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Peptide Variants of Viral CTL Epitopes Mediate Positive Selection and Emigration of Ag-Specific Thymocytes In Vivo

Masha Fridkis-Hareli, Pedro A. Reche, and Ellis L. Reinherz

During development, thymocytes carrying TCRs mediating low-affinity interactions with MHC-bound self-peptides are positively selected for export into the mature peripheral T lymphocyte pool. Thus, exogenous administration of certain altered peptide ligands (APL) with reduced TCR affinity relative to cognate Ags may provide a tool to elicit maturation of desired TCR specificities. To test this “thymic vaccination” concept, we designed APL of the viral CTL epitopes gp33–41 and vesicular stomatitis virus nucleoprotein octapeptide N52–59 relevant for the lymphocytic choriomeningitis virus-specific P14- and vesicular stomatitis virus-specific N15-TCRs, respectively, and examined their effects on thymocytes in vivo using irradiation chimeras. Injection of APL into irradiated congenic (Ly-5.1) mice, reconstituted with T cell progenitors from the bone marrow of P14 RAG2−/− (Ly-5.2) or N15 RAG2−/− (Ly-5.2) transgenic mice, resulted in positive selection of T cells expressing the relevant specificity. Moreover, the variants led to export of virus-specific T cells to lymph nodes, but without inducing T cell proliferation. These findings show that the mature T cell repertoire can be altered by in vivo peptide administration through manipulation of thymic selection. The Journal of Immunology, 2004, 173: 1140–1150.

Naive T cells expressing a highly diverse TCR repertoire are generated in the thymus from bone marrow (BM) lymphoid precursors (reviewed in Ref. 1). Upon entering the thymus, T cell progenitors proliferate and undergo a complex series of gene rearrangement events leading to cell surface TCR expression and subsequent differentiation (reviewed in Refs. 2 and 3). It has been demonstrated that peptides bound to the MHC molecules within the thymus control both positive and negative selection (reviewed in Ref. 4). During selection of the TCR repertoire, thymocytes that carry TCRs having low-affinity interactions with MHC-bound self-peptides are positively selected, and are exported into the pool of mature peripheral lymphocytes. In contrast, thymocytes bearing those TCRs that recognize self-peptides with high affinity are eliminated (3).

Single amino acid substitutions in either the MHC or the peptide dramatically alter recognition by T cells (5, 6). Analysis of crystal structures of αβ TCR/class I MHC complexes have demonstrated that peptide specificity of T cells is primarily determined by the interaction between the CDR of the TCR-Vα and -Vβ domains and the peptide side chains, which protrude of the peptide-binding groove of MHC molecules toward the two TCR-CDR3 loops. Structural studies of peptide/MHC complexes (pMHC) have provided detailed information about the conformation of peptide when bound to MHC molecules (reviewed in Refs. 7 and 8). The peptide-binding groove of MHC molecules is composed of two helices on top of an eight-strand anti-parallel β-pleated sheet. The peptide-binding groove contains various binding pockets, the shape and charge of which are dependent on the highly polymorphic amino acids characteristic of a given MHC allele, which in turn selectively determines the spectrum of peptides that may bind to it (reviewed in Ref. 9 and references therein).

Much of the recent work on thymic selection was influenced by experiments that examined T cell responses to peptide analogues derived from the antigenic peptide by substitution of amino acid residues involved in interactions with the TCR. Such peptide analogues, so-called altered peptide ligands (APL), can generate qualitatively different T cell responses compared with those produced by the antigenic peptide (10). In particular, some APL were shown to act as TCR antagonists and inhibit T cell responses to the antigenic peptide (11). Several studies have shown that antagonist peptides are capable of positively selecting (12, 13), negatively selecting (14), or otherwise altering (15) selection of thymocytes. Thymic selection processes have also been addressed in structural terms using TCR-transgenic mice. For example, in N15 transgenic mice carrying a TCR specific for the vesicular stomatitis virus nucleoprotein octapeptide N52–59 (VSV8) in the context of H-2Kb, a weak agonist peptide variant inducing positive selection has been identified (16). This variant is identical with the VSV8 peptide except for substitution of leucine for valine at the P4 peptide residue (L4). The cognate viral peptide ligand, VSV8, triggers negative selection. Another TCR transgenic mouse model, P14, expressing a TCR specific for the Dψ-restricted immunodominant lymphocytic choriomeningitis virus epitope gp33–41 has been developed (17). This system has been widely used to study the effect on thymocyte development of mutations in gp33–41 peptides that interact either with the binding pockets of Dψ (18) or with the TCR contact residues, using fetal thymic organ culture (FTOC; reviewed in Ref. 19). The crystal structure of gp33/H-2Dψ shows that conserved single mutations at positions 4 or 6 of the peptide are solvent exposed and presumably function as TCR contacts (20, 21). In yet a third TCR transgenic mouse model, F5, where the TCR recognizes a nucleoprotein peptide of the influenza virus...
NP366–379 in the context of H-2D\(^b\) (22, 23), the peptide antagonist mediated positive selection in FTOC (23, 24), whereas the cognate peptide itself led to deletion of CD4\(^+\)CD8\(^+\) (double-positive (DP)) thymocytes (25).

