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Thymoproteasome Shapes Immunocompetent Repertoire of CD8$^+$ T Cells

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SUMMARY

How self-peptides displayed in the thymus contribute to the development of immunocompetent and self-protective T cells is largely unknown. In contrast, the role of thymic self-peptides in eliminating self-reactive T cells and thereby preventing autoimmunity is well established. A type of proteasome, termed thymoproteasome, is specifically expressed by thymic cortical epithelial cells (cTECs) and is required for the generation of optimal cellularity of CD8$^+$ T cells. Here, we show that cTECs displayed thymoproteasome-specific peptide-MHC class I complexes essential for the positive selection of major and diverse repertoire of MHC class I-restricted T cells. CD8$^+$ T cells generated in the absence of thymoproteasomes displayed a markedly altered T cell receptor repertoire that was defective in both allogeneic and antiviral responses. These results demonstrate that thymoproteasome-dependent self-peptide production is required for the development of an immunocompetent repertoire of CD8$^+$ T cells.

INTRODUCTION

Most T lymphocytes are generated in the thymus. By entering the thymus and interacting with the microenvironment of the thymic cortex, lymphoid progenitor cells are induced to develop into thymocytes that express T cell receptor (TCR), as well as coreceptors CD4 and CD8 (double-positive, DP) (Scollay et al., 1988). Newly generated DP thymocytes that express a virgin set, or the germline repertoire, of TCRs are motile, seeking TCR engagement by interacting with peptide-major histocompatibility complex (MHC) expressed in the cortical microenvironment (Bousso et al., 2002; Li et al., 2007). DP thymocytes that receive weak signals of low-avidity (i.e., affinity × number per cell) TCR engagement are induced to survive and further develop into mature T cells that express large amounts of TCRs and either one of CD4 or CD8 (single-positive, SP) (Ashton-Rickardt et al., 1993, 1994; Hogquist et al., 1994; Sebzda et al., 1994; Takahama et al., 1994; Alam et al., 1996). This process is referred to as positive selection and is assumed to contribute to the enrichment of an immunocompetent, i.e., useful and self-protective, repertoire of self-MHC-restricted foreign-antigen-reactive T cells (Kisielow et al., 1988; von Boehmer, 1994; Allen, 1994; Starr et al., 2003). In contrast, DP thymocytes that receive strong signals of high-avidity TCR engagement are deleted, a process referred to as negative selection (Kappler et al., 1987; Palmer, 2003). It is well appreciated that negative selection is essential for eliminating self-reactive T cells and thereby preventing autoimmunity (Strasser, 2005; Siggs et al., 2006).

Unlike negative selection, the physiological and pathological importance of positive selection is still controversial. Positive selection was originally identified as the thymic process that determines the MHC-restriction specificity of T cells (Bevan, 1977; Zinkernagel et al., 1978) and is assumed to contribute to enriching an inherently rare T cell repertoire that is useful in the body harboring a given combination of MHC polymorphisms. However, it was shown that the germline TCR repertoire before positive and negative selection is inherently conserved to be MHC reactive (Zerrahn et al., 1997; Merkenschlager et al., 1997). It was also shown that a single MHC-peptide ligand identified in B lymphoma cells could induce positive selection of a diverse repertoire of T cells (Ignatowicz et al., 1996; Fukui et al., 1997). Based on these results, along with the structural analysis of TCR-MHC-peptide interactions, it is proposed that the specificity of TCR for peptides is not demanding during positive selection and that rather than positive selection, it is the subsequent negative selection that establishes the MHC-restriction specificity and the peptide specificity of peripheral T cells (Marrack and Kappler, 1997; Huseby et al., 2005; Dai et al., 2008; Huseby et al., 2008). However, those T cells generated in mice expressing single MHC-peptide ligands show markedly reduced cellularity and an unusual TCR repertoire that occasionally causes autoimmunity (Ignatowicz et al., 1996; Huseby et al., 2005; Oono et al., 2001). Thus, it is unclear whether the positive selection detectable in those single MHC-peptide-expressing mice represents positive selection occurring in the normal body. More importantly, it remains unanswered whether

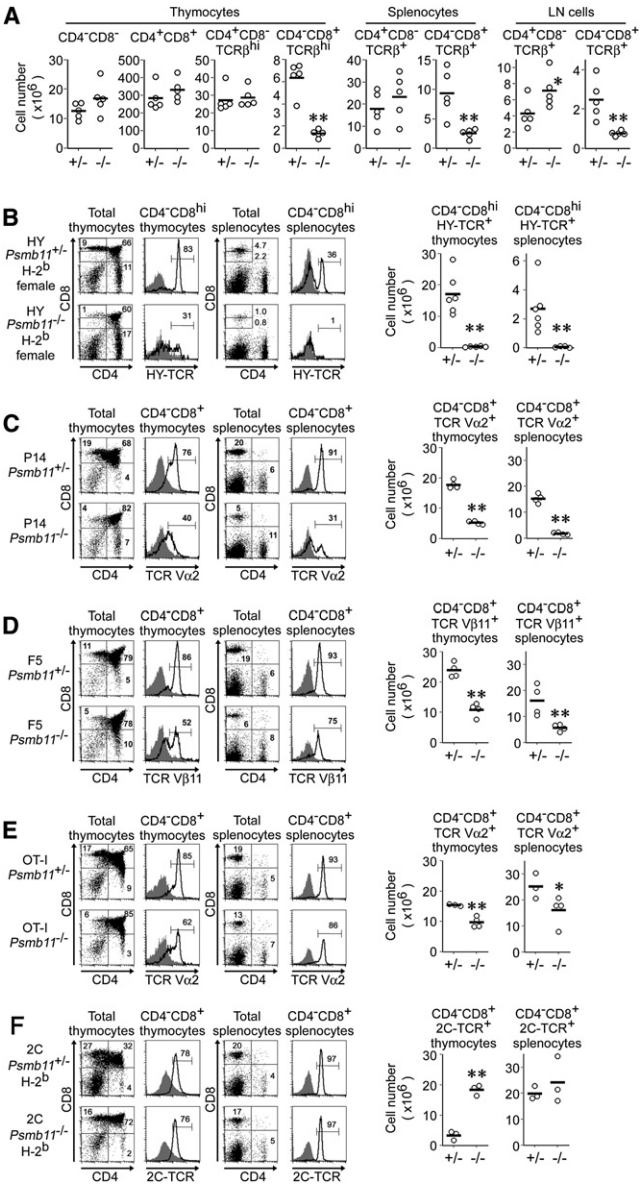

Figure 1. β5t Regulates Positive Selection of Diverse, but Not All, TCR Specificities of CD8⁺ T Cells

(A) Numbers (per mouse) of thymocytes, splenic T cells, and lymph node T cells in indicated populations were determined by flow cytometry in 3- to 6-week-old *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice. Data of individual mice (circles) and means (bars) are shown (n = 5).

(B–F) Thymocytes and splenocytes from HY-TCR-transgenic H-2^b female mice (B), as well as P14-TCR- (C), F5-TCR- (D), OT-I-TCR- (E), and 2C-TCR- (F) transgenic H-2^b mice, were analyzed by flow cytometry for CD4 and CD8. Histograms show TCR expression profiles (solid lines) obtained by staining with an antibody specific for HY-TCR (T3.70) (B), TCR Vα2 (C), TCR Vβ11 (D), TCR Vα2 (E), or 2C-TCR (1B2) (F), overlaid with control staining profiles (shaded lines), of the indicated cell populations. Numbers indicate percentage of cells within indicated areas. Graphs indicate cell numbers (per mouse) of indicated populations in individual mice (circles) and their means (bars) (n = 3 to 6). *p < 0.05; **p < 0.01. See also Figure S1.

self-peptides displayed in the thymus play a role in positive selection of an immunocompetent repertoire of T cells.

We previously identified β5t, a proteasome subunit that is specifically expressed in thymic cortical epithelial cells (cTECs) (Murata et al., 2007). Proteasomes are multicatalytic protease complexes that are responsible for the degradation of cytoplasmic proteins and the production of antigen peptides presented by MHC class I molecules (Brown et al., 1991; Rock et al., 1994). The β5 catalytic subunits of the proteasome are responsible for its chymotrypsin-like activity, producing peptides that possess at their carboxyl termini hydrophobic residues that can bind efficiently to MHC class I molecules (Heinemeyer et al., 1993; Fehling et al., 1994; Rock and Goldberg, 1999). β5t-containing proteasomes, termed thymoproteasomes, exhibit low chymotrypsin-like activity compared with the other types of proteasomes, i.e., β5-containing standard proteasomes or β5i-containing immunoproteasomes (Murata et al., 2007). Interestingly, β5t-containing cTEC-specific thymoproteasomes are essential for the generation of the optimal cellularity of CD8$^+$ T cells (Murata et al., 2007). However, the mechanism by which thymoproteasomes regulate T cell development has yet to be disclosed.

In this study, we examined how thymoproteasomes regulate T cell development. Our results showed that thymoproteasomes were essential for the positive selection of major and diverse, but not all, repertoire of CD8$^+$ T cells. We also found that thymoproteasomes conferred on cTECs the ability to express MHC class I -peptide complexes that were capable of generating major repertoire of CD8$^+$ T cells. cTECs in thymoproteasome-deficient mice compensatively assembled immunoproteasomes and expressed an altered set of MHC class I -peptide complexes that fail to positively select most repertoires of CD8$^+$ T cells. In addition, CD8$^+$ T cells generated in the absence of thymoproteasomes displayed an altered TCR repertoire that was defective in allogeneic and antiviral responses. Thus, this study reveals a unique role of cTEC-specific protein degradation that is essential for the cTEC-specific production of self-peptide-MHC class I complexes, and these complexes are required for the development of an immunocompetent and self-protective repertoire of CD8$^+$ T cells.

