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TCR Affinity for In Vivo Peptide-Induced Thymic Positive Selection Fine-Tunes TCR Responsiveness of Peripheral CD8$^+$ T Cells

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The affinity for TCR interactions with self-peptide/MHC complexes (pMHC) in the thymus critically affects immature thymocytes that newly express TCRs. Previous fetal thymus organ culture experiments have indicated that difference in the affinity for thymic TCR/pMHC interactions not only determines thymocyte fate between positive and negative selection, but also affects Ag responsiveness of positively selected thymocytes. In the current study, we examined whether TCR/pMHC affinity during positive selection in the thymus would further affect Ag responsiveness of mature T cells in the periphery. To do so, OVA peptide variants were in vivo administered to TAP1-deficient OT-I/TCR-transgenic mice in which T cell development was otherwise arrested at CD4$^+$CD8$^+$ thymocytes because of the lack of self-pMHC presentation in thymic APCs. We found that a group of peptide variants induced the transient generation of OT-I CD8$^+$ T cells in the thymus and the periphery. We also noticed that the affinity threshold for positive and negative selection detected in adult mice in vivo was higher than that measured in fetal thymus organ culture experiments in vitro. Interestingly, we further found that the affinity for positively selecting peptides proportionally affected TCR responsiveness of peripheral naive CD8$^+$ T cells. These results indicate that in vivo administration of a peptide can promote T cell selection in the thymus and the affinity for TCR/pMHC interaction during positive selection fine-tunes Ag responsiveness of peripheral T cells. ***The Journal of Immunology*****, 2019, 203: 881–887.**

Self-antigen recognition in the thymus determines the fate of newly generated T cells. The interaction between TCR expressed by developing thymocytes and self-peptide/MHC complexes (pMHC) displayed in the thymus critically affects the developmental outcome of thymocytes, determining their survival or absence (i.e., positive and negative selection) and their lineage direction to become functionally different cells (e.g., CD4 helper and CD8 killer). Studies using fetal thymus organ culture of TCR-transgenic thymocytes have indicated that a low-affinity interaction between TCR and pMHC promotes thymocyte maturation to give rise to functionally competent T cells (i.e., positive selection), whereas a high-affinity interaction causes the absence of self-reactive T cells (i.e., negative selection) (1–3). A narrow range of the TCR/pMHC affinity sets the threshold for positive and negative selection of developing thymocytes, contributing to the enrichment of functionally potent and self-protective T cells while excluding potentially harmful self-reactive T cells from a mature T cell pool (4, 5).

Recent experiments have indicated that TCR/pMHC affinity during positive selection in the thymus further affects TCR responsiveness of mature thymocytes. Within the window of the affinity for positively selecting TCR/pMHC interaction, a relatively high-affinity–mediated positive selection promotes the generation of mature thymocytes that express a large amount of cell-surface CD5 and that exhibit high TCR responsiveness, compared with mature thymocytes generated by a low-affinity–mediated positive selection (6). Fetal thymus organ culture experiments have demonstrated a direct link between TCR/pMHC affinity during positive selection and TCR responsiveness of mature thymocytes (6). A further link with peripheral T cell function was indirectly suggested by the amount of cell-surface CD5 molecules (7–9), which is strongly affected by TCR signals during and after thymic positive selection (10). Indeed, TCR signals that influence CD5 expression levels in T cells are not limited during positive selection in the thymus, but are widely distributed during subsequent T cell development, homeostasis, and immune response (7–10). Whether or not TCR/pMHC affinity during positive selection in the thymus remains influential to CD5 expression levels and TCR responsiveness of mature T cells in the periphery has not been addressed.

In the current study, we examined the effect of in vivo administration of various OVA antigenic peptide (OVAp) variants in OVA-Ag–specific, OT-I/TCR-transgenic, TAP1-deficient mice in which T cell development was otherwise arrested at CD4$^+$CD8$^+$ thymocytes because of the lack of positive-selection–inducing

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Abbreviations used in this article: OVAp, OVA antigenic peptide; pMHC, peptide/MHC complex.

self-pMHC presentation in the thymus (11, 12). Our results show the following: 1) the injection of a group of peptide variants induced the generation of a cohort of OT-I $CD8^+$ T cells in the thymus and the periphery, 2) the affinity threshold for positive and negative selection by the peptide injection experiments in adult mice in vivo was higher than that previously measured in fetal thymus organ culture experiments in vitro, and 3) the affinity for positively selecting peptides proportionally affected Ag responsiveness of $CD8^+$ T cells in the periphery. Thus, our results indicate that the in vivo administration of a peptide can modulate Ag-specific T cell repertoire selection in the thymus and that the affinity for TCR/pMHC interaction during positive selection influences TCR responsiveness of mature T cells in the periphery.

Materials and Methods

Mice

TAP1-deficient, OT-I/TCR-transgenic mice (4, 11) were maintained under specific pathogen-free conditions in the Institute of Advanced Medical Sciences at the University of Tokushima. All animal experiments were performed with approval from the Animal Experimentation Committee at the University of Tokushima.

In vivo peptide administration

OVA aa 257–264 peptide SIINFEKL (OVAp) and its variants EIINFEKL (E1), SIIQFEHL (Q4H7), SIITFEKL (T4), SIIQFERL (Q4R7), and SIIQFEKL (Q4) as well as vesicular stomatitis virus 8 (VSV8) aa 52–59 peptide RGYVYQGL were purchased from GenScript. TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old were i.p. injected with 25–100 μg of peptide in 100 μl of PBS. Mice were analyzed on the indicated day after the peptide administration.

