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TCR affinity for thymoproteasome-dependent positively selecting peptides conditions antigen responsiveness in CD8$^+$ T cells

Kensuke Takada[1], **Francois Van Laethem**[2], **Yan Xing**[3], **Kazuyuki Akane**[4], **Haruhiko Suzuki**[4], **Shigeo Murata**[5], **Keiji Tanaka**[6], **Stephen C. Jameson**[3], **Alfred Singer**[2], and **Yousuke Takahama**[1,*]

[1]Division of Experimental Immunology, Institute for Genome Research, University of Tokushima, Tokushima 770-8503, Japan

[2]Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

[3]Center for Immunology, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55414, USA

[4]Department of Immunology, Graduate School of Medicine, Nagoya University, Nagoya 466-8550, Japan

[5]Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan

[6]Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan

Abstract

In the thymus, low-affinity T cell antigen receptor (TCR) engagement facilitates positive selection of a useful T cell repertoire. Here we report that TCR responsiveness of mature CD8$^+$ T cells is fine-tuned by their affinity for positively selecting peptides in the thymus and that optimal TCR responsiveness requires positive selection on MHC class I-associated peptides produced by the thymoproteasome, which is specifically expressed in the thymic cortical epithelium. Thymoproteasome-independent positive selection of monoclonal CD8$^+$ T cells results in aberrant TCR responsiveness, homeostatic maintenance, and immune responses to infection. These results demonstrate a novel aspect of positive selection, in which TCR affinity for positively selecting peptides produced by thymic epithelium determines the subsequent antigen responsiveness of mature CD8$^+$ T cells in the periphery.

The thymus is an organ that produces functionally competent T cells, which play an essential role in the protection of vertebrates, including humans, from infectious diseases and malignant neoplasms. In the thymus, newly generated thymocytes exhibit a diverse range of T cell antigen receptor (TCR) recognition specificities following irreversible recombination of V(D)J genomic sequences in the nucleus. Subsequently, thymocytes are

*Correspondence to: takahama@genome.tokushima-u.ac.jp.

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positively and negatively selected to yield a potentially useful and self-tolerant repertoire of mature T cells[1]. Negative selection, together with the generation of regulatory T cells, contributes to the establishment of self-tolerance in T cells through high-affinity TCR engagement, whereas positive selection rescues a subset of immature thymocytes from default death through low-affinity TCR engagement and induces their differentiation into mature T cells[2,3]. The concept that positive selection selects only T cells bearing TCRs with potentially useful specificities for self-major histocompatibility complex (MHC)-associated foreign antigens, thereby affecting the antigen recognition repertoire, has been widely accepted[4,5]. However, it is unknown whether positive selection also plays a role in dictating the functional competency in individual T cells.

T cell positive selection is heavily dependent on the thymus microenvironment. Different types of antigen-presenting cells are distributed in the cortex and medulla of the thymus, and positive selection is primarily mediated in the thymic cortex by the engagement of TCRs expressed by cortical $CD4^+CD8^+$ thymocytes with self-peptide-MHC complexes expressed by cortical thymic epithelial cells (cTECs)[6–8]. cTECs harbor unique antigen-processing properties, which contribute to efficiently inducing positive selection by providing a set of MHC-associated self-peptides distinct from those in other antigen presenting cells, such as medullary TECs and dendritic cells[9,10]. A unique form of proteasomes, thymoproteasome, which contains a unique proteolytic β5 subunit, β5t (encoded by the *Psmb11* gene) that is specifically expressed in cTECs, was recently identified[11–13]. Proteasomes play an essential role in the cleavage of cytoplasmic proteins and the production of peptides presented by MHC class I molecules (MHC-I)[14]. In thymoproteasome-deficient mice, cTECs instead express β5i-containing immunoproteasomes, and thereby express a normal amount of MHC-I in association with an altered set of self-peptides. Consequently, in these mice, the $CD8^+$ T cell compartment is reduced to approximately 25% of that in normal mice and exhibits an altered TCR repertoire, whereas the $CD4^+$ T cell compartment remains unaffected[11,12]. Therefore, thymoproteasome-expressing cTECs produce a unique set of MHC-I-associated self-peptides and are essential for optimal positive selection to form a normal repertoire of $CD8^+$ T cells[13]. However, whether thymoproteasome-dependent positive selection contributes to the formation of functionally competent $CD8^+$ T cells only by selectively inducing the survival of a repertoire of thymocytes with low-affinity TCR specificities or also by influencing the functional capability within individual T cells after the positive selection is unknown.

The present study examined the development and function of monoclonal TCR-expressing $CD8^+$ T cells in a thymoproteasome-deficient thymic microenvironment. The use of monoclonal T cells allowed direct examination of the functional development of individual T cells, excluding the possible effects of repertoire alteration during T cell development. Our results demonstrate that monoclonal TCR-expressing $CD8^+$ T cells selected in the absence of thymoproteasomes exhibit diminished TCR responsiveness. Thymoproteasome-independent positive selection results in defective maintenance of the peripheral naïve T cell compartment and alteration of immune responses to pathogens. Our results further indicate that TCR affinity of positively selecting MHC-I-associated peptides directly impacts antigen responsiveness in positively selected $CD8^+$ T cells. Thus, TCR affinity of positively

selecting peptides presented by thymoproteasome-expressing thymic epithelium preconditions antigen responsiveness of individual $CD8^+$ T cells, in addition to shaping TCR recognition specificities.

RESULTS

Positive selection affects TCR responsiveness

To investigate the role of thymoproteasomes in the formation of functional competency in individual T cells, we first characterized monoclonal OT-I-TCR-expressing Rag2-deficient $CD8^+$ T cells, which react with K^b-associated ovalbumin peptide antigen (residues 257–264) (SIINFEKL, OVAp) and are positively selected and functionally competent in H-2^b mice[15], generated in the absence of thymoproteasomes. The generation of monoclonal OT-I-TCR^{hi} $CD4^-CD8^+$ thymocytes was undiminished even when the thymus microenvironment lacked thymoproteasomes (Fig. 1a), suggesting that OT-I-TCR-expressing T cells could be positively selected even in the absence of thymoproteasomes. Interestingly, however, OT-I-TCR-expressing $CD4^-CD8^+$ thymocytes and naïve $CD8^+CD44^{lo}$ T cells generated in thymoproteasome-deficient thymuses exhibited impaired TCR responsiveness. Namely, T cell responses to stimulation with anti-CD3 plus or minus anti-CD28 antibodies or OVAp–H-$2K^b$ tetramers, as measured by the upregulation of the activation markers CD69 and CD25, were significantly diminished in OT-I-TCR^+ $CD8^+$ T cells generated in thymoproteasome-deficient thymuses (Fig. 1b, 1c, Supplementary Fig. 1). The increase in cell size, as measured by the increase in forward scatter intensity, was also diminished (Fig. 1d). Reduced TCR responsiveness was not limited to OT-I-TCR-expressing $CD8^+$ T cells; naïve $CD8^+$ T cells bearing other TCRs generated in the absence of thymoproteasomes, including P14-TCR and F5-TCR, which are specific for the H-$2D^b$-associated lymphocytic choriomeningitis virus gp33 peptide antigen (residues 32–42) and H-$2D^b$-associated influenza nucleoprotein NP68 peptide antigen (residues 366–374), respectively, and are positively selected and functionally capable in H-2^b mice[16,17], also exhibited diminished TCR responsiveness (Fig. 1e, 1f).