RESULTS

β5t Regulates Positive Selection of Major Repertoire of CD8⁺ T Cells

In β5t-deficient (*Psmb11*$^{-/-}$) mice, the numbers of CD8$^+$ T cells in the spleen and lymph nodes were markedly reduced to 27% and 31% (ratio between averages, n = 5), respectively, of those in normal mice (Figure 1A). The number of CD4$^-$CD8$^+$ single-positive (CD8SP) thymocytes was also reduced to 21% (n = 5) of the control, whereas the numbers of CD4$^-$CD8$^-$ double-negative (DN), CD4$^+$CD8$^+$ DP, and CD4$^+$CD8$^-$ (CD4SP) thymocytes were unchanged (Figure 1A), indicating that β5t specifically regulates the CD8 lineage, but not the CD4 lineage of T cell development beyond the DP stage. Other lineages of immune cells, including TCRγδ$^+$ cells, NK cells, NKT cells, macrophages, dendritic cells, B cells, and CD8αα$^+$ intraepithelial lymphocytes, showed no decreases in their numbers in *Psmb11*$^{-/-}$ mice (Figure S1A available online). In order to examine how β5t specifically affects CD8$^+$ T cell development, *Psmb11*$^{-/-}$ mice were crossed with TCR-transgenic mice. In HY-TCR-transgenic

mice, MHC class I H-2D^b-restricted male-antigen-specific TCR drives the positive and negative selection of CD8-lineage T cells in female and male H-2^b mice, respectively. We found that the generation of CD8SP thymocytes and splenic CD8$^+$ T cells in H-2^b female HY-TCR-transgenic mice was severely impaired by the lack of β5t (to 2.2% and 2.6%, respectively, of control cell numbers, n = 4–6) (Figure 1B). In contrast, β5t deficiency has no effect on the decreased numbers of DP thymocytes and CD8^{hi} splenic T cells in H-2^b male HY-TCR-transgenic mice (Figure S1B). Furthermore, the arrested T cell development at DP stage in null-selector H-2^d HY-TCR-transgenic mice was also unaltered by the absence of β5t (data not shown). Together, the results indicate that β5t affects positive selection rather than negative or null selection of HY-TCR-transgenic T cells. In contrast, the generation of CD4SP thymocytes and splenic CD4 T cells in I-A^b-restricted pigeon cytochrome c-specific AND-TCR-transgenic mice and I-A^b-restricted ovalbumin-specific OT-II-TCR-transgenic mice was not diminished in the absence of β5t (Figures S1F and S1G), indicating that β5t is dispensable for the positive selection of MHC class II -restricted TCR-transgenic T cells. β5t deficiency neither affected the negative selection of thymocytes in two additional MHC class I -restricted TCR-transgenic models nor caused any signs of autoimmune diseases in various organs (Figure S1C–S1E). These results indicate that the β5t-containing thymoproteasome specifically regulates positive selection, rather than negative selection, of CD8$^+$ T cells, rather than CD4$^+$ T cells.

Similar to HY-TCR-transgenic T cells, the numbers of splenic T cells in two other MHC class I -restricted TCR-transgenic mice, namely, lymphocytic choriomeningitis virus-specific P14-TCR-transgenic mice and influenza virus-specific F5-TCR-transgenic mice, were markedly reduced in the absence of β5t (12%, n = 4 and 36%, n = 4, respectively, relative to control, i.e., β5t$^+$ mice, Figures 1C and 1D). Accordingly, the generation of CD8SP thymocytes in these TCR-transgenic mice was severely impaired by the lack of β5t (Figures 1C and 1D), indicating that β5t regulates the positive selection of CD8$^+$ T cells with multiple TCR specificities. Interestingly, however, MHC class I -restricted ovalbumin-specific OT-I-TCR-transgenic and allogeneic H-2L^d-specific 2C-TCR-transgenic CD8$^+$ T cells were less severely affected in *Psmb11*$^{-/-}$ mice (Figures 1E and 1F). The number of OT-I-TCR-transgenic splenic CD8$^+$ T cells in *Psmb11*$^{-/-}$ mice was reduced to 64% (n = 3 to 4) of that in control OT-I-TCR-transgenic mice (Figure 1E). Most notable was that the number of 2C-TCR-transgenic splenic CD8$^+$ T cells in *Psmb11*$^{-/-}$ mice was 122% (n = 3) of that in control 2C-TCR-transgenic mice carrying β5t (Figure 1F). These results indicate that the development of CD8$^+$ T cells that express individual specificities of MHC class I -restricted TCRs is differentially dependent on β5t. Along with the finding that the majority of CD8$^+$ T cells are lost in *Psmb11*$^{-/-}$ mice (Figure 1A), these results also indicate that the β5t-containing thymoproteasome regulates the positive selection of major and diverse, but not all, repertoires of MHC class I -restricted CD8$^+$ T cells.

β5t Regulates cTEC-Mediated Positive Selection of CD8$^+$ T Cells within Thymic Cortex

To determine the cells that are responsible for the β5t-mediated regulation of T cell development, we examined positive selection among various TCR specificities in bone marrow chimeras (Figure 2A). To do so, hematopoietic progenitor cells from TCR-transgenic mice were reconstituted in irradiated *Psmb11*$^{-/-}$ mice. In the thymus of β5t-deficient mice, positive selection of HY-TCR-, P14-TCR-, and F5-TCR-transgenic T cells was markedly diminished, whereas positive selection of OT-I-TCR-transgenic and 2C-TCR-transgenic T cells was affected little (Figure 2A). Thus, the differential β5t dependence of positive selection among various TCR specificities was reproduced in these bone marrow chimeras reconstituted in the thymus of irradiated *Psmb11*$^{-/-}$ mice (Table 1). In contrast, β5t deficiency in bone marrow donor cells did not diminish the positive selection of HY-TCR-transgenic T cells in the thymus of β5t-sufficient mice (Figure 2A), indicating that β5t in nonhematopoietic stromal cells, but not bone marrow-derived hematopoietic cells, is responsible for positive selection of the repertoire of CD8SP thymocytes. Furthermore, the specific deficiency of β5t in cTEC-enriched thymic stromal cells markedly diminished the capability to induce the generation of CD8$^+$ T cells from isolated DP thymocytes of P14-TCR-transgenic mice, but not 2C-TCR-transgenic mice, in reaggregated fetal thymus organ culture (Figure 2B). Along with the results showing that β5t is exclusively expressed in cTECs (Murata et al., 2007 and data not shown), these results indicate that thymoproteasome-expressing cTECs regulate the positive selection of major and diverse, but not all, repertoire of CD8$^+$ T cells. The β5t independency of positive selection weakly correlated with the signaling intensity of transgenic TCR, which was measured by the amount of surface CD5 and CD8 expression (Figure S5).

In thymic medulla, newly generated SP thymocytes interact with various cells, including mTECs, which promiscuously express various tissue-specific antigens (Derbinski et al., 2001; Anderson et al., 2002; Kyewski and Derbinski, 2004). The CCR7-mediated migration of SP thymocytes to the medulla is essential to trim the cortically generated T cell repertoire to establish self-tolerance (Takahama, 2006; Kurobe et al., 2006; Nitta et al., 2009). Thus, it is possible that the β5t regulation of repertoire formation may involve the migration of positively selected thymocytes to the medulla and the negative selection induced in thymic medulla. However, we found that the development of CD8SP thymocytes was defective even in *Psmb11*$^{-/-}$ *Ccr7*$^{-/-}$ mice, similar to *Psmb11*$^{-/-}$ mice (Figure 2C), indicating that defective positive selection in the absence of β5t reflects neither CCR7-mediated migration of positively selected thymocytes nor negative selection in thymic medulla. We also found that the formation of thymic medulla including Aire-expressing medullary epithelial cells was not impaired in β5t-deficient mice (Figure S2). Thus, β5t-containing thymoproteasome regulates the positive selection of major CD8$^+$ T cell repertoires within thymic cortex without the contribution of subsequent negative selection in thymic medulla.

The reduced number of CD8$^+$ T cells in bone marrow chimeras reconstituted in irradiated *Psmb11*$^{-/-}$ mice was not markedly altered when bone marrow cells were isolated from mice deficient for β2-microglobulin (β2 m), a component of MHC class I molecules (Figure 2D). Thus, the impaired but detectable positive selection in β5t-deficient mice is not due to MHC class I -restricted antigen presentation by bone marrow-derived cells, including dendritic cells and T-lineage cells, which were

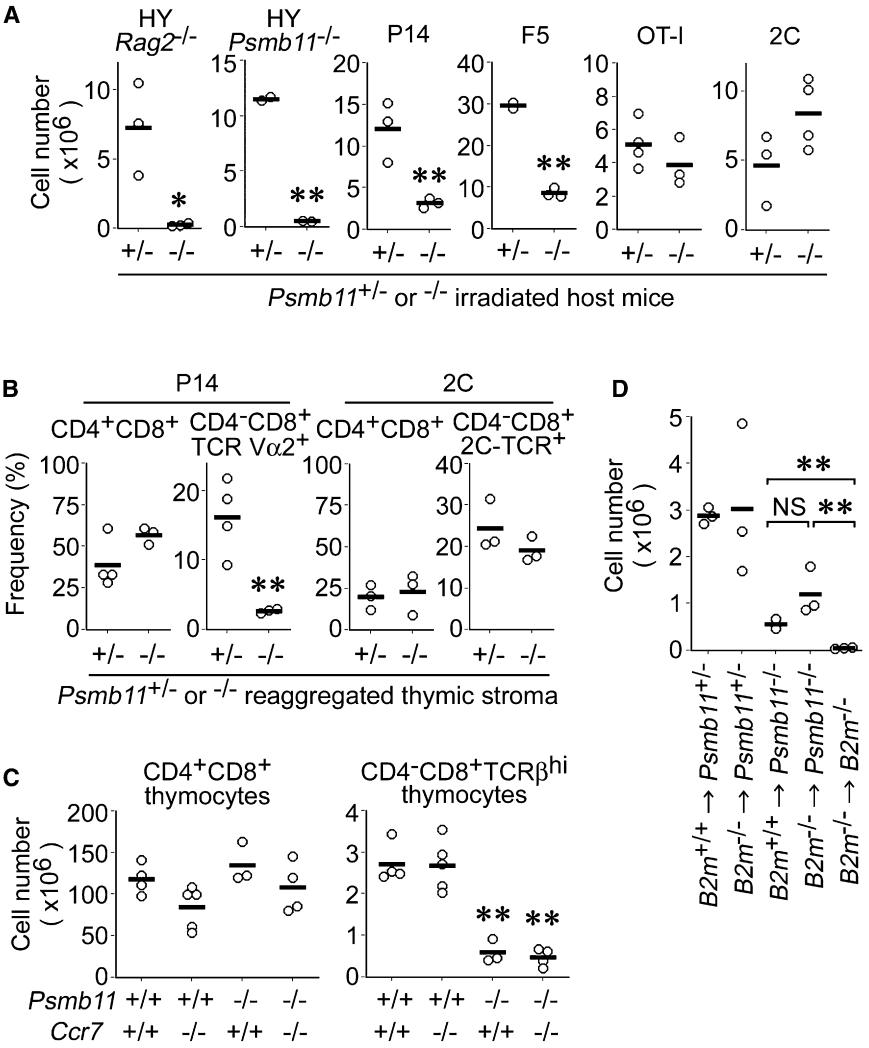

Figure 2. β5t in cTECs Regulates Positive Selection of Major TCR Specificities of CD8⁺ T Cells

(A) T cell-depleted bone marrow cells from indicated donor mice were transferred into lethally irradiated *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ H-2^{b} host mice. Thymocytes were analyzed 5 weeks after the reconstitution. Graphs indicate numbers (per mouse) of CD4^{-}CD8^{+}TCR^{hi} thymocytes in individual mice (circles) and their means (bars) (n = 2 to 4).