Fetal thymus organ culture

Fetal thymus lobes isolated from TAP1-deficient, OT-I/TCR-transgenic mice on embryonic day 15.5 were organ cultured for 4 d as described (13). Indicated peptides were included in the culture at 20 μM. Cells were analyzed by multicolor flow cytometry.

Thymus section analysis

Coronal sections of frozen thymus at 10-μm thickness were stained for H&E. Microscopic images were analyzed for cortical and medullary regions using Adobe Photoshop, as previously described (14, 15).

Flow cytometry and cell sorting

Single-cell suspensions isolated from indicated mouse organs were stained on ice with Abs specific for CD4 (RM4-5; eBioscience), CD5 (53-7.3; eBioscience), CD8α (53-6.7; eBioscience), CD8β (YTS 156.7.7; Absolute Ab), CD25 (3C7; BioLegend), CD44 (IM7; eBioscience), CD62L (MEL-14; BioLegend), CD69 (HI.2F3; BioLegend), CD122 (5H4; BioLegend), CD132 (TUGm2; BioLegend), and Vα2 (B20.1; BioLegend). For the staining with Ab specific for CCR7 (4B12; eBioscience), cells were incubated at 37°C for 30 min before the staining with other Abs. To isolate naive $CD8^+$ T cells, spleen cells were stained with biotinylated Abs specific for CD4, CD44, and B220 (RA3-6B2; BioLegend), and cells that did not bind to the Abs were enriched with magnetic bead–conjugated streptavidin (Miltenyi Biotec). Multicolor flow cytometry and cell sorting were performed on FACSVerse and FACSAria II (BD Biosciences), respectively.

T cell stimulation

Cells were placed in culture wells precoated with anti-CD3ε Ab (145-2C11; eBioscience) at the indicated concentrations and 10 μg/ml of anti-CD28 Ab (37.51; eBioscience). Where indicated, cells were cultured with OVAp-K^b peptide MHC tetramers (MBL) at the indicated dilutions. Cells were stimulated at 37°C for 3 h and CD69 expression was measured by flow cytometry.

Statistical analysis

Statistical significance was evaluated using the two-tailed unpaired Student *t* test. All p values <0.05 were considered statistically significant.

Results

Effects of OVA peptide variants in TAP1-deficient, OT-I/TCR-transgenic fetal thymus organ cultures

To examine the effects of antigenic peptide variants in vivo, we first verified the effects of those peptides in TAP1-deficient, OT-I/TCR-transgenic fetal thymus organ cultures in vitro. It was previously reported that OVA peptide variants could promote positive and negative selection in β2m-deficient, OT-I/TCR-transgenic fetal thymus organ cultures (1, 4). In β2m-deficient, OT-I/TCR-transgenic mice, β2m deficiency causes the lack of cell-surface MHC class I molecules and, thereby, the loss of positive selection for the generation of $CD8^+$ T cells, which is dependent on TCR recognition of self-pMHC class I complexes in the thymus (1, 4). The addition of OVA peptide variants in the fetal thymus organ cultures identified that E1 and Q4H7 peptides, which exhibit low affinity for OT-I/TCR along with MHC class I molecule K^b, promote positive selection for the generation of $CD4^-CD8^+$ thymocytes, whereas high-affinity peptides, Q4R7 and Q4, as well as the original OVAp, induce negative selection to delete or developmentally deviate immature thymocytes (4). The intermediate-affinity peptide T4 exhibited the threshold between positive and negative selection (4).

Similarly, we detected the arrest of thymocyte development at the $CD4^+CD8^+$ stage in TAP1-deficient, OT-I/TCR-transgenic fetal thymus organ cultures (Fig. 1A) in which the lack of peptide transporter TAP1 causes the defective expression of self-pMHC class I complexes on the cell surface and the defective

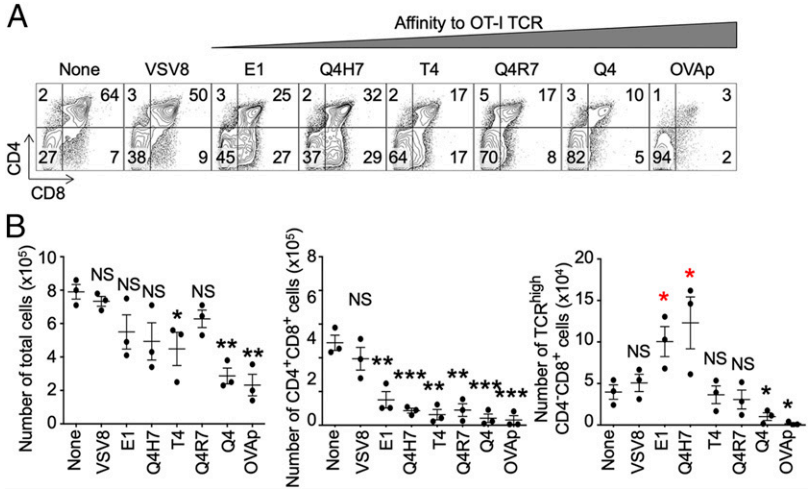

FIGURE 1. E15.5 fetal thymus of TAP1-deficient, OT-I/TCR-transgenic mice was cultured in the presence or absence of indicated peptides (20 μM). VSV8 peptide with an irrelevant specificity was used as control. (**A**) Representative CD4 and CD8β profiles of PI^- viable thymocytes are shown. Numbers indicate the frequencies of cells in the quadrants. (**B**) Numbers (means ± SEs; $n = 3$) of total, $CD4^+CD8^+$, and TCR^{high} $CD4^-CD8^+$ thymocytes are plotted. Statistically significant increase in cell number is highlighted by red asterisks. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

positive selection for the generation of CD8$^+$ T cells (11, 12). In agreement with previous reports, only the low-affinity peptides, E1 and Q4H7, with the affinity below the reported threshold between positive and negative selection, promoted the appearance of CD4$^-$CD8$^+$ thymocytes in TAP1-deficient, OT-I/TCR-transgenic fetal thymus organ cultures (Fig. 1A, 1B).