In accordance with the reduced expression of activation markers such as CD69 and CD25, calcium flux elicited by TCR crosslinking was diminished and delayed in both $CD69^+$ and $CD69^-$ subpopulations of $CD4^-CD8^+$ thymocytes and peripheral $CD8^+CD44^{lo}$ naïve T cells generated in the absence of thymoproteasomes (Fig. 2a). In addition, TCR-induced phosphorylation of extracellular signal-regulated kinase (ERK) 1 and 2 was also diminished (Fig. 2b, Supplementary Fig. 2). We further assessed the tyrosine-phosphorylation status in whole cell lysates of naïve OT-I-TCR^+ $CD8^+$ T cells with or without TCR stimulation. Basal and TCR-induced phosphorylation of TCRζ, which was visualized by partially phosphorylated p21 and fully phosphorylated p23, respectively[18,19], was reduced in OT-I-TCR^+ $CD8^+$ T cells generated in the absence of thymoproteasomes (Fig. 2c). These results indicate that TCR responsiveness in monoclonal $CD8^+$ T cells is impaired when positively selected in the absence of thymoproteasomes.

Positive selection affects T cell maintenance

Given that monoclonal TCR-expressing $CD8^+$ T cells are functionally diminished when positively selected in the absence of thymoproteasomes, we further examined how the loss of thymoproteasomes in cTECs would affect the competence of the peripheral immune system. Similar to the observations in polyclonal T cells[12,20], monoclonal OT-I-TCR^+ $CD8^+$ T cells generated in the absence of thymoproteasomes displayed an increased frequency of $CD44^{hi}CD122^{hi}$ "memory-like" cells (Fig. 3a, Supplementary Fig. 3a). Such increased frequency of memory-like cells was not observed in the thymus (Supplementary Fig. 3b) or in newborn mice (Supplementary Fig. 3c); however, it was because of the selective reduction of $CD44^{lo}$ naïve cells in secondary lymphoid organs (Fig. 3b). $CD44^{hi}$ memory-like cells were also reduced in newborn β5t-deficient mice but recovered during ontogeny (Supplementary Fig. 3d), which possibly reflected the enhanced homeostatic conversion from naïve cells (Supplementary Fig. 3e). The selective reduction of naïve cells in thymoproteasome-deficient mice was uniformly observed in various antigen-specific $CD8^+$ T cells, detected by tetramer-based enrichment[21–23] (Fig. 3c, Supplementary Fig. 3f). Thus, $CD8^+$ T cells positively selected in thymoproteasome-deficient thymus are unable to maintain naïve T cell compartment in the periphery.

Despite the striking reduction of naïve $CD8^+$ T cells in β5t-deficient mice, increased frequency of memory-like cells was limited in adult $CD8^+$ T cells and did not occur in the $CD4^+$ T cell compartment (Supplementary Fig. 4)[12,20], implying that homeostatic factors specific for $CD8^+$ T cells might promote lymphopenia-induced proliferation and conversion to memory-type cells during the establishment of the peripheral T cell pool. CD122 (IL2Rβ, encoded by the *Il2rb* gene), a cytokine receptor component shared by IL-2 and IL-15, is expressed in naïve $CD8^+$ but not $CD4^+$ T cells, and IL-15 contributes to the generation of memory-like $CD8^+$ T cells in unprimed mice[24]. Reconstitution of lethally irradiated β5t-deficient mice with CD122-deficient bone marrow cells significantly rescued the phenotypic abnormality of peripheral $CD8^+$ T cells in β5t-deficient mice (Fig. 3d). These results indicate that thymoproteasome-dependent positive selection is essential for the establishment of a homeostatic naïve cell-enriched $CD8^+$ T cell pool through the regulation of CD122-dependent generation of memory-like cells.

Positive selection affects responses to infection

To further understand the consequence of thymoproteasome deficiency in the formation of dynamic immune system, monoclonal $CD8^+$ T cells generated in the absence of thymoproteasomes were evaluated in pathogen-induced immune responses. OT-I-TCR^+ $CD8^+$ T cells were obtained from bone marrow chimeras where β5t-sufficient or β5t-deficient mice were reconstituted with OT-I-TCR-transgenic bone marrow cells. These β5t-dependent or -independent OT-I-TCR^+ T cells ($CD45.2^+CD90.2^+$) were co-transferred with normal OT-I-TCR^+ T cells ($CD45.2^+CD90.1^+$) into recipient mice ($CD45.1^+CD45.2^-$) that were subsequently infected with OVA-expressing *Listeria monocytogenes* (LM-OVA) (Supplementary Fig. 5a). Upon LM-OVA infection, normally generated OT-I-TCR^+ $CD8^+$ T cells give rise to "long-term memory" and "short-lived effector" cells[25]. However, OT-I-TCR^+ $CD8^+$ T cells derived from thymoproteasome-deficient thymuses preferentially gave rise to $KLRG1^{hi}CD44^{hi}$ "short-lived effector" cells, unlike normal thymus-derived OT-I-

TCR$^+$ CD8$^+$ T cells (Fig. 4, Supplementary Fig. 5b). Therefore, thymoproteasome-dependent positive selection is essential for functionally conditioning individual CD8$^+$ T cells with respect to mediating normal immune responses to the infection.

Thymoproteasomes affect positively selecting peptides

The results so far indicate that thymoproteasome-dependent positive selection is required for conditioning optimal responsiveness of individual CD8$^+$ T cells in the periphery. To understand the mechanism that underlies the thymoproteasome-dependent conditioning of functional capability in individual CD8$^+$ T cells during positive selection in the thymus, we next examined CD69 and C-C chemokine receptor 7 (CCR7) expression profiles in thymocytes. These markers can be used to categorize developing thymocytes into five stages according to selection, migration, and maturation[26] (Fig. 5a). CD69$^-$CCR7$^-$ thymocytes are cortical thymocytes that have not yet received TCR signals (stage I), whereas CD69$^+$CCR7$^-$ thymocytes have recently received TCR selection signals in the cortex[27] (stage II). CD69$^+$ thymocytes begin to express CCR7 (stage III) and thereby migrate to the thymus medulla[28]. CD69$^+$CCR7^{hi} medullary thymocytes (stage IV) down-regulate CD69 expression before becoming competent to emigrate into the circulation (stage V)[26]. We did not observe significantly retarded progression of OT-I-TCR-expressing monoclonal thymocytes from stage I to stage V even in the absence of thymoproteasomes (Fig. 5b, Supplementary Fig. 6). However, in the thymoproteasome-deficient thymus, recently signaled stage II thymocytes expressed lower amounts of CD5 and CD69 compared to those developing in the normal thymus (Fig. 5c, 5d). The expression of CD5 and CD69 was reduced also in P14-TCR- and F5-TCR-expressing stage II thymocytes (Fig. 5e, 5f). CD5 and CD69 expression in thymocytes correlate with the affinity of the TCR to its peptide-MHC ligand[29,30]. These results indicate that TCR affinities for peptide-MHC-I complexes that induce positive selection of OT-I-TCR-expressing monoclonal CD8$^+$ T cells are diminished in the thymus in the absence of thymoproteasomes, although positive selection-inducing TCR engagement occurs in the thymoproteasome-deficient thymus.

