Positive Selection of CD4$^+$ T Cells Is Induced In Vivo by Agonist and Inhibited by Antagonist Peptides

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Abstract

The nature of peptides that positively select T cells in the thymus remains poorly defined. Here we report an in vivo model to study the mechanisms of positive selection of CD4$^+$ T cells. We have restored positive selection of TCR transgenic CD4$^+$ thymocytes, arrested at the CD4$^+$CD8$^+$ stage, due to the lack of the endogenously selecting peptide(s), in mice deficient for H2-M and invariant chain. A single injection of soluble agonist peptide(s) initiated positive selection of CD4$^+$ transgenic T cells that lasted for up to 14 days. Positively selected CD4$^+$ T cells repopulated peripheral lymphoid organs and could respond to the antigenic peptide. Furthermore, coinjection of the antagonist peptide significantly inhibited agonist-driven positive selection. Hence, contrary to the prevailing view, positive selection of CD4$^+$ thymocytes can be induced in vivo by agonist peptides and may be a result of accumulation of signals from TCR engaged by different peptides bound to major histocompatibility complex class II molecules. We have also identified a candidate natural agonist peptide that induces positive selection of CD4$^+$ TCR transgenic thymocytes.

Key words: positive selection • agonist peptide • CD4$^+$ cells • thymus • MHC class II

Introduction

T cell ontogeny is a multistep process resulting in the generation of mature peripheral CD4$^+$ helper and CD8$^+$ cytotoxic lymphocytes which recognize antigenic peptides presented by MHC class II or I proteins, respectively. During development of the T cell lineage thymocytes are subject to negative and positive selection processes that shape a repertoire of mature T cells that is tolerant to self-peptide–MHC complexes and at the same time diversified enough to mount an effective immune response (1, 2). Selection is based on the interactions of immature CD4$^+$CD8$^+$ thymocytes with peptide–MHC complexes on the surface of thymic stromal cells and results in the three possible outcomes. Thymocytes will die by apoptosis from neglect if their TCRs do not interact with peptide–MHC strongly enough to transduce a signal for positive selection (3). Thymocytes, bearing receptors that interact strongly with self-peptide–MHC, die from activation-induced apoptosis (4, 5). Finally, thymocytes that interact weakly with peptide–MHC undergo positive selection and survive to differentiate into single positive CD4$^+$ or CD8$^+$ cells (6, 7). While the ability of antigenic (i.e., agonistic) peptides to induce apoptosis of CD4$^+$CD8$^+$ thymocytes in vivo is well established, the relationship between antigenicity of peptides and their ability to induce positive selection is controversial (8–10). Conflicting results concerning this issue have been obtained in a variety of in vitro systems (11–14), but until now no suitable and relatively simple system to study the identity and properties of peptides that mediate positive selection in vivo has been available. Here we describe for the first time an in vivo experimental model in which administration of soluble peptide(s) results in positive selection of mature, peripheral CD4$^+$ T cells. The peptide ligands that initiate positive selection of CD4$^+$ T cells in this model have agonist activity, whereas antagonist peptides only inhibited this process. Using protein sequence search algorithms we have identified an endogenous agonist peptide which also induced positive selection of transgenic T cells. This implies that natural positive selection of CD4$^+$ thymocytes may be driven by high not only low potency ligands.

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Materials and Methods

Mice. The TCR genes were cloned from T cell hybridoma specific for analogues of the pigeon cytochrome C (PCC[43–58])* peptide and expressed in VA-hCD2 cassette (15–17). All TCR transgenic mice (TCR^{Tg}) were made by comicroinjection of the respective TCR-α and -β constructs into fertilized eggs of F1 (C57BL/6×CBA/Ca) mice. TCR^{Tg} mice were crossed to C57BL6/TCR α chain knockout mice (TCR-α^-; The Jackson Laboratory) and to mice deficient in H2-M (H2-M^-; provided by E. Bikoff [Harvard University, Cambridge, MA] and R. Germain [National Institutes of Health, Bethesda, MD]) and Ii (Ii^-; provided by L. van Kaer, Vanderbilt University, Nashville, TN) to obtain TCR^{Tg}TCR-α^-H2-M^-Ii^-.