These results agreed with previous results and verified the effects of graded-affinity OVA peptide variants on thymocyte development in TAP1-deficient, OT-I/TCR-transgenic mice in fetal thymus organ culture experiments.

In vivo administration of OVA peptide variants in TAP1-deficient, OT-I/TCR-transgenic mice generates a cohort of CD4$^-$CD8$^+$ mature thymocytes

We then examined the effects of antigenic peptide variants in vivo by i.p. injecting various OVA peptide variants into TAP1-deficient, OT-I/TCR-transgenic adult mice in which thymic positive selection for generating CD8$^+$ T cells is arrested (Fig. 2A). We noticed that the administration of high-affinity peptides Q4 and OVAp caused the loss of CD4$^+$CD8$^+$ thymocytes rapidly by day 2 (Fig. 2A), in agreement with the negative selection of CD4$^+$CD8$^+$ thymocytes in fetal thymus organ culture (Fig. 1). Unlike fetal thymus organ culture, however, we failed to detect a clear generation of CD4$^-$CD8$^+$ thymocytes by the administration of low-affinity peptide E1. Instead, we noticed that the previously reported intermediate-affinity threshold peptides, T4 and Q4R7, elevated the frequency of CD4$^-$CD8$^+$ thymocytes on day 4. The increase in the frequency of CD4$^-$CD8$^+$ thymocytes reflected the increase in the absolute number of mature TCR-Vα2^{high} CD4$^-$CD8$^+$ thymocytes (Fig. 2B), whereas those TCR^{high} CD4$^-$CD8$^+$ thymocytes did not contain CD25^{high} proliferating cells (Fig. 3A), indicating that T4 and Q4R7 promoted the de novo generation of OT-I/TCR^{high} CD8$^+$ T cells rather than induced the outgrowth of a small number of pre-existing TCR^{high} CD4$^-$CD8$^+$ mature thymocytes or caused the accumulation of TCR^{low} CD4$^-$CD8$^+$ immature precursor cells. We also noted that T4 and Q4R7 enlarged the size of the thymic medullary region on day 4, further supporting that T4 and Q4R7 promoted the thymic generation of OT-I/TCR^{high} CD8$^+$ mature thymocytes (Fig. 3B). In addition to T4 and Q4R7, the low-affinity peptide Q4H7 also promoted the generation of mature TCR-Vα2^{high} CD4$^-$CD8$^+$ thymocytes, albeit at a low cellularity (Fig. 2A, 2B). The peptide-induced generation of TCR^{high} CD4$^-$CD8$^+$ thymocytes was apparent on day 4 and day 5 after the peptide administration and disappeared on day 7 or later (Fig. 2A, 2B), indicating that the peptide-induced positive selection was transient. The decrease in CD4$^+$CD8$^+$ thymocytes by the high-affinity peptides was also transient, and the cell number recovered to normalcy by day 7 (Fig. 2A, 2B). We also noted that the 2-fold increase or decrease in the dosage of injected peptides did not markedly alter the effects onto TAP1-deficient, OT-I/TCR-transgenic CD4$^+$CD8$^+$ and CD4$^-$CD8$^+$ thymocytes on day 4 (Fig. 3C).

These results indicate that the in vivo administration of antigenic peptide variants promotes the transient generation of a cohort of TCR^{high} CD4$^-$CD8$^+$ mature thymocytes. The affinity threshold for positive and negative selection in vivo appears different from and higher than that detected in vitro.

In vivo administration of OVA peptide variants in TAP1-deficient, OT-I/TCR-transgenic mice generates a cohort of CD8$^+$ T cells in the periphery

We next examined whether the peptide-mediated positive selection in the thymus in vivo would lead to the generation of T cells in the periphery. To do so, we analyzed spleen cells in TAP1-deficient, OT-I/TCR-transgenic mice that were injected with OVA peptide variants (Figs. 4, 5), and found that T4 and Q4R7 promoted a significant ($p < 0.05$) increase in the cellularity of TCR-Vα2$^+$ CD8$^+$ OT-I/TCR-transgenic CD44^{low}CD62L^{high} naive T cells on day 9 (Figs. 4, 5A, 5B). The increase was transient, as the significant increase in the number of OT-I/TCR-transgenic naive