Positively selecting peptides influence TCR responsiveness

The above results indicate that thymoproteasomes influence TCR affinities for peptide-MHC-I complexes that induce positive selection of OT-I-TCR$^+$ T cells. Thymoproteasomes produce a unique set of MHC-I-associated peptides expressed by cTECs[12,13,20], so that it was assumed that thymoproteasome-expressing cTECs present a set of MHC-I-associated self-peptides that exhibit an optimal affinity to positively select OT-I-TCR. Therefore, we examined whether the difference in TCR affinity of MHC-I-associated peptides would directly contribute to conditioning functional capabilities of CD8$^+$ T cells. To test the effects of positively selecting MHC-I-associated peptides independent of thymoproteasome-deficiency, fetal thymuses from Tap1-deficient (and β5t-sufficient) OT-I-TCR-transgenic mice were organ-cultured in the presence of various peptides capable of inducing positive selection of OT-I-TCR$^+$ CD8$^+$ T cells. In particular, the F-actin capping protein A peptide Cappa1 (residues 92–99, ISFKFDHL)[31], the β-catenin peptide Catbn (residues 329–336, RTYTYEKL)[32], and the OVAp variant peptide E1 (EIINFEKL)[15] are capable of inducing positive selection of OT-I-TCR$^+$ CD8$^+$ T cells when associated with MHC-I K^b; however, they are recognized with different affinities by OT-I-TCR (Cappa1 < Catbn < E1)[33]. Indeed,

all these peptides were capable of promoting positive selection of OT-I-TCR^{hi} CD4^{-}CD8^{+} thymocytes (Fig. 6a). We found that these peptides promoted positive selection of OT-I-TCR^{hi} CD4^{-}CD8^{+} thymocytes that expressed graded amounts of CD5 in proportion to the graded TCR affinities (Fig. 6b). Importantly, up-regulation of CD69 and CD25 in OT-I-TCR^{hi} CD4^{-}CD8^{+} thymocytes in response to TCR stimulation differed among the cells that were positively selected with different peptides and exhibited a linear relationship with the affinities of the MHC-I-peptide complexes used to induce positive selection (Fig. 6c). These results demonstrate that TCR affinities of positive selection-inducing MHC class-I-associated peptides directly contribute to preconditioning the functional capabilities of monoclonal TCR-expressing CD8^{+} T cells.

DISCUSSION

The present study addressed how the thymus epithelium contributes to the establishment of functionally competent T cells through positive selection. Our results reveal that thymoproteasome-expressing cTECs optimize functional competency in monoclonal CD8^{+} T cells. Experiments using monoclonal T cells demonstrate functional conditioning in individual T cells, excluding the possible influence of selection processes according to altered choice in different TCR specificities. Thus, the present study proposes that thymic epithelium-dependent positive selection preconditions TCR responsiveness in individual T cells (T cell education) in addition to positively selecting a fraction of immature thymocytes for potentially useful TCR specificities (survival of the fittest T cells),. Our results reveal that thymoproteasome-dependent positive selection contributes to the formation of functionally competent T cells by fine-tuning TCR responsiveness, whereas the lack of thymoproteasomes results in the impairment in antigen responsiveness of positively selected T cells.

We previously reported that thymoproteasome-deficient mice poorly respond to allogeneic cells and viral infection, and interpreted that the defects in those responses might be due to defective TCR repertoire of polyclonal CD8^{+} T cell pool generated in the absence of thymoproteasomes[12]. In contrast, the present results obtained from monoclonal TCR-expressing T cells demonstrate that individual CD8^{+} T cells positively selected in thymoproteasome-deficient mice are functionally abnormal in the maintenance and immune responses in the periphery. Thus, this study provides a previously unknown mechanism that underlies the defective immune responses in thymoproteasome-deficient mice.

Our results demonstrate that positively selecting MHC-I-associated peptides directly affect TCR responsiveness of positively selected CD8^{+} T cells and that functional responsiveness of CD8^{+} T cells exhibits a linear relationship with the TCR affinity of positive selection-inducing MHC-I-peptide complexes. CD5 expression in T cells has been extensively studied as a marker for self-recognition intensity by T cells in the thymus and in the periphery[19,29,30]. Recent studies suggested that CD5^{hi} T cells, presumably enriched with high self-reactivity T cells, dominate *in vivo* immune responses to foreign antigens in CD4^{+} and CD8^{+} compartments[19,34] Comparison of CD5^{hi} versus CD5^{lo} TCR-transgenic T cells also suggested a positive correlation between the CD5 expression amount and *in vitro* TCR responses[35]. In contrast, the present results provide clear evidence that the TCR affinity for

positive selection-inducing ligands directly influences the functional capability of mature $CD8^+$ T cells. Furthermore, our results revealing that thymoproteasome-expressing cTECs are essential for the establishment of functional competency in mature $CD8^+$ T cells indicate that positive selection-mediated optimization of T cell responsiveness represents a physiological process that occurs in the thymic microenvironment.

Thymoproteasomes produce a unique set of MHC-I-associated peptides expressed by cTECs[12,13,20,36]. Our results demonstrate that thymoproteasome-expressing cTECs optimize the functionality of $CD8^+$ T cells and that the TCR affinity of positive selection-inducing ligands correlates with the TCR responsiveness of mature T cells. These results collectively suggest that thymoproteasome-expressing cTECs precondition T cell responsiveness by optimizing TCR affinity in positively selecting peptides. It should therefore be important to further investigate how thymoproteasomes preferentially produce MHC-I-associated peptides that are optimal for inducing positive selection of $CD8^+$ T cells. Our results also indicate that the CD5 expression amount in $CD69^+CCR7^-$ thymocytes is reduced when positively selected in the absence of thymoproteasomes. During thymocyte development, TCR sensitivity is fine-tuned by multiple molecules, including CD5[37,38]. Molecular signals that preset thymoproteasome-dependent functional conditioning of T cells may include the signals mediated by CD5. It is interesting to note that CD5 expression amounts at later differentiation stages ($CD4^-CD8^+$ thymocytes and naïve $CD8^+$ T cells) generated in thymoproteasome-deficient thymus are variably affected among distinct TCR-expressing cells (data not shown). CD5 expression at those mature stages is even slightly elevated in polyclonal situation[20,36]. The mechanism for the changes in CD5 expression amounts in these later stages is unknown but may involve the affinity of TCR engagements by self-MHC-peptide complexes that positively selected T cells subsequently encounter in the thymic medulla and the periphery.