(B) CD4^{+}CD8^{+} thymocytes were isolated from P14-TCR-transgenic (left) or 2C-TCR-transgenic (right) mice with a cell sorter and reaggregated with *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ H-2^{b} fetal thymic stromal cells that contained 30%–40% cTECs, <3% mTECs, and 60%–70% non-TEC stromal cells, isolated from 2-deoxyguanosine-treated fetal thymus lobes. CD4^{+}CD8^{+} thymocytes before the culture had > 99% purity. The indicated cell populations were analyzed 4 days after reaggregated thymus organ culture. Graphs indicate percentages of indicated populations in individual reaggregated thymuses (circles) and their means (bars) (n = 3 to 4).

(C) Numbers of CD4^{+}CD8^{+} or CD4^{-}CD8^{+}TCRβ^{hi} thymocytes (per thymus lobe) in 4- to 6-week-old *Psmb11*$^{+/+}$*Ccr7*$^{+/+}$, *Psmb11*$^{+/+}$*Ccr7*$^{-/-}$, *Psmb11*$^{-/-}$*Ccr7*$^{+/+}$, or *Psmb11*$^{-/-}$*Ccr7*$^{-/-}$ mice.

(D) Thymocytes isolated from indicated bone marrow chimera mice were analyzed by flow cytometry for the expression of CD4, CD8, and TCRβ. Shown are the numbers of CD4^{-}CD8^{+}TCRβ^{high} thymocytes (n = 2 to 3). The number of CD8SP thymocytes in *Psmb11*$^{-/-}$ mice reconstituted with *B2m*$^{-/-}$ bone marrow cells (*B2m*$^{-/-}$ → *Psmb11*$^{-/-}$) was not significantly ($p \geq 0.05$) different from that in *Psmb11*$^{-/-}$ mice reconstituted with *B2m*$^{+/+}$ bone marrow cells (*B2m*$^{+/+}$ → *Psmb11*$^{-/-}$) but was significantly ($p < 0.01$) larger than that in *B2m*$^{-/-}$ mice reconstituted with *B2m*$^{-/-}$ bone marrow cells (*B2m*$^{-/-}$ → *B2m*$^{-/-}$), indicating that the generation of CD8SP thymocytes in *Psmb11*$^{-/-}$ mice was independent of bone-marrow-derived MHC class I molecules. *$p < 0.05$; **$p < 0.01$; NS, not significant. See also Figure S2.

previously shown to be capable of inducing positive selection under certain experimental conditions (Zinkernagel and Althage, 1999; Li et al., 2005; Choi et al., 2005; Kirberg et al., 2008). Rather, the reduced production of CD8^{+} T cells in *Psmb11*$^{-/-}$ mice is likely due to the defective and inefficient capability of thymoproteasome-deficient cTECs to induce positive selection.

cTECs Display a Thymoproteasome-Specific Set of MHC Class I-Associated Peptides

The surface expression of H-2K and H-2D MHC class I molecules, as well as I-A MHC class II molecules, on cTECs in *Psmb11*$^{-/-}$ mice was not markedly diminished (Figure 3A), although the surface expression of K^{b}, D^{b}, and L^{d} on β5t-deficient cTECs was slightly altered to 93% (n = 5), 89% (n = 5), and 103% (n = 3) of that in control cTECs carrying β5t (Table 2). Similar to cTECs from normal mice, β5t-deficient cTECs were fully capable of presenting SIINFEKL antigen peptide to stimulate OT-I-TCR-transgenic CD8^{+} T cells (Figure 3B). Thus, β5t-deficient cTECs are competent in presenting antigen peptides to MHC class I -restricted T cells. The majority of proteasomal β5-type subunits expressed in β5t-deficient cTECs were β5i, rather than β5 (Figure 3C), whereas β1i and β2i, rather than β1 or β2, were predominant in cTECs (Murata et al., 2007). Thus, unlike normal cTECs that predominantly express β5t-containing thymoproteasomes, β5t-deficient cTECs compensatively assemble β1i-, β2i-, and β5i-containing immunoproteasomes. Indeed, most MHC class I H-2L^{d} molecules expressed by cTECs exhibited the peptide-bound "folded" form (as detected by 30-5-7 antibody), rather than the peptide-empty "open" form (as detected by 64-3-7 antibody), irrespective of the presence or absence of β5t (Figure 3D). Thus, most MHC class I molecules expressed by cTECs in the presence or absence of β5t are associated with peptides, similarly to those expressed by other cells, such as mTECs.

In order to explore the nature of MHC class I -associated peptides in cTECs, we then examined the expression of epitopes

Table 1. Development of CD8$^+$ T Cells that Express Individual Specificities of MHC Class I -Restricted TCR Is Differentially Dependent on β5t

				% *Psmb11*$^{-/-}$/*Psmb11*$^{+/-}$[b]			
		MHC	Antigen	Thymocytes		Splenocytes	
TCR	Vα and Vβ	Restriction	Peptide[a]	Mice[c]	BMC[c]	Mice	BMC
Bulk	Bulk	Bulk	Bulk	21.0	19.6	27.3	17.8
HY	Vα17 Vβ8.2	D^b	Smcy	2.2	3.7	2.6	5.8
P14	Vα2 Vβ8.1	D^b	LCMV gp33	29.7	26.1	12.4	12.3
F5	Vα4 Vβ11	D^b	Flu NP68	45.3	28.9	35.5	14.9
OT-I	Vα2 Vβ5	K^b	Ovalbumin	63.4	76.0	63.9	56.0
2C[d]	Vα3 Vβ8.2	K^b	(L^d + α-KGDH)	553.2	181.1	121.7	47.7

[a] Smcy is a Y-chromosome-encoded H-Y antigen. LCMV gp33 is glycoprotein 33 of lymphocytic choriomeningitis virus. Flu NP68 is influenza virus nucleoprotein 68.
[b] 100 × (average CD8$^+$ SP T cell number in *Psmb11*$^{-/-}$ mice/average CD8$^+$ SP T cell number in *Psmb11*$^{+/-}$ mice).
[c] Mice, data obtained from TCR-transgenic mice as shown in Figure 1. BMC, data from bone marrow chimera mice as shown in Figure 2A.
[d] 2C-TCR-transgenic T cells in H-2^b mice are positively selected by K^b and are reactive to allogeneic L^d molecules that are associated with the endogenous protein α-ketoglutarate dehydrogenase (α-KGDH).

produced by the complexes of MHC class I molecules and limited varieties of peptides. We found that the expression of one of these epitopes directly detected by the TCR-like monoclonal antibody 25-D1.16 (Porgador et al., 1997; Mareeva et al., 2008), which recognizes a fraction of H-2K^b molecules associated with a population of peptides (Porgador et al., 1997), was markedly higher in cTECs from *Psmb11*$^{-/-}$ mice than in those from control mice (Figures 3E–3G). The surface expression of this epitope on mTECs, thymocytes, B cells, and dendritic cells was not different between *Psmb11*$^{-/-}$ and control mice (Figures 3E and 3F and Figure S3A), indicating that β5t specifically regulates the surface expression of this epitope on cTECs. Likewise, the cell-surface expression of another epitope of the H-2K^b complex associated with a different population of peptides, detected by 22-C5.9 (Porgador et al., 1997), was specifically altered in cTECs from *Psmb11*$^{-/-}$ mice compared to those from control mice (Table 2 and Figure S3B). The cTEC-specific difference in the expression of these epitopes between *Psmb11*$^{-/-}$ and control mice suggests that the repertoire of MHC class I -associated peptides expressed by cTECs is specifically regulated by β5t-containing thymoproteasomes and that cTECs in normal mice display a repertoire of MHC class I-associated peptides that are uniquely generated by thymoproteasomes.

Functionally Incompetent Repertoire of CD8$^+$ T Cells in β5t-Deficient Mice

Finally, we examined the function and repertoire of lymphopenic CD8$^+$ T cells that were generated in the absence of β5t. We found that CD8$^+$ T cells generated in *Psmb11*$^{-/-}$ mice were capable of proliferation and granzyme B production in response to TCR and CD28 stimulation (Figures 4A and 4B). OT-I-TCR-transgenic CD8$^+$ T cells generated in *Psmb11*$^{-/-}$ mice were fully capable of proliferating in the presence of SIINFEKL peptide (Figure 4C). Homeostatic proliferation of CD8$^+$ T cells isolated from *Psmb11*$^{-/-}$ mice was not impaired in vivo in both irradiated mice and RAG2-deficient (*Rag2*$^{-/-}$) mice (Figure 4D). Thus, CD8$^+$ T cells generated in the absence of thymoproteasomes are functionally potent to maintain in vivo survival in lymphopenic environments and to proliferate and become cytotoxic T-lymphocytes (CTL) in response to TCR stimulation.