Variants of peptides derived from infectious agents or tumor Ags could, in principle, mediate positive selection and export of specific T cells from the thymus. As such, these APL might be candidates for manipulating the thymic repertoire in vivo, controlling the generation of naive and memory T cells within the peripheral lymphoid compartment. This “thymic vaccination approach” would aim to deliver, by parenteral administration, Ags that would either prevent or reverse thymocyte maturation and emigration in vivo in two well-defined systems.

**Materials and Methods**

**Mice**

N15 tg \(^{+/+}\) RAG2\(^{-/-}\) H-2\(^b\) mice were generated as described previously (26). P14 tg \(^{+/+}\) RAG2\(^{-/-}\) H-2\(^a\) transgenic mice were obtained from Taconic Farms (Germantown, NY). Congenic strains C57BL/6 (Ly-5.2) and C57BL/6 (Ly-5.1) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were sex matched and used at 3–4 wk of age for peptide injections and at 7–11 wk of age for other manipulations. Mice were maintained and bred under sterile barrier conditions at the animal facility of the Dana-Farber Cancer Institute (Boston, MA).

**Peptide synthesis**

The peptide gp33–41 (KAVYNFATC) and its variants, Y4S/F6A (KAVYNFETC) and A7E (KAVYNFETC), were synthesized by standard solid phase methods with the modification of C to M (C9M) to prevent dimer formation mediated by free sulfhydryl-groups. N52-59 (VSV8, RGYVYQGL; Ref. 27) and its variant, L4, (RGYLYQGL) were also made –41 (KAVYNFATC) and its variants, Y4S/F6A C9M, or A7E C9M peptides were produced using the method previously described (28). For immunofluorescence analysis, 1 \(\times\) 10\(^6\) cells (thymocytes, splenocytes, or lymph node cells) were incubated with FITC anti-CD8\(\alpha\) mAb for 1 h at 4°C, followed by addition of 0.5 \(\mu\)g of PE-labeled tetramers gp33–41\(\text{COM}^D\), Y4S/F6A\(\text{COM}^D\), or A7E\(\text{COM}^D\) and CyChrome-anti-CD4 and incubation for another hour. After two washes, cells were analyzed on a FACScan as described above.

**Miscellaneous assays**

**Results**

**Design and initial characterization of gp33–41 variant peptides**

gp33–41 is the cognate peptide Ag of the P14 TCR (Ve2 and YB8) and triggers negative selection of P14-bearing DP thymocytes (reviewed in Ref. 19). Structural variants of gp33–41 were designed to influence the outcome of thymocyte selection by altering the affinity of the pMHC ligand interactions with the TCR. No change was made in the peptide anchor residues that occupy the binding pockets of H-2\(^D\), thus ensuring proper peptide presentation in the context of MHC. Indeed, the crystal structure of the gp33–41/H-2\(^D\) complex shows that the side chains of amino acid residues at peptide positions p1, p4, p6, p7, and p8 are exposed to the solvent (20, 21). To design a variant with reduced affinity for the P14 TCR, we have introduced two types of mutations: in one mutant, both centrally disposed p4 and p6 residues have been modified (Tyr (Y) to Ser (S) at p4 and Phe (F) to Ala (A) at p6). In the other, Ala (A) was substituted with Glu (E) at p7. Both variants were synthesized in two alternative forms, one with the natural amino acid Cys (C) at the anchor residue p9, and the other with Met (M) at p9, thus avoiding any potential peptide dimerization mediated by free SH-groups. This modification was previously shown to stabilize the binding of gp33–41 peptide to H-2\(^D\) (32). The sequence of gp33–41 and the variant peptides and the relevant gp33–41\(\text{D}^\text{M}\) structure are shown in Fig. 1A.

Next, we verified whether these gp33–41 variant peptides were able to bind to H-2\(^D\) molecules using RMA-S cells. To this end, RMA-S cells were incubated with the peptides listed in Fig. 1A, and the extent of staining with anti-H-2\(^D\) Abs on the surface of peptide-loaded RMA-S cells was measured by FACS. The binding profiles are shown in Fig. 1B. Variant peptides Y4S/F6A\(\text{COM}\) and A7E\(\text{COM}\) bound equally well to H-2\(^D\) molecules, and in a similar fashion compared with the cognate gp33–41\(\text{COM}\) epitope, suggesting that amino acid substitutions at peptide residues p4, p6, and p7 indeed do not affect peptide binding, and, by extension, peptide presentation to T cells.

To evaluate the functional potential of T cells in mice injected with gp33–41 variant peptides, splenocyte, and lymph node T cell responses to the above peptides were examined for proliferation and cytokine secretion. Both splenocytes (Fig. 1C) and lymph node T cells (data not shown) proliferated in vitro in response to the gp33–41\(\text{COM}\) peptide, reaching a peak response at 10\(^{-10}\) M. The highest response to the A7E\(\text{COM}\) mutant peptide was achieved at the peptide concentration of 10\(^{-8}\) M (Fig. 1C). It should be noted that when a similar assay was performed using the gp33–41 variant peptides, the potency of either of these peptides at the peak of response was reduced by two logs relative to gp33–41\(\text{COM}\), namely, 10\(^{-8}\) M for the gp33–41 and 10\(^{-6}\) M for the A7E mutant (data not shown). In contrast, incubation of T cells with the Y4S/F6A\(\text{COM}\) peptide resulted in essentially no response at any peptide concentration, possibly due to low affinity interactions with the
TCR. Consistent with the proliferation data, an assay for intracellular cytokine staining with anti-IFN-γ or -IL-2 Abs showed the highest levels of both cytokines when splenocytes were incubated with the gp33–41 C9M, slightly lower levels in the presence of A7E C9M and no cytokine secretion in the presence of Y4S/F6A C9M peptide (Fig. 1D). Collectively, our results suggest that the Y4S/F6A C9M variant peptide (and Y4S/F6A, data not shown) does not elicit responses of mature T cells from P14 RAG2−/−/− mice.