A recent study assessed immune responses of OT-I-TCR-expressing $CD8^+$ T cells positively selected by thymic hematopoietic cells but not thymic epithelial cells[34]. Those hematopoietic cell-selected T cells expressed reduced amounts of CD5 and exhibited impaired proliferation upon LM-OVA infection, in agreement with previous studies indicating that cTECs are required for optimal positive selection[6–8]. On the other hand, our results reveal that OT-I-TCR^+ thymocytes positively selected in thymoproteasome-deficient thymuses are reduced in CD5 expression specifically at the $CD69^+CCR7^-$ stage but not at the mature $CD8^+$ T cell stage and abnormally generate short-lived effector cell subsets with normal proliferation upon LM-OVA infection. In future studies, it will be interesting to see whether these effects extend to differences in the production of protective memory $CD8^+$ T cells. Therefore, thymoproteasome-deficient cTECs appear more efficient than thymic hematopoietic cells as positive selecting-inducing antigen presenting cells in preconditioning functional competency of $CD8^+$ T cells. Further characterization of cTECs should aid in fully elucidating the mechanisms underlying T cell positive selection in the thymus.

Our results demonstrate that the TCR affinities of positive-selection-inducing peptides directly contribute to the functional capabilities of monoclonal T cells. We therefore think that TCR engagement during positive selection directly affects the functional capabilities of

mature T cells. However, it is also possible that previously unappreciated diversity in preselected thymocytes contributes to the functional diversity of T cells.

Finally, the present results report that naïve $CD8^+$ T cells generated in the thymoproteasome-deficient thymus are hypo-responsive and inefficient in the *in vivo* maintenance of cellularity, whereas our previous report described that OT-I-TCR-expressing $CD8^+$ T cells generated in the absence of β5t could respond to antigen stimulation[12]. The previous experiments were carried out by using total $CD8^+$ T cells that contained both naïve and memory-phenotype cells, whereas the present experiments examine the responsiveness of isolated naïve $CD8^+$ T cells, excluding the contribution of memory-phenotype cells. As memory-phenotype T cells are highly reactive to TCR engagement[23,39], the relative enrichment of memory-phenotype cells in $CD8^+$ T cells from β5t-deficient mice can explain the unreduced responses reported in the previous study.

In conclusion, our results demonstrate a novel aspect of positive selection, in which the thymus epithelium contributes to the "education" of T cell responsiveness. Positive selection contributes to the establishment of functional competency in individual T cells, and this feature of positive selection is ascribed to the thymus epithelium and the antigen-presentation machineries specific to cTECs. Interestingly, an important role in functional education during development has been proposed for natural killer (NK) cells[40]. Self MHC-I determines the expression pattern of inhibitory receptors that specifically recognize different MHC-I alleles in NK cells, whereas the responsiveness of individual NK cells is fine-tuned, rather than switched on or off, by receptor signal intensity during development[40–42]. Taken together, functional education may represent a process common to both acquired and innate immune cells.

On-line methods

Mice

β5t-deficient[11], Rag2-deficient[43], Tap1-deficient[44], OT-I-TCR-transgenic[15], P14-TCR-transgenic[16], F5-TCR-transgenic[17], and B6.SJL-*Ptprc*^{a} (B6-Ly5.1)[45] mice were previously described. C57BL/6 mice and Rag1-deficient OT-I-TCR-transgenic mice were purchased from Japan SLC and Taconic, respectively. Mice were bred and maintained at our facility under specific pathogen free conditions. All animal experiments were performed with approval from the Animal Experimentation Committee of the University of Tokushima.

Bone marrow chimeras

Bone marrow cells were magnetically depleted of T cells using anti-CD90.2-conjugated magnetic beads and columns (Miltenyi Biotec). Recipient mice were irradiated with 10.0 Gy and injected intravenously with 1×10^7 bone marrow cells. Chimeric mice were used in experiments 7 to 10 weeks after the bone marrow transplantation.

Flow cytometry and cell sorting

Single-cell suspensions were stained with fluorochrome-labeled monoclonal antibodies for 30 min on ice. Data were acquired on FACSAria and FACSVerse flow cytometers (BD

Biosciences) and analyzed using FlowJo software (TreeStar). For cell isolation, cells were incubated with biotinylated antibodies and enriched by depleting antibody-bound cells with streptavidin-conjugated magnetic beads and columns (Miltenyi Biotec). Cells were subsequently sorted on FACSAria (BD Biosciences).

In vitro T cell stimulation

Cells were placed in culture plates pre-coated with anti-CD3ε a (145-2C11; eBioscience) at concentrations of 0.1 to 10 μg/ml in the presence or absence of anti-CD28 antibody (37.51; eBioscience) at 10 μg/ml. Where indicated, peptide-MHC tetramers (MBL) were added at concentrations of 0.1–5 nM.

Calcium flux

Cells were loaded with 1.8 mM Indo-1 (Molecular Probes) at 31 °C and subsequently incubated at 4 °C with 5 μg/ml biotinylated anti-TCRβ (H57-597; BD Biosciences) and fluorochrome-labeled antibodies. Cells were warmed to 37 °C for 2 min and subsequently analyzed by flow cytometry. The temperature was maintained at 37 °C throughout the analysis. Avidin (1 μg/ml) was added 30 seconds after the initiation of data acquisition.

ERK phosphorylation

Cells were incubated with peptide-MHC tetramers (MBL) at 1.25 nM for 1–5 min at 37 °C, followed by fixation with 4% formaldehyde and staining with phospho-ERK1/2-specific monoclonal antibody (197G2; Cell Signaling Technology) according to the manufacturer's protocol.

Tyrosine phosphorylation

Cells were incubated with peptide-MHC tetramers at 1.25 nM for 5 min at 37 °C. Cells were then solubilized on ice in lysis buffer containing 1% Nonidet P-40, 140 mM NaCl, 10 mM Tris-HCl (pH 7.2), 2 mM EDTA, 1 mM NaF, 1 mM orthovanadate, and protease inhibitor cocktail (Roche). Lysates were resolved on 4–12% gradient SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore). Membranes were incubated with an anti-phosphotyrosine antibody (4G10; Millipore), anti-mouse IgG secondary antibodies conjugated with horseradish peroxidase (Promega), and Western Lightning Plus ECL reagent (PerkinElmer).