CD8$^+$ T cells in *Psmb11*$^{-/-}$ mice exhibited increased frequency of cells that highly expressed CD44 and Ly6C (Figure 4E). The frequency of CD44^{hi} CD8$^+$ T cells in *Psmb11*$^{-/-}$ mice increased with the ontogeny (Figure 4F) and this increase coincided with the severer decrease in absolute number of CD44^{lo} CD8$^+$ T cells than CD44^{hi} CD8$^+$ T cells (Figure 4G). Both CD44^{lo} and CD44^{hi} CD8$^+$ T cells in *Psmb11*$^{-/-}$ mice were capable of proliferative response to TCR and CD28 stimulation (Figure 4H). However, CD44^{lo} CD8$^+$ T cells isolated from wild-type mice did not undergo excessive homeostatic expansion upon intravenous administration in *Psmb11*$^{-/-}$ mice, unlike in lymphopenic *Rag2*$^{-/-}$ mice (Figure 4I), suggesting that the increase in frequency of CD44^{hi} cells is unlikely due to the homeostatic expansion of CD44^{lo} cells and subsequent phenotype conversion into CD44^{hi} cells in *Psmb11*$^{-/-}$ mice. Thus, the marked in vivo persistence of CD44^{hi} memory-type CD8$^+$ T cells compared to CD44^{lo} naive CD8$^+$ T cells (Tanchot et al., 1997; Murali-Krishna et al., 1999) and possible defect in the maintenance of CD8$^+$ T cells generated in *Psmb11*$^{-/-}$ mice (for example, by defective TCR interactions with self MHC class I-peptide complexes because of the alteration in TCR repertoire) may contribute to the increased ratio of CD44^{hi} cells over CD44^{lo} cells in CD8$^+$ T cells of *Psmb11*$^{-/-}$ mice and the slightly elevated responses of CD8$^+$ T cells from *Psmb11*$^{-/-}$ mice.

Indeed, the distribution of TCR-Vβ and TCR-Vα in CD8$^+$ T cells was altered in *Psmb11*$^{-/-}$ mice (Figure 5A), whereas no substantial difference was detected in the TCR-V distribution in CD4$^+$ T cells between *Psmb11*$^{-/-}$ and control mice (Figure 5A), indicating that the T cell repertoire was altered specifically in CD8$^+$ T cells. Thus, CD8$^+$ T cells generated in *Psmb11*$^{-/-}$ mice carry undiminished TCR responsiveness but an altered TCR repertoire. The functional consequence of the altered repertoire of CD8$^+$ T cells in *Psmb11*$^{-/-}$ mice was examined by measuring immune response to foreign antigens. We found that CD8$^+$ T cells isolated from *Psmb11*$^{-/-}$ mice showed markedly diminished proliferative responses to allogeneic antigens (Figure 5B), which were dependent on β2 m-associated MHC class I

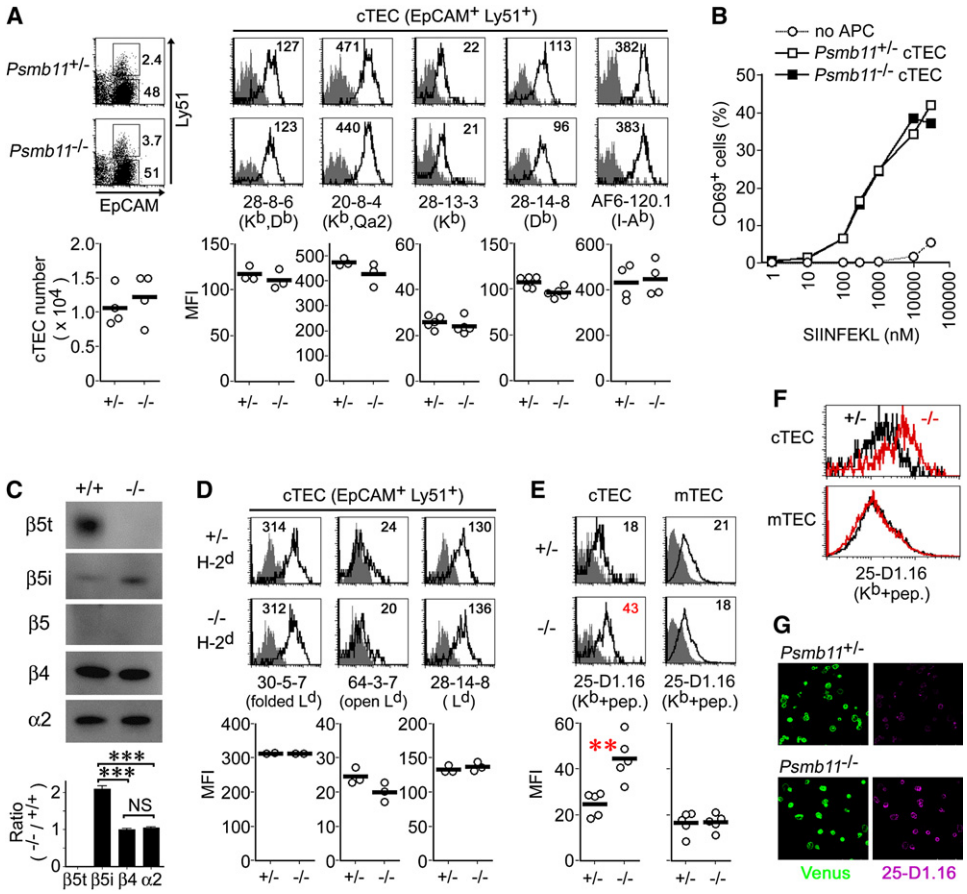

Figure 3. cTECs Produce β5t-Specific Peptide-MHC Class I Complexes

(A) Surface expression of MHC class I and class II molecules on cTECs from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ H-2^{b} mice. CD45^{-}TER119^{-} thymic stromal cells from 2-week-old mice were stained for EpCAM, Ly51, and indicated antibodies specific for MHC class I or class II molecules. Histograms show staining profiles (solid lines) overlaid with control staining profiles (shaded histograms) of EpCAM^{+}Ly51^{+} cTECs. Numbers in histograms indicate mean fluorescence intensity (MFI). Graphs show MFI of the histograms with indicated antibodies in individual measurements (circles) and their means (bars). Shown on the left are representative dot plots for EpCAM and Ly51 expression in CD45^{-} thymic stromal cells and the number of EpCAM^{+}Ly51^{+} cTECs in *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice. Numbers in dot plots indicate the frequency of cells within the box. The expression of D^{b} was significantly ($p < 0.05$) reduced in *Psmb11*$^{-/-}$ mice.

(B) CD8^{+} T cells from the spleen of OT-I-TCR-transgenic mice were cocultured in the absence or presence of CD45^{-}I-A^{+}Ly51^{+} cTECs from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ H-2^{b} mice. cTECs were pretreated with indicated concentrations of SIINFEKL peptide. Twenty hours later, the frequency of CD69^{+} cells in CD8^{+} T cells was analyzed by flow cytometry.

(C) Proteasomal components in β5t-deficient cTECs. Ly51^{+} cells were purified from the thymus of *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice, lysed, and subjected to immunoblot analysis with the indicated antibodies. β4 and α2 were used as controls. Means and standard errors (n = 3) of the ratios of the chemiluminescence signals in *Psmb11*$^{-/-}$ cTECs to those in *Psmb11*$^{+/+}$ cTECs are also shown. ***$p < 0.001$; NS, not significant. The signals for β5 were not detectable in cTECs from either *Psmb11*$^{+/+}$ or *Psmb11*$^{-/-}$ mice.

(D–F) CD45^{-}TER119^{-} thymic stromal cells were prepared from 2-week-old *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ H-2^{d} (D) or H-2^{b} (E) mice (F) and stained for EpCAM, Ly51, and indicated MHC class I molecules. Monoclonal antibodies used were 30-5-7 (specific for the peptide-bound "folded" form of L^{d}), 64-3-7 (specific for the peptide-empty "open" form of L^{d}), 28-14-8 (specific for L^{d} irrespective of peptide binding), and 25-D1.16 (specific for K^{b} associated with a limited variety of peptides). Histograms show staining profiles (solid lines) overlaid with control staining profiles (shaded histograms) of EpCAM^{+}Ly51^{+} cTECs or EpCAM^{+}Ly51^{-} mTECs (D and E). Staining profiles of EpCAM^{+}Ly51^{+} cTECs and EpCAM^{+}Ly51^{-} mTECs isolated from *Psmb11*$^{-/-}$ (red lines) and *Psmb11*$^{+/-}$ (black lines) mice are also indicated (F). Numbers in histograms indicate MFI. Graphs show the MFI of the histograms with indicated antibodies in individual measurements (circles) and their means (bars). **$p < 0.01$.

(G) Venus-expressing (green) cTECs isolated from β5t$^{+/-}$ or β5t$^{-/-}$ H-2^{b} mice were stained with 25-D1.16 (red).

See also Figure S3.

molecules (Figure 5C). The decrease in allogeneic response was not limited to a single combination of allogeneic stimulation but was shared by various allogeneic combinations (Figures 5B and 5C and Figure S4A). However, a certain combination of allogeneic response (H-2^{d} anti-H-2^{bq4}) of CD8^{+} T cells was not severely defective in *Psmb11*$^{-/-}$ mice (Figure S4A), in agreement with the possibility that CD8^{+} T cells generated in the absence of thymoproteasomes are functionally potent in response to TCR engagement but are defective in the formation of a functionally competent TCR repertoire. The allogeneic response of CD8^{+} T cells from *Psmb11*$^{-/-}$ mice was reduced even in isolated CD44^{lo} naive T cells (Figure S4B), suggesting that the defective

Table 2. Surface Expression of MHC Molecules on cTECs and mTEC in β5t-Deficient Mice

Mouse Examined	Target Molecule	Detecting Antibody	% *Psmb11*$^{-/-}$/*Psmb11*$^{+/-}$[a]	
			cTECs	mTECs
H-2^b	K^b, D^b	28-8-6	94.4	101.5
	K^b, Qa2	20-8-4	89.9	93.5
	K^b	28-13-3	93.4	105.0
	D^b	28-14-8	89.1	100.1
	K^b + peptides[b]	25-D1.16	180.2[b]	101.3
	K^b + peptides[b]	22-C5.9	139.1[b]	95.4
	I-A^b	AF6-120.1	103.3	102.6
H-2^d	L^d	28-14-8	102.8	100.3
	L^d (folded)[c]	30-5-7	99.8	95.6
	L^d (open)[c]	64-3-7	81.5	92.0

[a] MFI values of indicated molecules with indicated antibodies were measured by flow cytometry as shown in Figure 3. Shown are 100 × (average MFI in *Psmb11*$^{-/-}$ mice/average MFI in *Psmb11*$^{+/-}$ mice). For the analysis with 22-C5.9, cTECs and mTECs were *Psmb11*-driven Venus$^+$ and Venus$^-$ CD45$^-$ thymic cells.