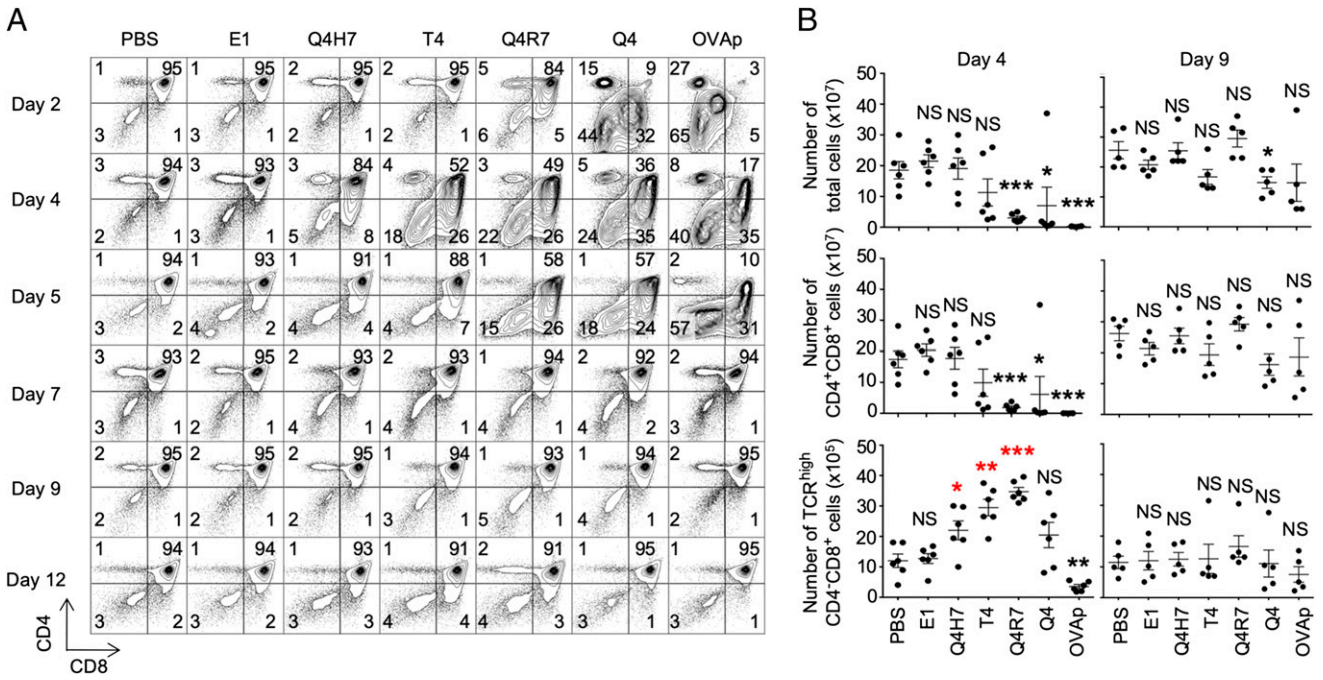

FIGURE 2. Indicated peptides were i.p. administered to TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old. (**A**) Representative CD4 and CD8 profiles of PI$^-$ viable thymocytes on indicated days after the administration of 50 μg of peptides are shown. Numbers indicate frequencies of cells in quadrants. (**B**) Numbers (means ± SEs; n = 5–6) of total, CD4$^+$CD8$^+$, and TCR^{high} CD4$^-$CD8$^+$ thymocytes are plotted. Statistically significant increase in cell number is highlighted by red asterisks. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

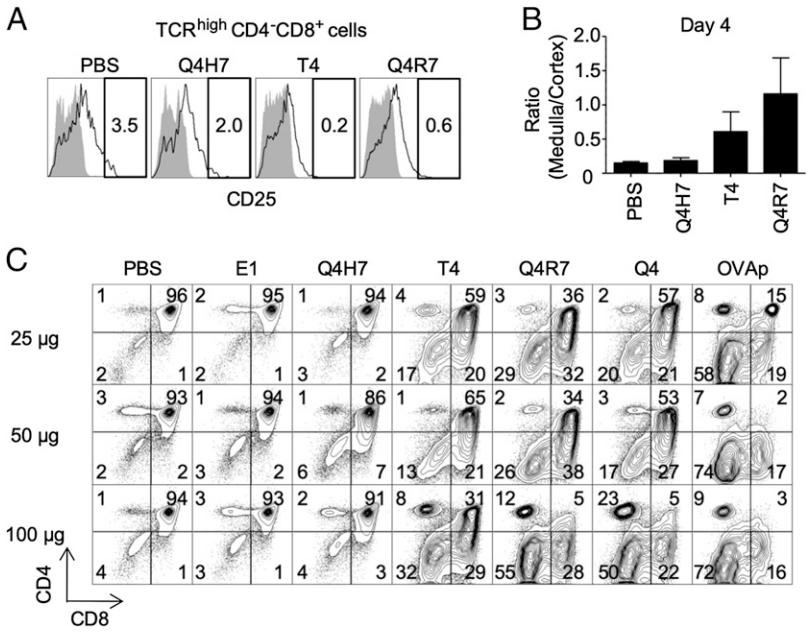

FIGURE 3. Indicated peptides were i.p. administered in TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old. (**A**) CD25 expression in TCR^{high} $CD4^{-}CD8^{+}$ thymocytes on day 4. Numbers indicate frequencies of cells in boxes. (**B**) Thymic sections were stained for H&E. Areas of the medullary regions normalized to areas of the cortical regions (means ± SEs; $n = 4–5$) are plotted. (**C**) Representative CD4 and CD8 profiles of PI^{-} viable thymocytes on day 4 after administration of indicated concentrations of peptides are shown. Numbers indicate frequencies of cells in quadrants.

T cells was detectable on day 9, but not day 4 or day 12 (Fig. 5A, 5B). The transiency was presumably due to the lack of self-pMHC required for the survival of newly generated T cells in TAP1-deficient mice. Meanwhile, the significant increase in the number of OT-I/TCR-transgenic naive T cells was systemic, as OT-I/TCR-transgenic naive T cells on day 9 was detectable not only in the spleen, but also in the lymph nodes (Fig. 5B, 5C). The increased OT-I/TCR-transgenic $CD44^{low}CD62L^{high}$ $CD8^{+}$ T cells on day 9 were CD25 $(IL\text{-}2R\alpha)^{low}$ CD122 $(IL\text{-}2R\beta)^{low}$ CD132 $(IL\text{-}2R\gamma)^{low}$ (Fig. 5D), indicating that T4 and Q4R7 promoted the de novo generation of OT-I/TCR-transgenic naive $CD8^{+}$ T cells rather than the outgrowth of a small number of preexisting $CD8^{+}$ T cells in the spleen.