Analysis of peptide-MHC tetramer-specific $CD8^+$ T cells

Tetramer-specific $CD8^+$ T cells were detected according to previously described procedures[21–23]. Single-cell suspensions prepared from spleens and lymph nodes were stained with phycoerythrin (PE)-conjugated peptide-MHC tetramers, including OVAp–H-$2K^b$, lymphocytic choriomeningitis virus glycoprotein 33 (gp33, KAVYNFATC)–H-$2D^b$, influenza nucleoprotein 373T (NP373T, ASNENMDTM)–H-$2D^b$, and human glycoprotein 100 (hgp100, KVPRNQDWL)–H-$2D^b$. All tetramers were purchased from MBL. Cells were then incubated with anti-PE microbeads (Miltenyi Biotec) and loaded onto magnetic columns (Miltenyi Biotec). Unbound and bound fractions were recovered and stained with anti-CD3ε (145-2C11), anti-CD4 (GK1.5), anti-B220 (RA3-6B2), anti-CD11b (M1/70),

anti-CD11c (N418), anti-F4/80 (BM8) (all from Biolegend), and anti-CD8α (K15) (Abcam) antibodies for flow cytometric analysis.

Homeostatic proliferation

Thymocytes were labeled with carboxyfluorescein diacetate succinimidyl ester (eBioscience) at 2 μM for 10 minutes at 37 °C and adoptively transferred to sublethally irradiated (6.0 Gy) recipient mice (1×10^6 SP thymocytes/recipient). CFSE dilution as well as CD4, CD8, and CD44 expression of donor cells in recipient spleens was analyzed 14 days after transfer.

LM-OVA infection

Bone marrow chimera-derived donor OT-I cells (5×10^4/recipient) were adoptively transferred with normal OT-I cells (5×10^4/recipient) into congenic recipient mice (Supplementary Fig. 5a). On the following day, recipient mice received intravenous injections of an attenuated strain of OVA-expressing *Listeria monocytogenes* (LM-OVA; ActA2; 3×10^6 colony-forming units/mouse)[46]. At day 5 and day 8 post-infection, donor cells in the blood were analyzed by flow cytometry and were identified according to the expression of CD45 congenic markers and K^b-OVAp tetramer binding.

Fetal thymus organ culture (FTOC)

FTOC was performed as described previously[12,31] using E15 thymus lobes from $Tap^{-/-}$ OT-I-TCR transgenic mice. Peptides were added at a concentration of 20 μM. On day 3, thymocytes were analyzed for the expression of CD4, CD8, and Vα2 by flow cytometry. Where indicated, cells were analyzed for CD5 expression and TCR responsiveness on day 5.

Statistics

Statistical significance was evaluated using the two-tailed unpaired or paired Student's *t* test. *P* values < 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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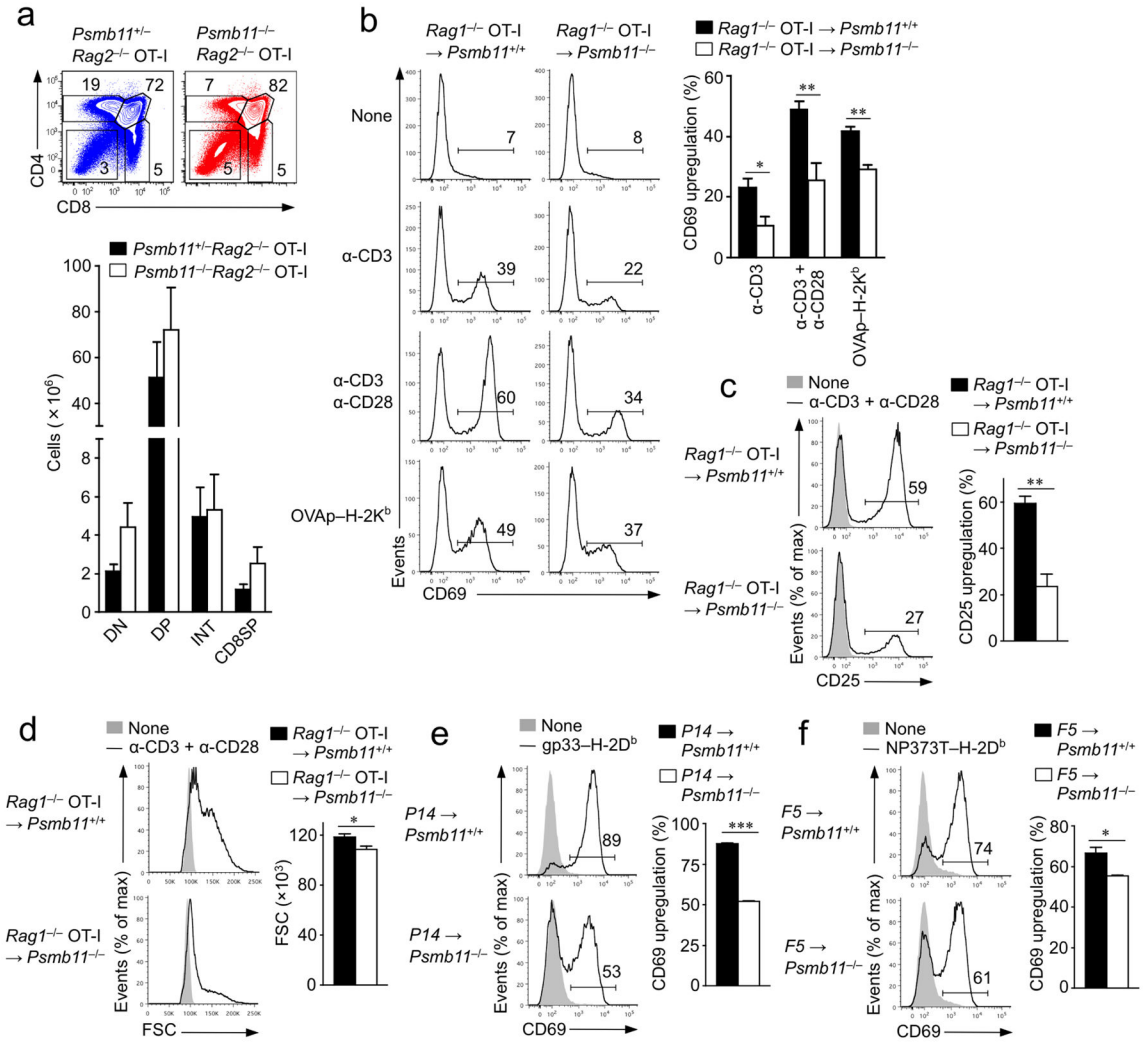

Figure 1.