[b] 25-D1.16 and 22-C5.9 are specific for K^b associated with a limited and mutually different variety of peptides (Porgador et al., 1997). The MFI values were significantly ($p < 0.05$) larger in *Psmb11*$^{-/-}$ cTECs than in *Psmb11*$^{+/-}$ cTECs.

[c] 30-5-7 is specific for the peptide-bound folded form of L^d, whereas 64-3-7 is specific for the peptide-empty open form of L^d (Lie et al., 1991).

CD8$^+$ T cell response in *Psmb11*$^{-/-}$ mice is due to the defective repertoire of naive CD8$^+$ T cells rather than the reduced frequency of naive cells in CD8$^+$ T cells. Finally, we found that upon influenza virus infection under the conditions where control mice could survive due to CD8$^+$ T cell responses, *Psmb11*$^{-/-}$ mice exhibited severe lethality (Figure 5D). These results indicate that CD8$^+$ T cells generated in the absence of thymoproteasomes are defective in mounting immune responses to allogeneic and viral antigens.

DISCUSSION

The present results demonstrate that cTECs display thymoproteasome-specific MHC class I-peptide complexes that are essential for the development of major and diverse repertoire of CD8$^+$ T cells. Thymoproteasome-deficient cTECs displayed altered MHC class I-peptide complexes that generated an altered TCR repertoire that was defective in allogeneic and antiviral responses. These results suggest that cTEC-specific production of MHC class I-associated self-peptides due to thymoproteasome-mediated protein degradation is essential for the development of an immunocompetent and self-protective CD8$^+$ T cell repertoire.

Chymotrypsin-like activity mediated by the β5 subunits of proteasomes is responsible for the production of peptides that carry carboxyl-terminal hydrophobic residues that efficiently associate with MHC class I molecules (Fehling et al., 1994; Rock and Goldberg, 1999). No carboxypeptidases other than proteasomes are detectable in the cells except lysosomes, whereas various aminopeptidases in the cytoplasm and the endoplasmic reticulum are involved in trimming the amino termini of proteasome-generated peptides (Reits et al., 2003; Yewdell et al., 2003). Indeed, naturally processed self-peptides eluted from many alleles of MHC class I molecules are highly biased to possess hydrophobic amino acids, and basic residues to a less extent, at the carboxyl terminus (Falk et al., 1991; Hunt et al., 1992; Young et al., 1995). On the other hand, β5t-containing thymoproteasomes exhibit selectively reduced chymotrypsin-like activity but normal trypsin-like and caspase-like activities, compared with other types of proteasomes, i.e., β5-containing standard proteasomes and β5i-containing immunoproteasomes (Murata et al., 2007). It is therefore reasonable to speculate that the specifically reduced chymotrypsin-like activity in thymoproteasomes is responsible for the generation of the cTEC-specific repertoire of cytoplasmic peptides. Even though the TAP complex, which is responsible for transporting cytoplasmic peptides into the lumen of the endoplasmic reticulum, prefers the hydrophobic carboxyl termini of the peptides (Momburg et al., 1994; Uebel et al., 1997; Burgevin et al., 2008), our results show that most of the MHC class I molecules expressed on the surface of cTECs irrespective of β5t expression are associated with the peptides rather than being peptide-empty. Thus, the thymoproteasome-mediated production of a cTEC-specific repertoire of cytoplasmic peptides likely results in the production of a unique repertoire of MHC class I-associated peptides specifically displayed by cTECs.

With regard to the nature of MHC class I-bound peptides expressed by cTECs, current technology of mass spectrometry analysis (which requires 10^8 to 10^9 cells) does not readily allow us to directly identify the sequences of MHC-bound peptides expressed by isolated cTECs (approximately 1×10^4 cells can be isolated per mouse). However, the present results obtained by the two different TCR-like monoclonal antibodies show that the expression of the epitopes, likely produced by the complexes of MHC class I molecules and endogenously produced peptides, was markedly and specifically altered in cTECs from β5t-deficient mice compared to those from control mice, suggesting that cTECs display a thymoproteasome-specific set of MHC class I-associated peptides. The peptides expressed by cTECs may be unique to cTECs and different from the peptides expressed by other cells because of the unique enzymatic activity of cTEC-specific thymoproteasome. Alternatively, the decreased chymotrypsin-like activity of thymoproteasomes may uniquely limit the variety of MHC class I-associated peptides displayed by cTECs. It is also possible that these antibodies may recognize a conformational status of K^b, which may be increased specifically in cTECs in the absence of β5t but be independent of the variety of associated peptides.

Our results show that the positive selection of various transgenic TCR is differentially affected by β5t deficiency in multiple magnitudes rather than in an all-or-none manner. It is thus speculated that the positive selection of a single TCR specificity is induced by multiple (thymoproteasome-dependent and thymoproteasome-independent) peptides associated with MHC class I molecules. A major fraction of positively selecting peptides may be uniquely generated by thymoproteasomes in cTECs, whereas some positively selecting peptides may be additionally generated in a thymoproteasome-independent manner.

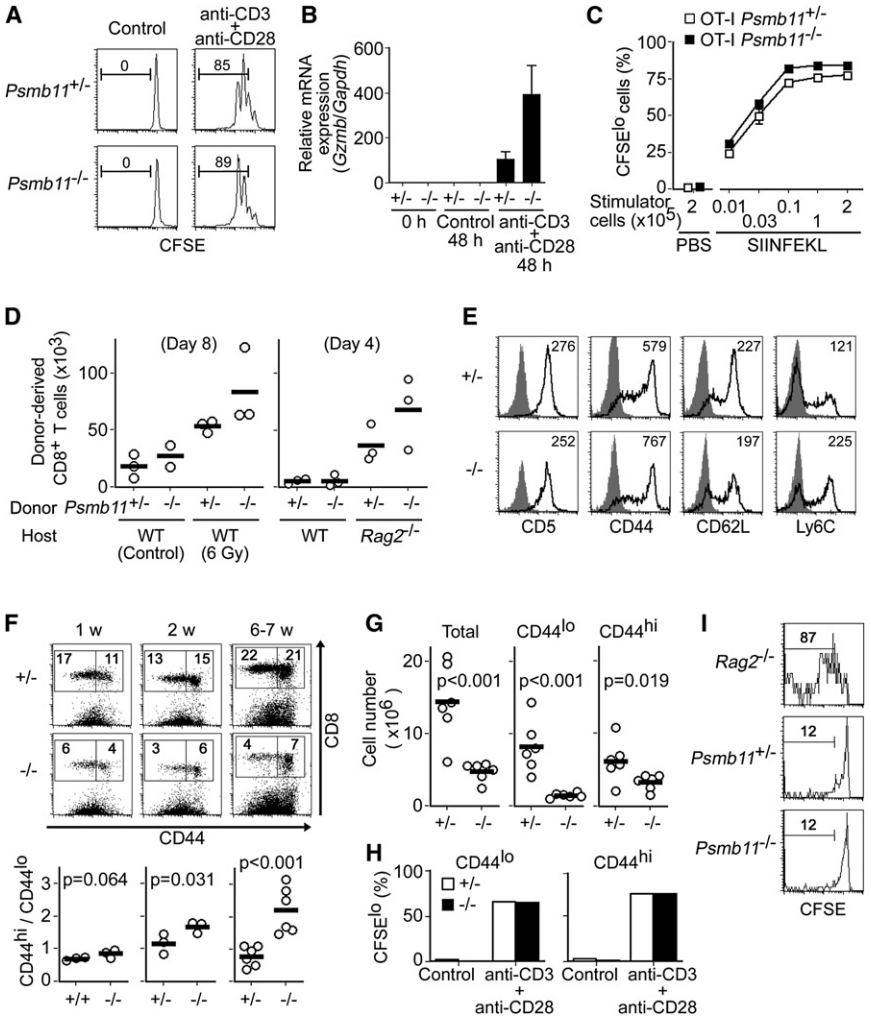

Figure 4. CD8$^+$ T Cells Generated in β5t-Deficient Mice Are Functionally Potent to Proliferate and Become Cytotoxic T-Lymphocytes in Response to TCR Stimulation In Vitro and to Maintain Survival In Vivo

(A and B) Splenic CD8$^+$ T cells were purified from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice, labeled with CFSE, and cultured with or without plate-bound anti-CD3 and anti-CD28 for 48 hr. (A) Histograms show CFSE fluorescence profiles, and numbers indicate the frequency of CFSE^{lo} cells. (B) mRNA expression of granzyme B determined by quantitative RT-PCR analysis was normalized to GAPDH mRNA expression, and those in *Psmb11*$^{+/-}$ CD8$^+$ T cells before the culture were set to 1.

(C) Splenic CD8$^+$ T cells (1 × 10^5) from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ OT-I-TCR-transgenic H-2^b mice were labeled with CFSE and cultured with PBS- or SIINFEKL-loaded C57BL/6 splenocytes for 40 hr. Graphs show means ± standard errors of the frequency of CFSE^{lo} cells (n = 3).

(D) Splenic CD8$^+$ T cells from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice were labeled with CFSE and intravenously injected into nonirradiated (control) or irradiated (6 Gy) wild-type mice or nonirradiated *Rag2*$^{-/-}$ mice (1 × 10^6 cells/host mouse). Graphs indicate numbers of CD8$^+$CFSE$^+$ splenocytes in individual mice (circles) and their means (bars) (n = 2 to 3).