Effect of gp33–41 variant peptides on thymocyte development in P14 RAG2−/−/− mice

To examine the effect of gp33–41 variant peptides on thymocyte development in P14 RAG2−/−/− mice, we developed a protocol for peptide injection in vivo. Earlier studies using N15 RAG2−/−/− transgenic mice showed that a single i.v. injection of VSV8 peptide leads to a severe depletion of DP thymocytes, whereas a variant of VSV8, L4 (V4L mutation at p4) mediates positive selection in FTOC (16). Here, in contrast, a single injection of gp33–41 C9M peptide into P14 RAG2−/− transgenic mice caused only a modest reduction in the percentage of the DP thymocytes, although the total number of thymocytes was reduced by approximately two-thirds (Fig. 2A, upper panel). Note that the down-regulation of both CD4 and CD8 on the DP thymocytes due to impending clonal deletion causes “spillover” into a single-positive (SP) CD8 gate, resulting in a higher percentage of SP CD8 cells as compared with the PBS control. However, injection of the double mutant Y4S/F6A C9M led to an unexpected tripling of total cell numbers, with no perturbation in subset distribution. In contrast, A7E C9M had no effect on the thymocyte number and only a slight increase in the percentages of DP or SP CD8 thymocyte subsets.

To next investigate the effect of variant peptides on the expression of thymocyte surface markers characteristic of maturation and/or activation states, cells were examined by triple-color immunofluorescence using various mAbs. A representative staining profile of SP CD8 thymocytes for the expression of β7 integrin, a marker linked to thymocyte emigration (33), is shown in Fig. 2A.
RAG2 had little influence on the percentage of DP thymocytes in P14−/− mice. Thymocytes from mice injected with gp33–41 variants. Upper panel, the CD4/CD8α profiles in thymocytes are altered in the presence of gp33–41 variant peptides. Thymocytes from P14 RAG2−/− mice at 3–4 wk of age, injected with gp33–41COM variants 18 h earlier (25 μg i.v.), were stained with CyChrome-anti-CD4, PE-anti-CD8α FITC-anti-β7 integrin. The percentages of DP and SP CD8 subsets after gating on 50,000 live cells are indicated. Lower panel, The histograms of β7 integrin expression on the gated SP CD8 thymocytes. The numbers represent the percentages of β7 integrin-positive cells. B, Expression of several T cell markers is altered on SP CD8 thymocytes from mice injected with gp33–41COM variant peptides. Thymocytes were stained with CyChrome-anti-CD4, PE-anti-CD8α, and FITC-anti-CD25, -CD44, -CD62L, -CD69, -β7 integrin, -Vβ8, -CD8β, and -CD5. Arrows up and down indicate up-regulation and down-regulation, respectively, as expressed by change in the MFI and/or percentage of positive cells; n.c., no change in the expression was detected as compared with control mice (injected with PBS). Results are representative of four independent experiments.

FIGURE 2. Modulation of thymocyte number and phenotype in P14 RAG2−/− mice by the Y4S/F6A COM variant. A, Triple-staining profiles of thymocytes from mice injected with gp33–41 variants. Upper panel, the CD4/CD8α profiles in thymocytes are altered in the presence of gp33–41 variant peptides. Thymocytes from P14 RAG2−/− mice at 3–4 wk of age, injected with gp33–41COM variants 18 h earlier (25 μg i.v.), were stained with CyChrome-anti-CD4, PE-anti-CD8α FITC-anti-β7 integrin. The percentages of DP and SP CD8 subsets after gating on 50,000 live cells are indicated. Lower panel, The histograms of β7 integrin expression on the gated SP CD8 thymocytes. The numbers represent the percentages of β7 integrin-positive cells. B, Expression of several T cell markers is altered on SP CD8 thymocytes from mice injected with gp33–41COM variant peptides. Thymocytes were stained with CyChrome-anti-CD4, PE-anti-CD8α, and FITC-anti-CD25, -CD44, -CD62L, -CD69, -β7 integrin, -Vβ8, -CD8β, and -CD5. Arrows up and down indicate up-regulation and down-regulation, respectively, as expressed by change in the MFI and/or percentage of positive cells; n.c., no change in the expression was detected as compared with control mice (injected with PBS). Results are representative of four independent experiments.