Monoclonal TCR-expressing T cells generated in β5t-deficient thymus exhibit impaired TCR responsiveness. (**a**) CD4 and CD8 profiles and numbers of $CD4^{-}CD8^{-}$ (DN), $CD4^{+}CD8^{+}$ (DP), $CD4^{+}CD8^{int}$ (INT), and $TCR^{hi}CD4^{-}CD8^{+}$ (CD8SP) thymocytes from control (*n* = 6) and β5t-deficient (*n* = 8) Rag2-deficient OT-I-TCR-transgenic mice. (**b**–**d**) Bone marrow chimera mice were prepared by reconstituting lethally irradiated *Psmb11*$^{+/+}$ or *Psmb11*$^{-/-}$ B6-Ly5.1 mice with T-cell-depleted bone marrow cells from Rag1-deficient OT-I-TCR-transgenic mice. $CD44^{lo}CD8^{+}$ $CD45.1^{-}CD45.2^{+}$ donor-derived T cells isolated from spleens and lymph nodes were cultured in the absence or presence of indicated reagents. (**b**) Cells were assessed for CD69 expression after 3 hours of culture (*n* = 4). CD69 upregulation % = % $CD69^{hi}$ cells in T cells by indicated stimulation – % $CD69^{hi}$ cells in unstimulated T cells. **(c)** CD25 upregulation in response to anti-CD3 + anti-CD28 antibodies were examined after 12 h of culture. CD25 upregulation was calculated as CD69 was in (**b**). (**d**) Cell size measured by forward scatter intensities in response to anti-CD3 + anti-CD28 antibodies after 24 h of culture. (**e** and **f**) P14-TCR-transgenic **(e)** and F5-TCR-transgenic **(f)** T cells were stimulated or unstimulated with specific tetramers. CD69 upregulation in $tetramer^{hi}$

$CD44^{lo}CD8^{+}$ T cells is shown ($n = 4$). Representative data from four (**b, c, f**) and two (**d, e**) independent experiments. Bars indicate average ± standard errors of the mean. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

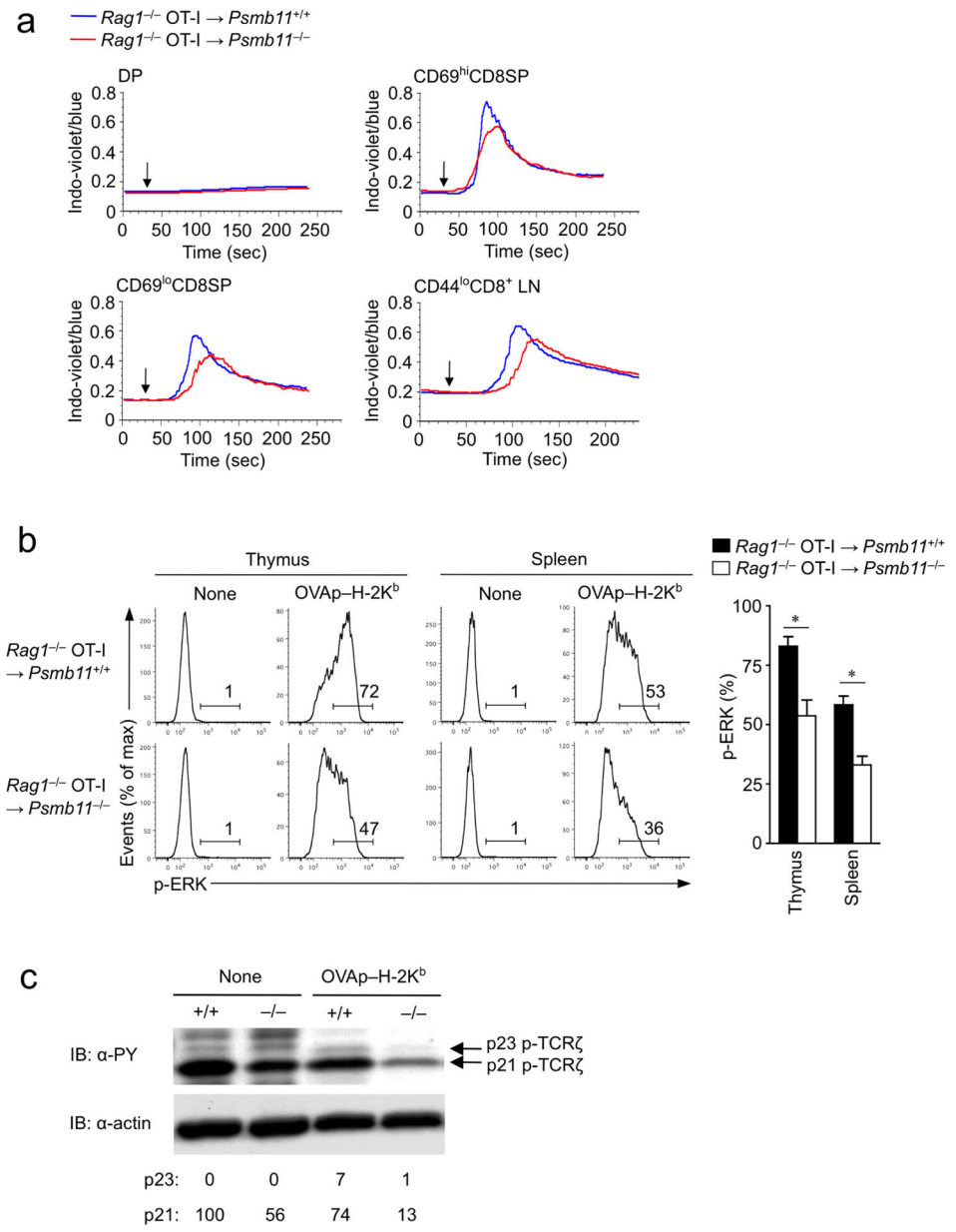

Figure 2.

Impaired proximal TCR signaling events in T cells generated in the β5t-deficient thymus. (**a**) Indicated Indo-1-loaded thymocyte or lymph node cell subpopulations were coated with a biotinylated anti-TCRβ antibody. Avidin was added at the time points indicated by arrows. TCR stimulation-induced calcium mobilization was plotted. (**b**) CD8SP thymocytes and CD44^{lo}CD8^{+} splenocytes from indicated mice were incubated with or without OVAp–H-2K^{b} tetramers for 2 minutes. ERK1 and 2 phosphorylation was analyzed in donor-derived OT-I-TCR-transgenic T cells (n = 4). (**c**) Donor-derived CD44^{lo}CD8^{+} OT-I-TCR-transgenic T cells were or were not stimulated with OVAp–H-2K^{b} tetramers. Cell lysates were subsequently assessed for tyrosine phosphorylation by immunoblotting. Numbers below the bands indicate the intensity normalized to that of p21 in unstimulated cells from β5t-

sufficient recipients. Representative data from two independent experiments are shown (**a**–**c**). Bars indicate average ± standard errors of the mean. *$P < 0.01$.