(E) The expression of CD5, CD44, CD62L, and Ly6C in CD8$^+$TCRβ$^+$ spleen cells from β *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice was analyzed by flow cytometry. Numbers in histograms indicate MFI.

(F) The expression of CD44 and CD8 in TCRβ$^+$ spleen cells from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice at indicated ages was analyzed by flow cytometry. Numbers in dot plots indicate the frequency of cells within the box. Graphs indicate the ratios of the numbers of CD44^{hi} CD8$^+$ T cells over CD44^{lo} CD8$^+$ T cells in individual mice (circles) and their means (bars, n = 3–6). *p < 0.05.

(G) Absolute numbers (circles) and their means (bars, n = 6) of total, CD44^{lo}, and CD44^{hi} subsets of CD8$^+$TCRβ$^+$ spleen cells in *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice at 6 to 7 weeks old were analyzed by flow cytometry.

(H) CD44^{lo} and CD44^{hi} CD8$^+$ T cells isolated from the spleen of *Psmb11*$^{+/-}$ (open bars) or *Psmb11*$^{-/-}$ (closed bars) mice were labeled with CFSE and cultured with or without plate-bound anti-CD3 and anti-CD28 antibodies for 48 hr. Graphs indicate the frequency of CFSE^{lo} cells.

(I) CD44^{lo} CD8$^+$ T cells (5 × 10^6) from wild-type mice were CFSE-labeled and intravenously administered into nonirradiated *Rag2*$^{-/-}$, *Psmb11*$^{+/-}$, or *Psmb11*$^{-/-}$ mice. Histograms show representative CFSE fluorescence profiles (n ≥ 5), and numbers indicate the frequency of CFSE^{lo} cells.

It is also interesting to note that the signaling intensity of transgenic TCR, which is measured by the amount of surface CD5 and CD8 expression (Park et al., 2007), weakly correlated with the presumable β5t independency of positively selecting ligands, suggesting that positively selecting peptides generated in cTECs by thymoproteasomes tend to exhibit low TCR signaling intensity. It is possible that the MHC class I -associated self-peptides uniquely displayed by cTECs may be rich in peptides that exhibit low-avidity TCR engagement, as previously suggested (Ashton-Rickardt et al., 1994). It should be emphasized that the order of β5t independency among the TCR-transgenic models (HY < P14 < F5 < OT-I < 2C) is not identical to the order of the TCR signaling intensity (HY < F5 < 2C ≤ P14 < OT-I) (Figure S5; Ernst et al., 1999; Ge et al., 2004; Park et al., 2007; Agenès et al., 2008), arguing against the possibility that β5t regulates the production or activity of a general costimulus for positive selection. Indeed, our results showing that β5t-deficient cTECs are fully capable of presenting various concentrations of antigen peptide to stimulate CD8$^+$ T cells contradict such possibility.

The present results also show that β5i-containing immunoproteasomes appear to be the dominant cellular proteasome in cTECs of β5t-deficient mice. Immunoproteasomes are able to produce a set of peptides that efficiently associate with MHC class I molecules at the carboxyl terminus (Rock and Goldberg, 1999). Indeed, our results suggest that cTECs in β5t-deficient mice express the MHC class I -peptide complex, whereas the altered set of MHC class I-peptide complex expressed by thymoproteasome-deficient and thereby immunoproteasome-dominant cTECs is inefficient in inducing the positive selection of major CD8$^+$ T cell repertoires. Thus, in order to generate an optimum CD8$^+$ T cell repertoire, cTECs may have to display a repertoire of MHC class I-bound peptides that inefficiently

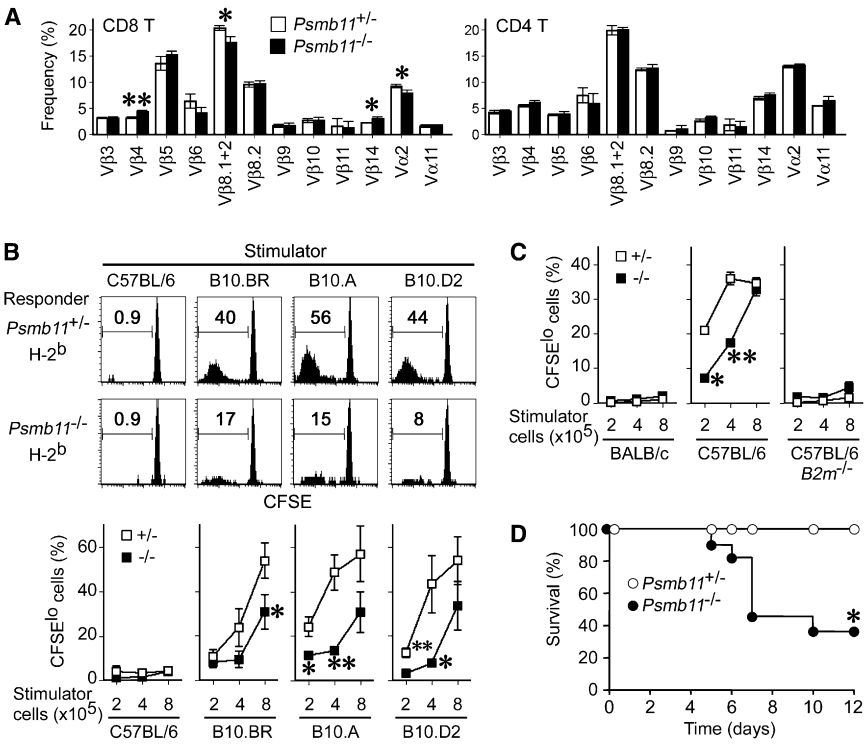

Figure 5. Altered TCR Repertoire and Defective Immune Responses of CD8$^+$ T Cells in β5t-Deficient Mice

(A) TCR-Vβ and TCR-Vα distribution in CD8$^+$TCRβ$^+$ spleen cells (CD8$^+$ T cells) or CD8$^-$TCRβ$^+$ spleen cells (CD4 T cells) from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ mice was determined by flow cytometry. Graphs show means ± standard errors of three independent measurements.

(B and C) Splenic CD8$^+$ T cells (1 × 10^5) from *Psmb11*$^{+/-}$ or *Psmb11*$^{-/-}$ H-2^b (B) or H-2^d (C) mice were labeled with CFSE and cocultured with irradiated splenocytes (2 ×, 4 ×, or 8 × 10^5) from indicated mouse strains for 4 days. Histograms show representative CFSE fluorescence profiles of CD8$^+$ T cells cultured with 4 × 10^5 stimulator cells. Numbers in histograms indicate the frequency of CFSE^{lo} cells. Graphs show means ± standard errors of at least three independent measurements.

(D) Eleven *Psmb11*$^{-/-}$ mice (closed circles) and 6 *Psmb11*$^{+/-}$ mice (open circles) were infected with 1,000 PFU of A/PR8 virus. Survival was monitored up to 12 days after the infection. Data were pooled from two experiments. The Mann-Whitney nonparametric *U*-test was used to compare survival between groups of mice. *p < 0.05; **p < 0.01.

See also Figure S4.

associate with MHC class I molecules and tend to provide low TCR signaling intensity.

Our results show that CD8$^+$ T cells in β5t-deficient mice are defective in allogeneic and antiviral responses. Upon TCR stimulation, CD8$^+$ T cells in β5t-deficient mice were capable of proliferation and granzyme B production. On the other hand, the TCR repertoire in V regions of CD8$^+$ T cells was altered in β5t-deficient mice. Allogeneic responses of CD8$^+$ T cells in β5t-deficient mice were defective in various, but not all, allogeneic combinations and were defective even when CD8$^+$ T cells were isolated into naive T cells. Thus, we think that the defect in immune responses of β5t-deficient mice is due to an altered repertoire of CD8$^+$ T cells generated in the absence of β5t-containing thymoproteasome. It is remarkable that the thymoproteasome, which may affect the MHC class I-associated peptide repertoire specifically in cTECs, is essential for shaping the immunocompetent TCR repertoire of CD8$^+$ T cells.

We found that the immune protection of β5t-deficient mice from influenza virus infection is defective. Cytotoxic T lymphocyte (CTL) activity was observed in mice from day 5 to day 15 after influenza virus infection (Kedzierska et al., 2006; Stambas et al., 2007) and that virus-specific CD8 CTLs are the key effectors of virus clearance in mice infected with influenza virus (Bender et al., 1992; Mozdzanowska et al., 1997; Doherty et al., 1997). Thus, we think that the death of β5t-deficient mice infected with influenza virus is a result of incompetent CTL responses in the mice due to an altered repertoire of CD8$^+$ T cells.

Based on the analysis of mice expressing B cell lymphoma-derived single MHC peptides, it was previously assumed that any peptide that causes low-avidity TCR engagement can trigger positive selection of thymocytes and that rather than positive selection, subsequent negative selection establishes repertoire formation of T cells (Ignatowicz et al., 1996; Huseby et al., 2005). It was additionally shown that the ability to induce positive selection experimentally was not limited to cTECs but also included fibroblasts and T-lineage cells (Pawlowski et al., 1993; Hugo et al., 1993; Zinkernagel and Althage, 1999; Martinic et al., 2003; Choi et al., 2005; Li et al., 2005). In fact, if positive selection should select developmental thymocytes solely according to low-avidity TCR engagement, any MHC-peptide complex expressed by any cell type could support the generation of a full repertoire of T cells. However, our results demonstrate that the development of an immunocompetent T cell repertoire requires positive selection by thymoproteasome-dependent MHC class I -peptide complexes specifically expressed by cTECs. Thus, the small number and unusual repertoire of T cells positively selected by the single MHC-peptides (Ignatowicz et al., 1996; Fukui et al., 1997) may resemble abnormal positive selection and incompetent T cell development detectable in β5t-deficient mice. Accordingly, similar to β5t-deficient cTECs, fibroblasts and hematopoietic cells can only induce positive selection of only a limited repertoire of T cells, such as 2C-TCR-transgenic T cells, but not HY-TCR-transgenic T cells (Zerrahn et al., 1999; Lilić et al., 2002). Instead, our results support the idea that self-peptides expressed by cTECs critically contribute to the establishment of an immunocompetent T cell repertoire (Singer et al., 1986).