(lower panel). Injection with the gp33–41COM peptide led to a decrease in the β7 integrin expression level on SP CD8 thymocytes as compared with the control PBS-injected mouse (mean fluorescence intensity (MFI) 50 vs 108, respectively), despite essentially no change in the percentage (40–41%) of anti-β7 integrin reactive cells. Both Y4S/F6A COM and A7E COM induced an increase in the percentage of this thymocyte population (61–70%) without changing β7 integrin levels on individual thymocytes. A complete analysis of thymocyte markers is summarized in Fig. 2B. Higher levels of CD44 and CD69 were observed on SP CD8 thymocytes injected with the gp33–41COM peptide. In contrast, Y4S/F6A COM had no effect on the above markers, but Vβ8 (P14-specific TCR), β7 integrin, and CD8β expression were up-regulated. There was an increase in the expression of CD44, CD8β, and β7 integrin on SP CD8 thymocytes of mice injected with the A7E COM variant. These results indicated that a single injection of gp33–41COM and Y4S/F6A COM affected both the cell numbers and expression of thymocyte markers, suggestive of early events in thymocyte activation. A7E injection did not alter cell numbers, but affected thymocyte marker expression. Such phenotypic changes may be reflective of molecular up- or down-regulation and/or selection of cellular subpopulations. Although not shown, alterations in cellular phenotypes were evident at the earliest interval examined postinjection (6 h) as well. While reducing absolute cell number, a single dose of gp33–41 had little influence on the percentage of DP thymocytes in P14 RAG2−/− mice. Therefore, we have injected gp33–41 variants every 24 h for 3 days and found that under these conditions the DP thymocyte depletion was pronounced, leading to a nearly total elimination of these thymocytes (Fig. 3A). This observation is consistent with that made in another H-2D b -restricted TCR transgenic system, F5, where multiple peptide injections were also required (34). In contrast, almost total elimination of N15 RAG2−/− DP thymocytes was achieved by a single K b -binding VSV8 cognate peptide injection (Refs. 16 and 26 and data not shown). Whether this difference is a result of greater CD8αβ coreceptor binding to H-2K b vs D b (28), the higher copy number of peptide complexes with K b vs D b molecules (28), or TCR affinity differences remains to be determined. Surprisingly, injection with the Y4S/F6A COM mutant resulted in a significant increase in the total number of thymocytes as well as DP thymocyte subpopulation. In contrast, the A7E COM variant had no effect on the thymocyte counts (Fig. 3A). The expression of the examined phenotypic markers on SP CD8 thymocytes followed a similar trend after third injection (data not shown) as compared with a single peptide injection (Fig. 2B).

The possibility that the unusual increase in the number of DP thymocytes following exposure to the Y4S/F6A peptide might be due to cellular proliferation and attendant DNA synthesis was examined by BrdU incorporation assay as shown in Fig. 3B. As expected, lower BrdU incorporation was detected in each of the thymocyte subpopulations (double-negative (DN), DP, and SP CD8) of mice injected with gp33–41COM, supporting previous observations on negative selection and apoptosis by this ligand (19). More importantly, no significant difference in the amount of BrdU incorporation was found in thymocytes of mice injected with either Y4S/F6A COM or A7E COM variants, compared with the control PBS-injected mice. As a consequence, we wondered whether Y4S/F6A peptide might increase the DP subpopulation by preventing apoptosis. To test this possibility, staining of cells from mice injected with gp33–41 variants with anti-annexin V mAb was performed (Fig. 4). Indeed, there was an increase in the percentage of annexin V + cells (12%) in mice injected with gp33–41COM, suggestive of apoptosis. In contrast, no such phenomenon was observed upon injection of either Y4S/F6A COM or A7E COM peptides. In those instances, values were comparable to the control mouse. We reasoned that if the Y4S/F6A peptide competes with apoptosis-inducing peptides for binding to H-2D b molecules on the cell
surface, then Y4S/F6A may "rescue" thymocytes from undergoing cell death. Competitive binding assays, in which P14 RAG2−/− mice were injected with the mixtures of the negatively selecting cognate peptide gp33–41 C9M and Y4S/F6A C9M variant, were performed in vivo. The results in Fig. 5 show that, as predicted, increasing the amount of Y4S/F6A peptide in the injection mixture resulted in a higher number of total and DP thymocytes. Thus, we infer that the Y4S/F6A variant may compete with other negatively

FIGURE 3. Quantitative changes in thymocyte subpopulations following multiple injections of the Y4S/F6A C9M variant. A, Cell numbers of thymocyte subpopulations in P14 RAG2−/− mice. P14 RAG2−/− mice at 3–4 wk of age were injected i.v. three times every 24 h with 25 μg of each peptide and sacrificed 18 h following the third injection. Thymocytes were double-stained with CyChrome-anti-CD4 and PE-anti-CD8α, and the expression of CD4 and CD8α was detected by flow cytometry after gating on 50,000 cells. The distributions of log10 cell counts ×10^6 are shown in box plots. The box in the plot extends from the first to the third quartiles of the data; the line in the middle of the box plot denotes the median. The lines above and below the boxes extend to the largest observation (respectively, smallest observation) that is below the third quartile plus 1.5× the interquartile range (respectively, the largest observation above the first quartile minus 1.5× the interquartile range). Individual points shown in the graphs are >1.5× the interquartile range from the nearest quartile. All plots were drawn in Stata version 8.0 for MS Windows (Microsoft, Redmond, WA). Data represent 10–12 independent experiments. B, gp33–41 C9M variant peptides do not alter DNA synthesis in residual thymocytes. P14 RAG2−/− mice were injected three times, as indicated in A. On the day of the last injection, mice were given 1 mg of BrdU twice at a 4-h interval, i.p. 18 h later, and thymocytes were triple-stained with CyChrome-anti-CD4, PE-anti-CD8α, and FITC-anti-BrdU. The histograms of BrdU staining on the gated DN, DP, and SP CD8 thymocytes are shown. The numbers represent the percentages of BrdU-positive cells. Results are representative of three independent experiments.