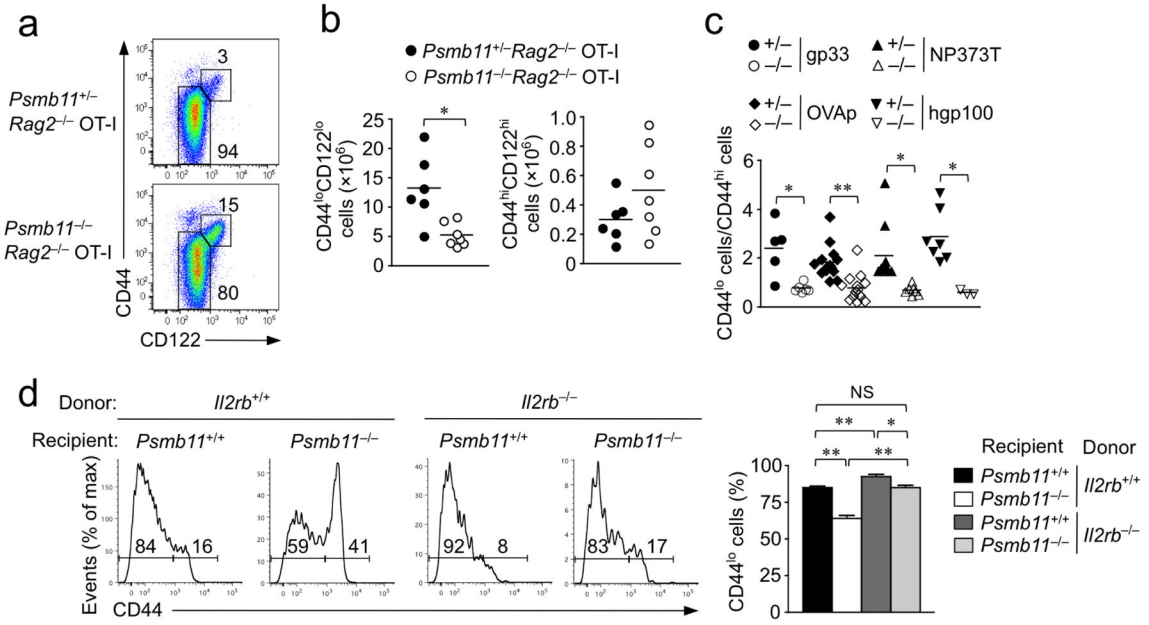

Figure 3.

β5t-dependent positive selection optimizes the peripheral maintenance of T cells. (**a** and **b**) Phenotypic analysis of $CD8^+$ OT-I-TCR-transgenic T cells in spleens and lymph nodes. (**c**) $CD8^+$ T cells specific for the indicated epitopes in polyclonal *Psmb11*$^{-/-}$ and control mice were analyzed to determine ratios of $CD44^{hi}$ to $CD44^{lo}$ cells following enrichment with different MHC-I tetramers. (**d**) Lethally irradiated *Psmb11*$^{+/+}$ or *Psmb11*$^{-/-}$ mice ($CD45.1^+$) were reconstituted with *Il2rb*$^{+/+}$ or *Il2rb*$^{-/-}$ bone marrow cells ($CD45.2^+$) mixed with $CD45.1^+$ bone marrow cells to ensure immune tolerance ($n = 5$). CD44 expression amounts in $CD45.1^-CD45.2^+$ donor-derived $CD8^+$ T cells are shown. Accumulated results from more than three mice/group in (**b** and **c**), and representative data from two independent experiments (**d**) are shown. Bars indicate average ± standard errors of the mean. $^{**}P < 0.001$, $^{*}P < 0.01$.

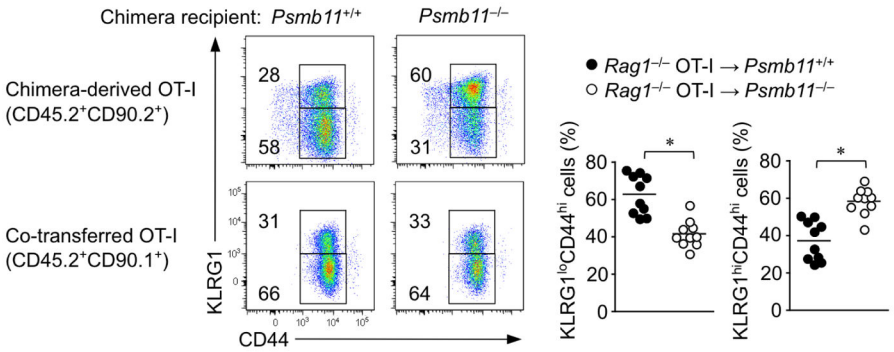

Figure 4.

β5t-dependent positive selection affects the immune response to infection. Graphical scheme of LM-OVA infection experiments is shown in Supplementary Fig. 5a. Expression of CD44 and KLRG1 in donor OT-I T cells from infected recipients 5 days after infection (n = 10). Cumulative data from two independent experiments are shown. Bars indicate average ± standard errors of the mean. *P < 0.001.

