but does not exclude data points) to plot the compensated data. Cell numbers for gated populations were calculated by multiplying total thymocyte number (counted by hemocytometer) by the frequency of that population in the flow cytometry sample.

Fetal thymic organ culture. E16-E17 embryos from timed pregnant mice were cultured in Costar transwell plates, essentially as previously described50. 500 µg M5/114 (anti–MHC class II) or control monoclonal antibody LTF-2 (both from Bio X Cell) was added to the culture medium for the 5 d of culture.

Statistical analysis. Statistical analysis of data (s.d., t-tests, one- and two-way ANOVA, as indicated in figure legends) was performed using GraphPad Prism version 5.0a for Mac OSX, (GraphPad Software, San Diego, California USA, http://www.graphpad.com). We used a pre-established criterion by which thymi with more than five times the percentage of CD8SP cells compared to comparable samples (indicating contamination with mature lymphocytes) were excluded. Samples size was based on past experience, and were sufficient to show significant differences. Animals were not randomly assigned. No investigator blinding was done.

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