FIGURE 4. Decreased apoptotic cell death in thymocytes of P14 RAG2−/− mice injected with the Y4S/F6A C9M variant 6 h previously. Treatment of P14 RAG2−/− mice with gp33–41 C9M resulted in reduction of thymocyte number (without gp33–41 C9M, 4.6 × 10^7 cells per thymus; with gp33–41 C9M, 3.2 × 10^7 cells per thymus). By contrast, P14 RAG2−/− mice injected with Y4S/F6A C9M variant showed increase in the number of thymocytes (7 × 10^7 cells per thymus), whereas injection with A7E C9M variant resulted in little changes in thymocyte numbers (4 × 10^7 cells per thymus). Total thymocytes were stained with FITC-anti-annexin V and assayed by flow cytometry. Dead cells were gated out using propidium iodide to reveal the proportion of live thymocytes undergoing early stages of apoptosis. The histograms of annexin V staining on the propidium iodide-negative live thymocytes are shown. Histograms of PBS-injected thymocyte staining (dotted lines) were superimposed on those of peptide-treated thymocyte profiles (solid lines). The numbers represent the percentages of annexin V− apoptotic thymocytes in mice injected with gp33–41 C9M variant peptides.
selecting peptides for binding to H-2D\(^{b}\) molecules expressed on thymic stroma either by binding to "empty" surface MHC class I molecules or, perhaps, by a cross-presentation mechanism (35).

**Y4S/F6A peptide interacts with the P14 TCR with low affinity**

To study the relative avidity of interactions between the P14 TCR and the gp33–41 variant peptides bound to H-2D\(^{b}\) molecules, we prepared tetramers of H-2D\(^{b}\) with each of the above peptides, and performed quantitative immunofluorescence analysis of thymocytes, splenocytes, and lymph node cells from P14 RAG2\(^{-/-}\) mice. The results of MFI staining of SP CD8 thymocytes from P14 RAG2\(^{-/-}\) mice with the three tetramers at different concentrations are depicted in Fig. 6. The *inset* shows representative staining profiles at a single comparable concentration of tetramer. Clearly, the strongest binding occurs with the tetramer containing the gp33–41 C9M peptide, as reflected by higher fluorescence intensity levels. Tetramer containing A7E C9M mutant bound with lower affinity, whereas no detectable binding was observed with the tetramer refolded with the Y4S/F6A C9M variant peptide. These results in conjunction with functional data (Fig. 1, B–D) suggest that the Y4S/F6A C9M mutant must interact with the P14 TCR with extremely poor affinity, if at all.

**Development of an in vivo model for thymocyte selection and emigration**

To date, the processes of T cell development involving the interactions between the P14 TCR and the gp33–41 variant peptides have been studied exclusively in the transgenic mouse system. Although the homogeneity of TCR-expressing cells on the RAG2\(^{-/-}\) background is an advantage for specific analysis, it remains difficult to identify the numerically small population of recent thymic emigrants (RTE). To overcome this problem, we have used irradiation chimeras using congenic mouse strains (expressing the CD45.1 marker in B6 and CD45.2 in P14 and N15 transgenic mice). Previously, using N15 transgenic mice carrying a TCR specific for the VSV8 peptide in the context of H-2K\(^{b}\), a weak agonist peptide variant L4, with the substitution of leucine for valine at the P4 peptide residue, inducing positive selection has been shown to occur by higher fluorescence intensity levels. Tetramer containing A7E C9M mutant bound with lower affinity, whereas no detectable binding was observed with the tetramer refolded with the Y4S/F6A C9M variant peptide. These results in conjunction with functional data (Fig. 1, B–D) suggest that the Y4S/F6A C9M variant peptide must interact with the P14 TCR with extremely poor affinity, if at all.

**Y4S/F6A and L4 variants of viral epitopes mediate positive selection and emigration of thymocytes**

Between 3–4 wk post-BM reconstitution, gp33–41 and its variant peptides were injected daily for 3 days and animals examined 24 h...
FIGURE 7. Kinetics of T cell development in irradiation chimeras of B6 Ly-5.1 mice reconstituted with BM from P14 RAG2−/− (Ly-5.2) mice. B6 Ly-5.1 mice at 8 wk of age were injected with lineage-negative BM cells from P14 RAG2−/− donors (1 × 10⁶ cells per mouse) 2–4 h following irradiation (7 Gy). Thymuses, spleens, and lymph nodes were obtained weekly and cells were triple-stained with CyChrome-anti-CD4, PE-anti-CD8α, and either FITC-anti-CD45.1 or FITC-anti-CD45.2. Results represent expression of donor (Ly-5.2) and recipient (Ly-5.1) phenotype on thymocytes, splenocytes, and lymph node cells. Mean ± SD of two to four independent experiments is shown.

later. Fig. 8A, upper four panels, shows the anti-CD4 and anti-CD8α profiles of all thymocytes in the recipient (both donor and host). Fig. 8A, lower eight panels, enumerate the donor (anti-CD45.2 reactive) cells in the DP and SP CD8 subpopulation as gated in the upper panel, with the absolute number of thymocytes given in the Fig. 8 inset (right). The number of thymocytes in irradiation chimeras injected with Y4S/F6A_CM was highest, whereas that of gp33–41_CM-injected mice was lowest. This difference recapitulates the effect of gp33–41_CM variant peptides vs gp33–41_CM on thymocytes from P14 RAG2−/− transgenic mice. Fig. 8A, inset, shows that the lowest DP numbers are in irradiation chimeras injected with gp33–41_CM peptide, whereas DP numbers are increased in mice injected with the Y4S/F6A_CM Variant. The number of SP CD8 thymocytes was also highest in chimeras injected with Y4S/F6A_CM Variant peptide (Fig. 8A, inset), suggesting that this ligand mediated positive selection of P14 RAG2−/−-specific T cells. As the increase in thymocyte numbers exceeds the donor-engrafted population, injection of Y4S/F6A_CM peptide may lead to the rescue of certain nontransgenic thymocytes from negative selection as well.