These results indicate that the in vivo administration of antigenic peptide variants promotes the transient generation of naive $CD8^{+}$ T cells in the periphery, subsequent to the transient generation of TCR^{high} $CD4^{-}CD8^{+}$ mature thymocytes in the thymus. The generation of peripheral $CD8^{+}$ naive T cells was dependent on the affinity of injected peptides. Similar to the generation of TCR^{high} $CD4^{-}CD8^{+}$ mature thymocytes, the affinity required for the generation of peripheral T cells was higher in in vivo peptide injection experiments than in in vitro fetal thymus organ culture experiments.

TCR affinity for thymic selection affects CD5 expression levels in peripheral T cells

We also wanted to examine whether TCR affinity among the peptide ligands that promote positive selection in vivo would affect the phenotype of positively selected T cells in the thymus and the periphery. It was shown that the ligand affinity for TCR engagement during positive selection at $CD4^{+}CD8^{+}$ thymocytes is proportionally correlated with the expression level of cell-surface CD5 in TCR-stimulated thymocytes, including positively selected CD4/CD8 single-positive thymocytes (10). However, it was unclear how TCR-ligand affinity during positive selection at $CD4^{+}CD8^{+}$ thymocytes would persistently affect CD5 expression level in T cells beyond the export from the thymus to the periphery. As shown in Fig. 5, CD5 expression levels in positively selected TCR^{high} $CD4^{-}CD8^{+}$ thymocytes detected on day 4 and in TCR^{high} $CD4^{-}CD8^{+}$ $CD44^{low}CD62L^{high}$ spleen naive T cells detected on day 9 were proportionally correlated with OT-I/TCR affinity of the injected peptide variants between Q4H7 and T4 (Fig. 6A, 6B). In contrast, the proportional correlation was less clear and not statistically significant in positively selected thymocytes and spleen naive T cells between T4-injected mice and Q4R7-injected mice (Fig. 6A, 6B), even though the affinity for OT-I/TCR is different between these two peptides (T4 < Q4R7). Unlike CD5 expression levels, the expression levels of TCR and CD8 in TCR^{high} $CD4^{-}CD8^{+}$ $CD44^{low}CD62L^{high}$ spleen naive T cells on day 9 were not significantly different between T4-injected mice and Q4R7-injected mice (Fig. 7).

These results indicate that cell-surface CD5 expression levels in positively selected $CD4^{-}CD8^{+}$ thymocytes and even in peripheral $CD4^{-}CD8^{+}$ naive T cells are proportionally correlated with TCR ligand affinity for positive selection when Q4H7 and T4 peptides are compared, although the proportional correlation between TCR

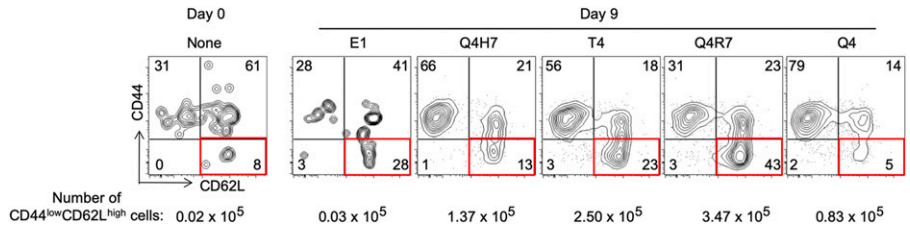

FIGURE 4. Indicated peptides (50 µg) were i.p. administered to TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old. Spleen cells were analyzed on indicated days after the administration of peptides. Representative CD44 and CD62L profiles of $CD8^{+}V\alpha2^{high}$ cells are shown. Numbers in quadrants indicate frequencies of cells in boxes. Numbers at the bottom indicate absolute numbers per mouse of $CD44^{low}CD62L^{high}$ $CD8^{+}V\alpha2^{high}$ cells (shown in red boxes).