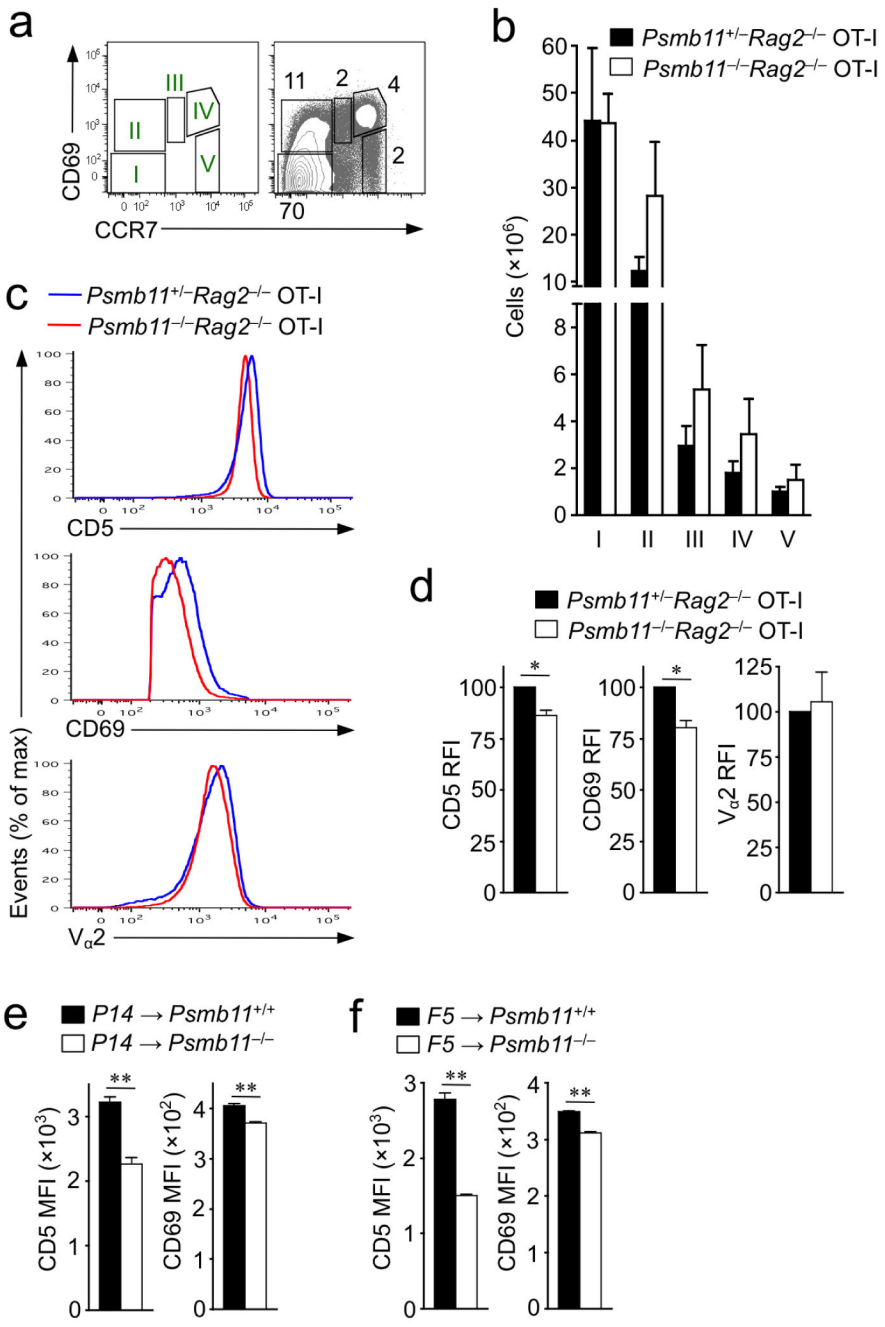

Figure 5.

In β5t-deficient mice, thymocytes are positively selected through reduced TCR signals. Total thymocytes from B6 mice (**a**) and indicated mice (**b**) were analyzed for CD69 and CCR7 expression. Five stages of thymocyte development (I–V) were defined as indicated. The expression of CD5, CD69, and Vα2-TCR in $CD69^{+}CCR7^{-}$ stage II thymocytes (**c**) was evaluated according to relative fluorescence intensity (RFI), or levels normalized to the mean fluorescence intensity in control mice (**d**). (**e** and **f**) P14-TCR-transgenic **(e)** or F5-TCR-transgenic **(f)** stage II thymocytes were analyzed for CD5 and CD69 expression by the

mean fluorescence intensity ($n = 4$). Representative data from six (**a** and **c**) and two (**e** and **f)** independent experiments. Accumulated data from more than five mice/group (**b** and **d**) are shown. Bars indicate average ± standard errors of the mean. $^{**}P < 0.001$, $^{*}P < 0.01$.

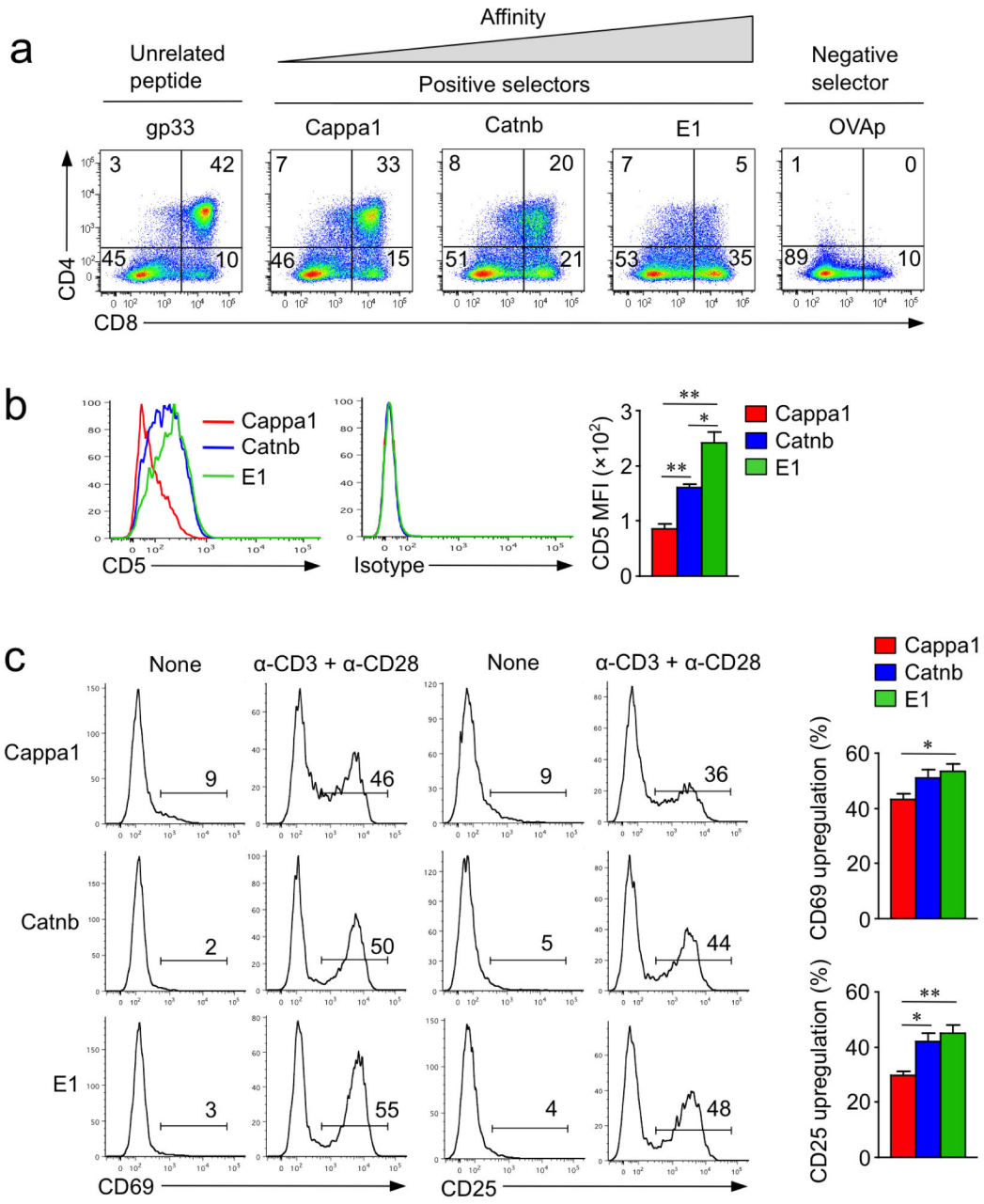

Figure 6.

Positively selecting peptides precondition antigen responsiveness in monoclonal T cells. Fetal thymus organ culture (FTOC) was performed with Tap1-deficient OT-I-TCR-transgenic thymus lobes in the presence of the indicated peptides. (**a**) Representative CD4 versus CD8 profiles of thymocytes. gp33 does not bind to OT-I-TCR and thereby served as the negative control. (**b**) CD5 expression in $V\alpha2^{hi}$ $CD69^{lo}CD62L^{hi}$ CD8SP thymocytes (n = 3/group). (**c**) Thymocytes harvested from FTOC were or were not stimulated with anti-CD3 and anti-CD28 antibodies. CD69 and CD25 expression was assessed in CD8SP thymocytes

(n = 3–4/group). Representative data from three independent experiments (**a** to **c**) are shown. Bars indicate average ± standard errors of the mean (**b** and **c**). $^{**}P < 0.01$, $^{*}P < 0.05$.