To appreciate whether APL might function in an analogous manner in other systems to modulate selection, we have generated N15 RAG2−/−–B6 irradiation chimeras, injected either VSV8, L4, or PBS and subjected the animals to comparable analysis. Here, as well, the number of thymocytes in irradiation chimeras injected with L4 was highest, whereas that of VSV8-injected mice was lowest (Fig. 8B, inset), similarly to the effect of gp33–41 variant peptides on thymocytes from P14 RAG2−/−–B6 chimeras. The CD4/CD8 donor thymocyte profiles (Fig. 8B, lower panel) showed the lowest percentage of DP and SP CD8 thymocytes in irradiation chimeras injected with VSV8 peptide, compared with mice injected with L4 or with PBS. In contrast, the number of both DP and SP CD8 donor thymocytes was highest in chimeras injected with L4 variant peptide, consistent with positive selection.

To determine whether Y4S/F6A and L4 lead to emigration of SP CD8 thymocytes from the thymus to the periphery, spleens and lymph nodes from the same P14 RAG2−/−–B6 and N15 RAG2−/−–B6 irradiation chimeras analyzed in Fig. 8 were examined using triple-color immunofluorescence with anti-CD45.2, anti-CD8α, and anti-Vα2 or -Vβ5 mAbs. The results are represented in Fig. 9A, where the percentages of donor T cells in the host lymph nodes of P14 RAG2−/−–B6 irradiation chimeras injected with gp33–41_CM variant peptides are shown. Note that treatment with gp33–41_CM leads to activation of the cognate P14 T cells, as judged by their size increase (Fig. 9A, upper panel) and down-regulation of the TCR (Vα2; Fig. 9A, lower panel), in line with previous observations in other TCR transgenic models (25). The greatest number of donor-type CD45.2-CΔ8/Vα2+ T cells (Fig. 9A, inset) was in the lymph nodes of Y4S/F6A_CM-injected chimeras, suggesting that donor-type thymocytes developing in the presence of Y4S/F6A_CM mature and emigrate to the lymph nodes. Similar increase in the numbers of donor-type cells in lymph nodes was observed 9 wk after injection of Y4S/F6A_CM peptide (data not shown). The functional analysis of donor-type CD8+ lymph node T cells in irradiation chimeras injected with the positively selecting Y4S/F6A_CM peptide showed ~2-fold higher proliferation levels in response to the cognate peptide gp33–41_CM in vitro, compared with cells from PBS control-engrafted chimeric mice, reflecting the 2-fold difference in the number of donor-type CD8+ T cells in lymph nodes of chimeras injected with the Y4S/F6A_CM peptide (data not shown). Note that Y4S/F6A_CM peptide induces little emigration to spleen relative to the PBS control. In contrast, in N15 RAG2−/−–B6 irradiation chimeras injected with the L4 variant, higher CD8+Vβ5.2+ donor-type T cell numbers were observed both in lymph nodes and spleens, several days (Fig. 9B, inset) or 9 wk (data not shown) after L4 injection. The possible basis for this difference is described below, perhaps related to differential K8 vs D9 peptide presentation.

Mature donor-type T cells do not proliferate in response to Y4S/F6A or L4 variant peptides in vivo

To investigate the basis for the higher T cell numbers of the donor phenotype in the peripheral lymphoid tissues of mice injected with the Y4S/F6A or L4 peptides, we measured cell divisions in re-
FIGURE 8. Y4S/F6A C9M and L4 variants of viral epitopes mediate positive selection of thymocytes. Irradiated (7 Gy) B6 Ly-5.1 mice (8–10 wk of age) were injected i.v. with 25 μg of each peptide ~4 wk after BM reconstitution. Thymocytes were triple-stained with CyChrome-anti-CD4, PE-anti-CD8α, and either FITC-anti-CD45.1 or FITC-anti-CD45.2. A, Upper panel, the CD4/CD8α profiles in thymuses reconstituted with P14 RAG2−/−-derived BM progenitors as affected by gp33–41 (C9M) variant peptides. The percentages of DP and SP CD8 subsets after gating on 50,000 live cells are indicated. The histograms are of the donor phenotype (CD45.2) expression on the gated DP (middle panel), or SP CD8 (lower panel) thymocytes. The numbers represent the percentages of CD45.2-positive cells. The inset (right) shows absolute numbers of donor DP and SP CD8 subpopulations, based on the total thymocyte counts and the percentages of CD45.2-positive cells. Asterisks indicate p < 0.05 relative to PBS control according to Student’s t test. Results represent mean ± SD of three independent experiments. B, The CD4/CD8α profiles (upper panel), histograms of the donor phenotype (CD45.2) expression on the gated DP (middle panel) and SP CD8 (lower panel) thymocytes in thymuses reconstituted with N15 RAG2−/−-derived BM progenitors as affected by VSV8 and its variant L4 peptide. Other details as in A.
injected with the A7E_{C9M} peptide, the percentage of CFSE^{+}CD8^{+} cells was higher than in gp33−41_{C9M}-injected mice, but lower than in Y4S/F6A_{C9M}-injected mice, consistent with the A7E_{C9M} variant inducing some degree of T cell proliferation. It must be noted that because the adoptive transfer is into irradiated recipients, donor-type cell proliferation is significant even in the absence of peptide administration (31, 36), based on the reduction in the intensity of CFSE staining of the control (PBS injected) chimeras (MFI = 121) as compared with the initial CFSE staining intensity of donor cells before injection (MFI = 9000).