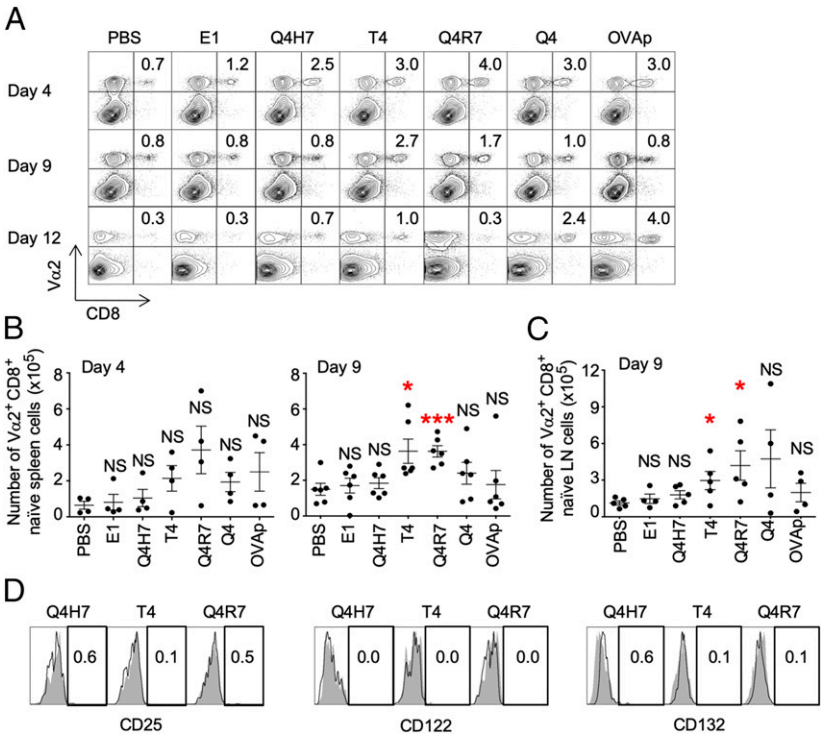

FIGURE 5. Indicated peptides (50 μg) were i.p. administered to TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old. (**A**) Spleen cells were analyzed on indicated days after the administration of peptides. Representative CD8 and Vα2 profiles of $CD44^{low}CD62L^{high}$ cells are shown. Numbers indicate frequencies of cells in boxes. (**B**) Numbers (means ± SEs) of $V\alpha2^+CD8^+CD44^{low}CD62L^{high}$ naive T cells in the spleen on day 4 ($n = 4$) and day 9 ($n = 6$) are plotted. (**C**) Numbers (means ± SEs; $n = 4$) of $V\alpha2^+CD8^+CD44^{low}CD62L^{high}$ naive T cells in the lymph nodes on day 9. Statistically significant increase in cell number is highlighted by red asterisks. $*p < 0.05$, $***p < 0.001$. (**D**) CD25, CD122, and CD132 expression in $V\alpha2^+CD8^+CD44^{low}CD62L^{high}$ naive T cells in the spleen on day 9. Numbers indicate frequencies of cells in boxes.

ligand affinity for thymic positive selection and CD5 expression level in T cells is not always clear and insignificant at the affinity between T4 and Q4R7 peptides.

TCR affinity for positive selection affects Ag responsiveness of peripheral T cells

We finally examined whether TCR ligand affinity during peptide-induced positive selection in vivo would affect TCR responsiveness of positively selected T cells. We found that CD69 upregulation response to TCR stimulation with anti-CD3 plus anti-CD28 Abs in positively selected $TCR^{high}CD4^-CD8^+$ thymocytes was proportionally correlated with TCR affinity among Q4H7, T4, and Q4R7 peptide variants injected for inducing positive selection (Fig. 8A). CD69 response to OVAp-K^b tetramer was similarly influenced by TCR affinity for positive-selection–inducing peptide variants, even though the difference in CD69 upregulation was more pronounced between Q4H7 and T4 peptides and less pronounced between T4 and Q4R7 peptides (Fig. 8B). TCR affinity for the injected peptide variants further affected TCR-stimulated CD69 responses in $CD4^-CD8^+$ $CD44^{low}CD62L^{high}$ naive spleen T cells in a similarly proportional manner (Fig. 8C, 8D). However, TCR responsiveness of $CD4^-CD8^+$ $CD44^{low}CD62L^{high}$ naive spleen T cells was not significantly different among the different time points between day 4 and day 12 after the peptide administration (Fig. 8E, 8F), despite that the peptide-promoted T cell generation was transient and maximum on day 9 after peptide injection (Fig. 5A, 5B). These results indicate that TCR responsiveness of positively selected $CD4^-CD8^+$ thymocytes, and peripheral $CD4^-CD8^+$ naive T cells is proportionally correlated with TCR affinity for in vivo injected positive-selection–inducing peptides.

Discussion

The results obtained in this study demonstrate that the in vivo administration of OVA peptide variants T4 and Q4R7 induces the generation of OT-I $CD8^+$ T cells in the thymus and the periphery. The peptide-induced generation of $CD8^+$ T cells was transient, as the thymic cohort was optimally detected 4 d after the peptide administration and the cohort in the secondary lymphoid organs, including the spleen and the lymph nodes, was optimally detected 9 d after the administration. The generated $CD8^+$ T cells were phenotypically normal, without overexpression of IL-2 receptor chains, reflecting the consequence of de novo positive selection in the thymus rather than the outgrowth of a small number of preexisting T cells or the accumulation of aberrant T cells. It was previously shown that the keratin 14 promoter–driven transgenic expression of another low-affinity peptide variant E1 in TAP1-deficient, OT-I/TCR-transgenic mice restored the generation of OT-I $CD8^+$ T cells in the thymus and the spleen (16). The keratin 14 promoter drives transgene expression in epithelial cells, including cortical thymic epithelial cells (16, 17), suggesting that E1-mediated positive selection in the thymic cortex is responsible for the restoration of OT-I $CD8^+$ T cells in vivo. These results together indicate that the in vivo delivery of a peptide could induce positive selection of T cells that interact with the pMHC at a low affinity in the thymus.

The in vivo administration of peptides offers promise as therapy for various human diseases, including cancer and diabetes (18, 19). Peptides are also included in nutritional supplements and functional diets (20, 21). Our results indicate that peptide administration in vivo can affect thymocyte development, including the alteration of the T cell repertoire by the positive selection of T cells that interact with the pMHC in the thymus at a low affinity. Careful consideration of possible peptide delivery into the thymus and the subsequent alteration of thymocyte selection and T cell development may help avoiding the adverse effects of peptide administration in humans.