The unique peptide-producing activity of cTECs may not be limited to MHC class I-associated peptides but also occur in MHC class II -associated peptides (Takahama et al., 2008), since cathepsin L and thymus-specific serine protease, which are lysosomal proteases that are highly expressed by cTECs, are required for the optimal generation of the CD4 T cell repertoire

(Nakagawa et al., 1998; Honey et al., 2002; Gommeaux et al., 2009). A recent report also showed that thymic epithelial cells, including cTECs, have high constitutive activity of autophagy, which contributes to MHC class II peptide-mediated T cell repertoire formation and self-tolerance (Nedjic et al., 2008). The unique features of cTECs in protein degradation and self-peptide presentation may govern the positive selection of T cells in both CD4 and CD8 lineages.

The present study reveals that positive selection induced by thymoproteasome-expressing cTECs is essential for the formation of an immunocompetent and self-protective repertoire of $CD8^+$ T cells. In the absence of thymoproteasome-mediated positive selection, mice exhibited severe defects in the allogeneic responses of $CD8^+$ T cells and in the survival to influenza virus infection. On the other hand, we detected no signs of autoimmunity in thymoproteasome-deficient mice. Thus, positive selection induced by thymoproteasomes and cTECs specifically governs the installment of diverse repertoire of $CD8^+$ T cells, which is important for rejecting allogeneic tissues and eradicating virus-infected cells, rather than the establishment of a self-tolerant T cell repertoire, which is separately governed by other mechanisms, including Aire-associated promiscuous gene expression by mTECs (Derbinski et al., 2001; Anderson et al., 2002). Together, this study suggests that positive selection induced by a thymoproteasome-specific repertoire of self-peptides is essential for the installment of an immunocompetent and self-protective repertoire of $CD8^+$ T cells. We propose that the cTEC expression of thymoproteasomes be considered for the therapeutic reconstitution of T cells by transplantation or regeneration of thymic stromal cells in the clinical setting.

EXPERIMENTAL PROCEDURES

Mice

The list of mice used in this study and the procedure for preparation of irradiated bone marrow chimeras are described in Supplemental Experimental Procedures. Animal experiments were performed after obtaining approval from the Animal Experimentation Committee of the University of Tokushima.

Flow Cytometry Analysis and Cell Sorting

Multicolor flow cytometry analysis and cell sorting were performed with FACS-Calibur and FACS-Vantage (BD Bioscience), as described previously (Ueno et al., 2005). Thymic stromal cells were prepared by digesting thymic fragments with collagenase, dispase, and DNase I (Roche), as described previously (Gray et al., 2006; Hikosaka et al., 2008). To sort thymic epithelial cells, thymic stromal cells were enriched by depleting $CD45^+$ cells and $TER119^+$ cells using magnetic-bead-conjugated antibodies specific for CD45 and TER119 (Miltenyi Biotec) prior to FACS cell sorting.

Reaggregated Thymus Organ Culture

The procedures for fetal thymus organ culture and reaggregated thymus organ culture were described previously (Ueno et al., 2005). DP thymocytes were sorted from adult TCR transgenic mice (purity >99%). Thymic stromal cells were prepared from E15.5 fetal thymus lobes that were cultured for 5 days in the presence of 2-deoxyguanosine. DP thymocytes (5×10^5) and thymic stromal cells (5×10^4) were reaggregated and organ-cultured for 4 days.

Analysis of Proteasomal Subunits

$Ly51^+$ cells were magnetically enriched from adult thymus using biotin-conjugated antibody specific for Ly51 and streptavidin-conjugated magnetic beads. Cells with >90% purity were lysed and subjected to immunoblot analysis.

Additional Experimental Procedures

See Supplemental Experimental Procedures for additional experimental procedures.

SUPPLEMENTAL INFORMATION

The Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2009.10.009.

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REFERENCES

Agenès, F., Dangy, J.P., and Kirberg, J. (2008). T cell receptor contact to restricting MHC molecules is a prerequisite for peripheral interclonal T cell competition. J. Exp. Med. *205*, 2735–2743.

Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., and Gascoigne, N.R. (1996). T-cell-receptor affinity and thymocyte positive selection. Nature *381*, 616–620.

Allen, P.M. (1994). Peptides in positive and negative selection: a delicate balance. Cell *76*, 593–596.

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395–1401.

Ashton-Rickardt, P.G., Van Kaer, L., Schumacher, T.N., Ploegh, H.L., and Tonegawa, S. (1993). Peptide contributes to the specificity of positive selection of $CD8^+$ T cells in the thymus. Cell *73*, 1041–1049.

Ashton-Rickardt, P.G., Bandeira, A., Delaney, J.R., Van Kaer, L., Pircher, H.P., Zinkernagel, R.M., and Tonegawa, S. (1994). Evidence for a differential avidity model of T cell selection in the thymus. Cell *76*, 651–663.

Bender, B.S., Croghan, T., Zhang, L., and Small, P.A., Jr. (1992). Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. *175*, 1143–1145.

Bevan, M.J. (1977). In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells. Nature *269*, 417–418.

Bousso, P., Bhakta, N.R., Lewis, R.S., and Robey, E. (2002). Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. Science *296*, 1876–1880.

Brown, M.G., Driscoll, J., and Monaco, J.J. (1991). Structural and serological similarity of MHC-linked LMP and proteasome (multicatalytic proteinase) complexes. Nature *353*, 355–357.

Burgevin, A., Saveanu, L., Kim, Y., Barilleau, E., Kotturi, M., Sette, A., van Endert, P., and Peters, B. (2008). A detailed analysis of the murine TAP transporter substrate specificity. PLoS ONE *3*, e2402. 10.1371/journal.pone.0002402.

Choi, E.Y., Jung, K.C., Park, H.J., Chung, D.H., Song, J.S., Yang, S.D., Simpson, E., and Park, S.H. (2005). Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. Immunity *23*, 387–396.

Dai, S., Huseby, E.S., Rubtsova, K., Scott-Browne, J., Crawford, F., Macdonald, W.A., Marrack, P., and Kappler, J.W. (2008). Crossreactive T Cells

spotlight the germline rules for alphabeta T cell-receptor interactions with MHC molecules. Immunity *28*, 324–334.

Derbinski, J., Schulte, A., Kyewski, B., and Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat. Immunol. *2*, 1032–1039.

Doherty, P.C., Topham, D.J., Tripp, R.A., Cardin, R.D., Brooks, J.W., and Stevenson, P.G. (1997). Effector $CD4^+$ and $CD8^+$ T-cell mechanisms in the control of respiratory virus infections. Immunol. Rev. *159*, 105–117.

Ernst, B., Lee, D.S., Chang, J.M., Sprent, J., and Surh, C.D. (1999). The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. Immunity *11*, 173–181.

Falk, K., Rötzschke, O., Stevanović, S., Jung, G., and Rammensee, H.G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature *351*, 290–296.

Fehling, H.J., Swat, W., Laplace, C., Kühn, R., Rajewsky, K., Müller, U., and von Boehmer, H. (1994). MHC class I expression in mice lacking the proteasome subunit LMP-7. Science *265*, 1234–1237.

Fukui, Y., Ishimoto, T., Utsuyama, M., Gyotoku, T., Koga, T., Nakao, K., Hirokawa, K., Katsuki, M., and Sasazuki, T. (1997). Positive and negative $CD4^+$ thymocyte selection by a single MHC class II/peptide ligand affected by its expression level in the thymus. Immunity *6*, 401–410.

Ge, Q., Bai, A., Jones, B., Eisen, H.N., and Chen, J. (2004). Competition for self-peptide-MHC complexes and cytokines between naive and memory $CD8^+$ T cells expressing the same or different T cell receptors. Proc. Natl. Acad. Sci. USA *101*, 3041–3046.

Gommeaux, J., Grégoire, C., Nguessan, P., Richelme, M., Malissen, M., Guerder, S., Malissen, B., and Carrier, A. (2009). Thymus-specific serine protease regulates positive selection of a subset of $CD4^+$ thymocytes. Eur. J. Immunol. *39*, 956–964.

Gray, D.H., Seach, N., Ueno, T., Milton, M.K., Liston, A., Lew, A.M., Goodnow, C.C., and Boyd, R.L. (2006). Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. Blood *108*, 3777–3785.

Heinemeyer, W., Gruhler, A., Möhrle, V., Mahé, Y., and Wolf, D.H. (1993). PRE2, highly homologous to the human major histocompatibility complex-linked RING10 gene, codes for a yeast proteasome subunit necessary for chrymotryptic activity and degradation of ubiquitinated proteins. J. Biol. Chem. *268*, 5115–5120.

Hikosaka, Y., Nitta, T., Ohigashi, I., Yano, K., Ishimaru, N., Hayashi, Y., Matsumoto, M., Matsuo, K., Penninger, J.M., Takayanagi, H., et al. (2008). The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. Immunity *29*, 438–450.

Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. (1994). T cell receptor antagonist peptides induce positive selection. Cell *76*, 17–27.

Honey, K., Nakagawa, T., Peters, C., and Rudensky, A. (2002). Cathepsin L regulates $CD4^+$ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. J. Exp. Med. *195*, 1349–1358.

Hugo, P., Kappler, J.W., McCormack, J.E., and Marrack, P. (1993). Fibroblasts can induce thymocyte positive selection in vivo. Proc. Natl. Acad. Sci. USA *90*, 10335–10339.

Hunt, D.F., Henderson, R.A., Shabanowitz, J., Sakaguchi, K., Michel, H., Sevilir, N., Cox, A.L., Appella, E., and Engelhard, V.H. (1992). Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science *255*, 1261–1263.

Huseby, E.S., White, J., Crawford, F., Vass, T., Becker, D., Pinilla, C., Marrack, P., and Kappler, J.W. (2005). How the T cell repertoire becomes peptide and MHC specific. Cell *122*, 247–260.

Huseby, E.S., Kappler, J.W., and Marrack, P. (2008). Thymic selection stifles TCR reactivity with the main chain structure of MHC and forces interactions with the peptide side chains. Mol. Immunol. *45*, 599–606.