CD44^{+}CD8^{+} splenocytes from N15 RAG2^{−/−} mice were similarly labeled with CFSE and transferred into irradiated congenic hosts, followed by injection of VSV8 or L4 peptides (Fig. 10B). In the presence of VSV8, no brightly CFSE^{+}CD8^{+} cells were detected in lymph nodes (Fig. 10B) or spleen (data not shown), suggesting that naive N15-specific T cells had undergone proliferation and AICD in response to the VSV8 ligand. In contrast, the number of CFSE^{+}CD8^{+} T cells in mice injected with the L4 peptide was similar to the control group, implying that this peptide caused neither substantial T cell expansion or AICD. These data suggested

**FIGURE 9.** The Y4S/F6A_{C9M} variant leads to increased emigration of CD45.2^{+}CD8^{+} cells to the lymph nodes of irradiation chimeras. Lymph node cells from Fig. 8 animals were triple-stained with CyChrome-anti-CD8α, PE-anti-Vα2, and either FITC-anti-CD45.1 or FITC-anti-CD45.2. A. Upper panel, the dot plot profiles of forward scatter/CD45.2 in lymph nodes of irradiation chimeras reconstituted with P14 RAG2^{−/−}-derived BM progenitors as affected by gp33−41_{C9M} variant peptides. The percentages of donor CD45.2^{+} cells after gating on 25,000 live cells are indicated. Lower panel, The CD8α/Vα2 dot plot profiles of the gated CD45.2^{+} T cells. The numbers represent the percentages of CD8α^{+}Vα2^{+}-positive cells. Inset, Absolute numbers of donor CD45.2^{+}CD8α^{+}Vα2^{+} splenocytes and lymph node cells, based on the total cell counts and the percentages of CD45.2-positive cells. Asterisks indicate $p < 0.05$ relative to PBS control according to the Student’s $t$ test. Results represent mean ± SD of three independent experiments. B. The dot plot profiles of forward scatter/CD45.2 (upper panel), and the CD8α/Vβ5 dot plot profiles of the gated CD45.2^{+} T cells (lower panel) in lymph nodes of irradiation chimeras reconstituted with N15 RAG2^{−/−}-derived BM progenitors as affected by VSV8 and its variant L4 peptide. The numbers represent the percentages of CD45.2^{+}CD8α^{+}Vβ5^{+}-positive cells. Other details as in A.
A7E C9M is a weak agonist of gp33 p6, a single mutation at p7 did not significantly affect tetramer staining. In contrast to substitutions of amino acids at p4 plus p6 of gp33/H-2Db, splenocytes with the mutation at p7 to interact with the P14 TCR, incubation of P14 thymocytes with A7E C9M led to thymocyte activation and subsequent increase in the expression of CD44 and CD69, in line with similar observations in other models (25, 42, 43), whereas the A7E C9M led to up-regulation of CD44, β7 integrin and CD8β (Fig. 2B).

The kinetics of reconstitution of irradiated hosts by thymocyte progenitors from the BM of P14- and N15-TCR transgenic mice was similar to the findings of Tanchot and Rocha (36). In addition, we show that thymocyte emigration is dependent on the affinity/avidity of pMHC/TCR interactions. Peripheral SP CD8 T cells of the donor phenotype, when transferred into the irradiated hosts later injected with Y4S/F6A C9M or L4 ligands, did not expand, as judged by CFSE staining. "Background" proliferation did occur in a peptide-independent manner in chimeric hosts due to availability of niches caused by irradiation (31). These results suggest that although the low affinity pMHC/TCR interactions are insufficient to trigger cell divisions, differentiation nevertheless follows.

The nature and the number of APL involved in positive selection of MHC class I-restricted T cells and their relationship to antigenic peptides has been controversial (reviewed in Ref. 3 and references therein). Although most of the above studies have been performed in vitro, little is known about mechanisms of positive selection of CD8 T cells in vivo. Affinity measurements support the idea that weakly selecting peptide ligand affinities are lower than those of negatively selecting ligands for TCRs, but additionally linked to their MHC binding/stability properties (44). A recent publication described an antagonist of H-2Kb-specific OT-I TCR and a variant of OVA 257-264 peptide, (E1), endogenously expressed by cortical epithelial cells of TAP-deficient mice, which mediated positive selection of CD8+ T cells in vivo (45). Our report supports the idea that weak pMHC class I/TCR interactions promote positive selection of SP CD8 thymocytes. Certainly, the 10,000-fold weaker functional N15 T cell stimulation by L4 vs VS8 peptide is consistent with the view (46). However, because Y4S/F6A C9M in complex with H-2D b has no detectable binding with the P14 TCR, we cannot exclude the possibility that this pMHC/TCR interaction is no greater than the basal level of P14 TCR binding to D b in general. Two recent studies in class II MHC-restricted TCR transgenic mouse systems are also consistent with the notion that weak pMHC ligands may foster positive selection (47, 48).