Our results show that the affinity threshold for positive and negative selection in in vivo peptide injection experiments is different from and higher than that measured in fetal thymus organ culture experiments in vitro. Previous fetal thymus organ culture experiments have shown that a set of low-affinity peptides, including E1 and Q4H7, promotes the positive selection of OT-I $CD8^+$ T cells, whereas a set of high-affinity peptides, including Q4R7, Q4, and OVAp, causes the negative selection of OT-I/TCR-expressing thymocytes (1, 4). It was also shown that the T4

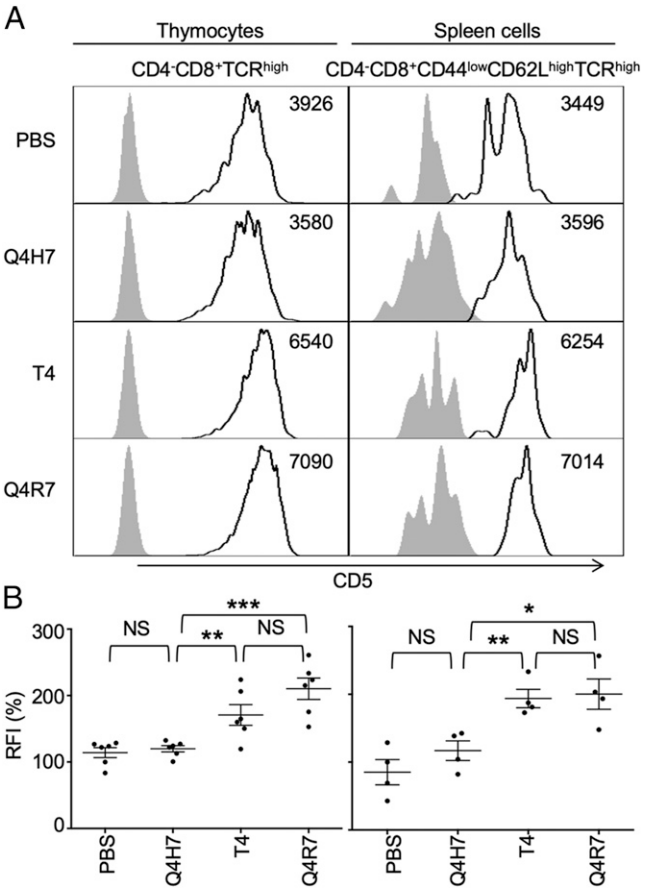

FIGURE 6. Indicated peptides (50 μg) were i.p. administered to TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old. (**A**) CD5 expression was analyzed on day 4 for $CD4^{-}CD8^{+}TCR^{high}$ thymocytes and on day 9 for $CD4^{-}CD8^{+}CD44^{low}CD62L^{high}TCR^{high}$ spleen cells. Shaded histograms indicate profiles with control IgG. Numbers indicate median fluorescent intensities of CD5 histograms. (**B**) Relative median fluorescence intensity (RFI) (means ± SEs; n = 4–6) of CD5 expression normalized to the median fluorescence intensity in PBS-injected group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

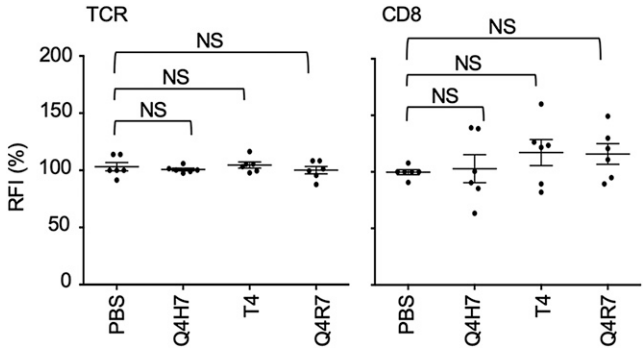

FIGURE 7. Indicated peptides (50 μg) were i.p. administered to TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old. Cell-surface expression of TCRβ and CD8α was analyzed on day 9 for $CD4^{-}CD8^{+}CD44^{low}CD62L^{high}TCR^{high}$ spleen cells. Relative median fluorescence intensity (RFI) (means ± SEs; n = 6) of TCRβ and CD8α expression normalized to the median fluorescence intensity in PBS-injected group. $p \geq 0.05$.

peptide exhibits an affinity threshold between positive and negative selection for OT-I thymocytes (4). As shown in Fig. 1 in this study, our results from fetal thymus organ culture experiments agree with published results, including the affinity threshold for OT-I thymocytes. In contrast, however, our results from in vivo peptide injection experiments show that T4 and Q4R7 promoted the positive selection of OT-I $CD8^{+}$ T cells, whereas the injection of Q4 and OVAp did not elevate the cellularity of OT-I $CD8^{+}$ T cells in the thymus, indicating that the affinity threshold detected from in vivo peptide injection experiments is higher than that measured in fetal thymus organ culture experiments in vitro. We speculate that the difference between the results of those in vivo and in vitro experiments could be due to the difference in the accessibility of the peptides to form the peptide MHC class I complexes displayed by cortical thymic epithelial cells and, thereby, the difference in the availability and the avidity of the peptide MHC class I complexes to induce thymocyte positive selection. Indeed, it was previously demonstrated that the different availability of selecting peptides, including the T4 threshold peptide used in this study, markedly affects the fate of developing thymocytes (4, 5, 22). It has been also shown that peptides tend to have short half-lives in vivo and, therefore, be low in bioavailability for function (18, 19, 23), which may contribute to the limited availability of selecting peptides in the thymus by in vivo administration. It is interesting to note that peptide E1, which induced positive selection in vivo by K14 promoter–mediated transgenic expression in thymic epithelial cells (16), did not promote positive selection in our peptide injection experiments. This difference may also result from the difference in the abundance and the avidity of E1-K^{b} complexes in the thymic cortex. The transgenic expression in thymic epithelial cells may provide a much higher abundance in the formation of E1-K^{b} complexes in the thymic cortex than the i.p. peptide injection. Thus, the affinity threshold between positive and negative selection shared by several TCR-transgenic thymocytes (5) may be applicable only in certain experimental conditions in which cortical thymic epithelial cells are sufficiently saturated with positive-selection–inducing pMHC, for example, by peptide addition in in vitro fetal thymus organ culture experiments or transgenic expression of the peptide in thymic epithelial cells. To clarify these issues, it would, therefore, be interesting to measure the amount of actual selecting pMHC in the thymus, particularly those expressed by cortical thymic epithelial cells, for example, by using Abs or TCR tetramers specific for those selecting pMHC (24, 25).