Ignatowicz, L., Kappler, J., and Marrack, P. (1996). The repertoire of T cells shaped by a single MHC/peptide ligand. Cell *84*, 521–529.

Kappler, J.W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. Cell *49*, 273–280.

Kedzierska, K., Venturi, V., Field, K., Davenport, M.P., Turner, S.J., and Doherty, P.C. (2006). Early establishment of diverse T cell receptor profiles for influenza-specific $CD8(^+)CD62L(^{hi})$ memory T cells. Proc. Natl. Acad. Sci. USA *103*, 9184–9189.

Kirberg, J., Bosco, N., Deloulme, J.C., Ceredig, R., and Agenès, F. (2008). Peripheral T lymphocytes recirculating back into the thymus can mediate thymocyte positive selection. J. Immunol. *181*, 1207–1214.

Kisielow, P., Teh, H.S., Blüthmann, H., and von Boehmer, H. (1988). Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature *335*, 730–733.

Kurobe, H., Liu, C., Ueno, T., Saito, F., Ohigashi, I., Seach, N., Arakaki, R., Hayashi, Y., Kitagawa, T., Lipp, M., et al. (2006). CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. Immunity *24*, 165–177.

Kyewski, B., and Derbinski, J. (2004). Self-representation in the thymus: an extended view. Nat. Rev. Immunol. *4*, 688–698.

Li, W., Kim, M.G., Gourley, T.S., McCarthy, B.P., Sant'Angelo, D.B., and Chang, C.H. (2005). An alternate pathway for CD4 T cell development: thymocyte-expressed MHC class II selects a distinct T cell population. Immunity *23*, 375–386.

Li, J., Iwanami, N., Hoa, V.Q., Furutani-Seiki, M., and Takahama, Y. (2007). Noninvasive intravital imaging of thymocyte dynamics in medaka. J. Immunol. *179*, 1605–1615.

Lie, W.R., Myers, N.B., Connolly, J.M., Gorka, J., Lee, D.R., and Hansen, T.H. (1991). The specific binding of peptide ligand to L^d class I major histocompatibility complex molecules determines their antigenic structure. J. Exp. Med. *173*, 449–459.

Lilić, M., Santori, F.R., Neilson, E.G., Frey, A.B., and Vukmanović, S. (2002). The role of fibroblasts in thymocyte-positive selection. J. Immunol. *169*, 4945–4950.

Mareeva, T., Martinez-Hackert, E., and Sykulev, Y. (2008). How a T cell receptor-like antibody recognizes major histocompatibility complex-bound peptide. J. Biol. Chem. *283*, 29053–29059.

Marrack, P., and Kappler, J. (1997). Positive selection of thymocytes bearing $\alpha\,\beta$ T cell receptors. Curr. Opin. Immunol. *9*, 250–255.

Martinic, M.M., Rülicke, T., Althage, A., Odermatt, B., Höchli, M., Lamarre, A., Dumrese, T., Speiser, D.E., Kyburz, D., Hengartner, H., and Zinkernagel, R.M. (2003). Efficient T cell repertoire selection in tetraparental chimeric mice independent of thymic epithelial MHC. Proc. Natl. Acad. Sci. USA *100*, 1861–1866.

Merkenschlager, M., Graf, D., Lovatt, M., Bommhardt, U., Zamoyska, R., and Fisher, A.G. (1997). How many thymocytes audition for selection? J. Exp. Med. *186*, 1149–1158.

Momburg, F., Roelse, J., Howard, J.C., Butcher, G.W., Hämmerling, G.J., and Neefjes, J.J. (1994). Selectivity of MHC-encoded peptide transporters from human, mouse and rat. Nature *367*, 648–651.

Mozdzanowska, K., Furchner, M., Maiese, K., and Gerhard, W. (1997). $CD4^+$ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. Virology *239*, 217–225.

Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J., and Ahmed, R. (1999). Persistence of memory CD8 T cells in MHC class I-deficient mice. Science *286*, 1377–1381.

Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y., and Tanaka, K. (2007). Regulation of $CD8^+$ T cell development by thymus-specific proteasomes. Science *316*, 1349–1353.

Nakagawa, T., Roth, W., Wong, P., Nelson, A., Farr, A., Deussing, J., Villadangos, J.A., Ploegh, H., Peters, C., and Rudensky, A.Y. (1998). Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. Science *280*, 450–453.

Nedjic, J., Aichinger, M., Emmerich, J., Mizushima, N., and Klein, L. (2008). Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. Nature *455*, 396–400.

Nitta, T., Nitta, S., Lei, Y., Lipp, M., and Takahama, Y. (2009). CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. Proc. Natl. Acad. Sci. USA *106*, 17129–17133.

Oono, T., Fukui, Y., Masuko, S., Hashimoto, O., Ueno, T., Sanui, T., Inayoshi, A., Noda, M., Sata, M., and Sasazuki, T. (2001). Organ-specific autoimmunity in mice whose T cell repertoire is shaped by a single antigenic peptide. J. Clin. Invest. *108*, 1589–1596.

Palmer, E. (2003). Negative selection—clearing out the bad apples from the T-cell repertoire. Nat. Rev. Immunol. *3*, 383–391.

Park, J.H., Adoro, S., Lucas, P.J., Sarafova, S.D., Alag, A.S., Doan, L.L., Erman, B., Liu, X., Ellmeier, W., Bosselut, R., et al. (2007). 'Coreceptor tuning': cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR. Nat. Immunol. *8*, 1049–1059.

Pawlowski, T., Elliott, J.D., Loh, D.Y., and Staerz, U.D. (1993). Positive selection of T lymphocytes on fibroblasts. Nature *364*, 642–645.

Porgador, A., Yewdell, J.W., Deng, Y., Bennink, J.R., and Germain, R.N. (1997). Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. Immunity *6*, 715–726.

Reits, E., Griekspoor, A., Neijssen, J., Groothuis, T., Jalink, K., van Veelen, P., Janssen, H., Calafat, J., Drijfhout, J.W., and Neefjes, J. (2003). Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. Immunity *18*, 97–108.

Rock, K.L., and Goldberg, A.L. (1999). Degradation of cell proteins and the generation of MHC class I-presented peptides. Annu. Rev. Immunol. *17*, 739–779.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell *78*, 761–771.

Scollay, R., Wilson, A., D'Amico, A., Kelly, K., Egerton, M., Pearse, M., Wu, L., and Shortman, K. (1988). Developmental status and reconstitution potential of subpopulations of murine thymocytes. Immunol. Rev. *104*, 81–120.

Sebzda, E., Wallace, V.A., Mayer, J., Yeung, R.S., Mak, T.W., and Ohashi, P.S. (1994). Positive and negative thymocyte selection induced by different concentrations of a single peptide. Science *263*, 1615–1618.

Siggs, O.M., Makaroff, L.E., and Liston, A. (2006). The why and how of thymocyte negative selection. Curr. Opin. Immunol. *18*, 175–183.

Singer, A., Mizuochi, T., Munitz, T.I., and Gress, R.E. (1986). Role of self antigen in the selection of the developing T cell repertoire. Prog Immunol. *6*, 60–66.

Stambas, J., Doherty, P.C., and Turner, S.J. (2007). An *in vivo* cytotoxicity threshold for influenza A virus-specific effector and memory CD8(+) T cells. J. Immunol. *178*, 1285–1292.

Starr, T.K., Jameson, S.C., and Hogquist, K.A. (2003). Positive and negative selection of T cells. Annu. Rev. Immunol. *21*, 139–176.

Strasser, A. (2005). The role of BH3-only proteins in the immune system. Nat. Rev. Immunol. *5*, 189–200.

Takahama, Y. (2006). Journey through the thymus: stromal guides for T-cell development and selection. Nat. Rev. Immunol. *6*, 127–135.

Takahama, Y., Suzuki, H., Katz, K.S., Grusby, M.J., and Singer, A. (1994). Positive selection of $CD4^+$ T cells by TCR ligation without aggregation even in the absence of MHC. Nature *371*, 67–70.

Takahama, Y., Tanaka, K., and Murata, S. (2008). Modest cortex and promiscuous medulla for thymic repertoire formation. Trends Immunol. *29*, 251–255.

Tanchot, C., Lemonnier, F.A., Pérarnau, B., Freitas, A.A., and Rocha, B. (1997). Differential requirements for survival and proliferation of CD8 naïve or memory T cells. Science *276*, 2057–2062.

Uebel, S., Kraas, W., Kienle, S., Wiesmüller, K.H., Jung, G., and Tampé, R. (1997). Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. Proc. Natl. Acad. Sci. USA *94*, 8976–8981.

Ueno, T., Liu, C., Nitta, T., and Takahama, Y. (2005). Development of T-lymphocytes in mouse fetal thymus organ culture. Methods Mol. Biol. *290*, 117–133.

von Boehmer, H. (1994). Positive selection of lymphocytes. Cell *76*, 219–228.

Yewdell, J.W., Reits, E., and Neefjes, J. (2003). Making sense of mass destruction: quantitating MHC class I antigen presentation. Nat. Rev. Immunol. *3*, 952–961.

Young, A.C., Nathenson, S.G., and Sacchettini, J.C. (1995). Structural studies of class I major histocompatibility complex proteins: insights into antigen presentation. FASEB J. *9*, 26–36.

Zerrahn, J., Held, W., and Raulet, D.H. (1997). The MHC reactivity of the T cell repertoire prior to positive and negative selection. Cell *88*, 627–636.

Zerrahn, J., Volkmann, A., Coles, M.C., Held, W., Lemonnier, F.A., and Raulet, D.H. (1999). Class I MHC molecules on hematopoietic cells can support intrathymic positive selection of T cell receptor transgenic T cells. Proc. Natl. Acad. Sci. USA *96*, 11470–11475.

Zinkernagel, R.M., and Althage, A. (1999). On the role of thymic epithelium vs. bone marrow-derived cells in repertoire selection of T cells. Proc. Natl. Acad. Sci. USA *96*, 8092–8097.

Zinkernagel, R.M., Callahan, G.N., Althage, A., Cooper, S., Klein, P.A., and Klein, J. (1978). On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? J. Exp. Med. *147*, 882–896.