Of importance is the observation that Y4S/F6A C9M led to an increase in the number of DP thymocytes, a phenomenon which has not been reported to occur during positive selection. However, the binding of Y4S/F6A C9M to D b might possibly prevent other endogenous negatively selecting thymic peptides from binding and interacting with the TCR. Consistent with this possibility, we show that Y4S/F6A C9M competes for binding to H-2D b with the negatively selecting cognate peptide gp33–41 (Fig. 5).

Collectively, our data show that cognate peptides can be modified to create variants that result in selection, directly or indirectly, of desired TCR specificities at the level of thymic development. This exogenous peptide administration offers a potential of expanding repertoire generation in vivo in a manner useful to the organism. Whether these peptide-specific T cells generate stronger defense mechanisms

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**FIGURE 10.** APL variants do not induce donor T cell divisions in the periphery of irradiated hosts. A, Sorted CD44+CD8+ CFSE-labeled P14 RAG2−/− splenocytes (Ly-5.2; 2 × 10⁶) were transferred to 7 Gy-irradiated B6 (Ly-5.1) mice. Four days later, gp33–41 C9M variant peptides were injected i.v. After an additional 4 days, spleen and lymph node cells were stained with CyChrome-anti-CD8. Numbers correspond to the percentages of CFSE−CD8+ cells after gating on 25,000 live cells. Note that because the absolute number of splenocytes and lymph node cells is comparable in all groups, the percentage values are also representative of cell numbers. MFI of labeled cells before injection was 8300–9000. One of three representative independent experiments is shown. B, A total of 2 × 10⁶-sorted CD44+CD8+ CFSE-labeled N15 RAG2−/− splenocytes (Ly-5.2) were transferred to 7 Gy-irradiated B6 (Ly-5.1) mice. Four days later, VS8V and its variant L4 peptide were injected i.v. and subjected to a similar protocol as in A. One of two independent experiments is shown.

that cognate peptide ligands gp33–41 and VS8V, interacting with the TCR with relatively high affinity, compared with their respective APL, induce activation of peripheral T cells, whereas peptide variants Y4S/F6A and L4, which bind TCR with low affinity and mediate positive selection, do not cause cell divisions.

**Discussion**

Analysis of Y4S/F6A and A7E peptides in H-2Db mice represents the first examination of the direct effects of amino acid substitutions at the P14 TCR contact residues on thymocyte selection and activation in vivo. The crystal structure of gp33/H-2Db suggests that single mutations at both peptide positions 4 (p4) or 6 (p6) directly affect TCR contacts (20, 21, 37). Those mutants of gp33–41 mediate positive selection presumably due to weaker pMHC/TCR interactions (19). In contrast to previous studies, in the present work mutations at p4 and p6 of gp33–41 were introduced in the same variant, to generate a positively selecting ligand with reduced TCR affinity to foster emigration of the developing thymocytes to the periphery. Indeed, Y4S/F6A C9M was found to interact with the P14 TCR on SP CD8 thymocytes with an affinity below our detection limit, as judged by tetramer staining. In contrast to substitutions of amino acids at p4 plus p6, a single mutation at p7 did not significantly affect thymocyte subset numbers. However, the A7E C9M variant altered the thymocyte phenotype (Fig. 2B). Our functional data (Fig. 1) suggest that A7E C9M is a weak agonist of gp33–41. Consistent with the ability of peptides with the mutation at p7 to interact with the P14 TCR, incubation of P14 thymocytes with A7S in vitro in FTOC at high molarity resulted in negative selection (38).

Y4S/F6A C9M induced up-regulation of the P14 TCR, the CD8β coreceptor, and the β7 integrin levels on SP CD8 thymocytes, characteristic of positive selection, with no change in the expression of other markers for activation/emigration/positive selection, including CD25, CD44, CD62L, and CD69. Characterization of RTE has been controversial. Up-regulation of several markers on SP CD8 thymocytes undergoing positive selection and emigration, including CD5 (39), β7 integrin, CD44 and L-selectin (CD62L) has been reported (33, 40), whereas others did not observe these changes (41). Our data further demonstrate the heterogeneity of the phenotypes of SP CD8 thymocyte subpopulations affected by positively selecting ligands, as well as difficulties in the precise characterization of the small subpopulation of RTE. In contrast, gp33–41 C9M led to thymocyte activation and subsequent increase in the expression of CD44 and CD69, in line with similar observations in other models (25, 42, 43), whereas the A7E C9M led to up-regulation of CD44, β7 integrin and CD8β (Fig. 2B).

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to fight viral infection or tumors in the normal, nontransgenic mouse, remains to be investigated. In this respect, exploring means of enhancing differentiation of thymocytes bearing desired TCRs, together with the understanding of the mechanism of thymocyte emigration to the periphery, would be of great importance. Several agents have been shown to inhibit thymic export (reviewed in Ref. 49), while a recent report described factors mediating emigration from the thymus (50).

In the future, combined approach of exposing the subject to a positively selecting APL, plus a thymic export-enhancing agent might provide a practical and efficient protective immunity.

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References