Most interestingly, our results demonstrate that TCR affinity for positive-selection–inducing OVA peptide variants influences TCR responsiveness of positively selected OT-I $CD8^{+}$ T cells in the thymus and the periphery. It was previously shown that TCR/pMHC affinity during positive selection proportionally affects TCR responsiveness of mature thymocytes (6). However, it was not shown that the affinity for positively selecting TCR/pMHC interaction further affects TCR responsiveness of mature T cells in the periphery, even though the possibility was indirectly suggested by the analysis of cell-surface CD5 expression levels in mature peripheral T cells (7–9). In contrast, our results directly demonstrate that the difference in TCR affinity among Q4H7, T4, and Q4R7 during thymic positive selection proportionally programs the intrinsic difference in TCR responsiveness of positively selected OT-I $CD8^{+}$ T cells in the spleen. These results may be relevant considering that TCR-mediated positive selection in the thymus actually corresponds to the primary response for newly generated T cells (26). The quality of Ag receptor engagement during the primary response (i.e., positive selection of newly generated T cells in the thymus) may well affect the subsequent functional behavior of peripheral T cells during immune responses.

The CD5 expression level in spleen naive OT-I $CD8^{+}$ T cells was also proportionally correlated with positive-selection–inducing TCR

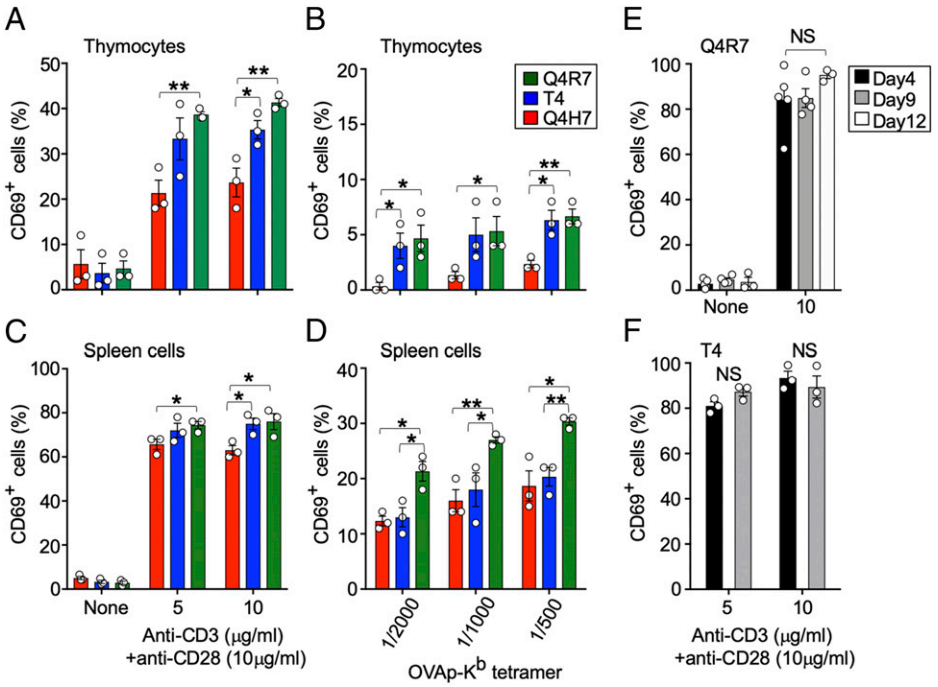

FIGURE 8. Indicated peptides (50 μg) were i.p. administered to TAP1-deficient, OT-I/TCR-transgenic mice at 4 wk old. TCR^{high} $CD4^{-}CD8^{+}$ thymocytes obtained on day 4 (**A** and **B**) and $TCR^{+}CD8^{+}CD44^{low}CD62L^{high}$ spleen naive T cells obtained on day 9 (**C** and **D**) were stimulated with indicated concentrations of plate-bound anti-CD3 Ab in the presence of anti-CD28 Ab (10 μg/ml) (A and C) or indicated dilutions of OVAp-K^{b} peptide/MHC tetramers (B and D) for 3 h. (**E** and **F**) $TCR^{+}CD8^{+}CD44^{low}CD62L^{high}$ spleen naive T cells from indicated days after the administration of Q4R7 (E) or T4 (F) peptide were stimulated with indicated concentrations of plate-bound anti-CD3 Ab in the presence of anti-CD28 Ab (10 μg/ml). Plotted are frequencies of CD69-expressing cells (means ± SEs; $n = 3$–5). $^{*}p < 0.05$, $^{**}p < 0.01$.

ligand affinity between Q4H7 and T4, whereas the proportional correlation was nonsignificant between T4 and Q4R7 for positive selection in the thymus. These results indicate that the difference in TCR affinity during positive selection does not always cause clear difference in CD5 expression level in naive T cells in the periphery.

In conclusion, our results indicate that the in vivo administration of a peptide can induce T cell positive selection in the thymus and that the affinity for TCR/pMHC interaction during positive selection in the thymus fine-tunes Ag responsiveness of mature T cells in the periphery.

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Disclosures

The authors have no financial conflicts of interest.

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