Flow Cytometry Analysis. Monoclonal antibodies specific for CD4(RM4-5), CD8(53-6.7), Vβ8(F23.1), CD69(H1.2F3), CD44 (IM7), and CD62L(MEL-14) were purchased from BD PharMingen and used according to the manufacturer's recommendations. Cells were analyzed using a FACSCalibur™ instrument (Becton Dickinson) and CELLQuest™ software. For intracellular staining, cells were first stained for CD4 and CD8, fixed in 2% paraformaldehyde, permeabilized in 0.1% Tween-20, and stained with anti–bcl-2 antibody (BD PharMingen) according to the manufacturer's instruction.

Antigen Response and Antagonism Assays of TCR Transgenic Cells. Proliferation of lymph node cells isolated from TCR^{Tg} TCR-α^- and TCR^{Tg}TCR-α^-H2-M^-Ii^- mice injected with the selecting peptide was measured in response to antigen. Response of the TCR^{Tg}TCR-α^- lymph node cells to different agonist peptides was measured by proliferation assay in a 96-well plate. 10^5 responder cells were stimulated with peptides presented by 5×10^5 irradiated C57BL6 or H2-M^-Ii^- splenocytes. Agonist and control peptides were used at concentrations of 0.01, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 μM. After 3 d cells were pulsed with 1 μCi of [^{3}H]thymidine added to each well and after 14–16 h thymidine incorporation was measured. The sequences of peptides used were: PCC50V (AEGFSYTVANKNKGIT), PCC50L (AEGFSYTLANKNKGIT), PCC50V54A (AEGFSYTVANKAKGIT), PCC46A49A50V54A (AEGASYAVANKAKGIT), PCC50F54A (AEGFSYTFANKAKGIT), neutral ceramidase (AGFFQYTLYILASEG), and IgGVH (NADFKTPATLTVDKA). All peptides were synthesized by fluorenyl methoxycarbonyl chemistry and purified by reversed-phase high performance liquid chromatography. To compare the antigenic response of $CD4^+$ TCR^{Tg} cells lymph node cells were collected from TCR^{Tg}TCR-α^- and TCR^{Tg}TCR-α^-H2-M^-Ii^- mice 2 wk after peptide injection. Single cell suspensions were prepared and $CD4^+$ cells were sorted using magnetic beads coated with anti-CD4 antibodies (MACS®). Purity of sorted cells exceeded 85%. 2×10^4 of purified $CD4^+$ cells per well was used in proliferation assay. Cells were stimulated with increasing concentrations of agonist peptides presented by APCs from wild-type or H2-M^-Ii^- mice. IgGVH-derived peptide was used as a negative control. Proliferation was measured after 3 d by thymidine incorporation by pulsing cells with 1 μCi of [^{3}H]thymidine for 16 h. Antagonism assay was done by prepulsing 5×10^5 relevant APCs per well for 2 h with 5 μM of the antigenic peptide PCC50V54A, washing, and then exposing them to varying concentrations of peptide analogues with 2×10^4 purified $CD4^+$ TCR^{Tg} lymphocytes as responder cells (18). For antagonism assays the percentage of proliferation inhibition was calculated as 100–100* (thymidine incorporation in the presence of antagonist/thymidine incorporation in the absence of antagonist; reference 18).

Peptide Injections and Surgical Procedures. TCR^{Tg}TCR-α^- and TCR^{Tg}TCR-α^-H2-M^-Ii^- were injected intraperitoneally with agonist and control peptides dissolved in PBS. Highly hydrophobic neutral ceramidase peptide was initially dissolved in DMSO followed by dilution in PBS. After indicated time mice were killed, thymus and lymph nodes were collected and single cell suspensions were prepared. Pups were injected with 2 μg of peptide subcutaneously 12–24 h after birth and thymi were transplanted into recipient mice 24 h after injection. 5–8-wk-old TCR^{Tg}TCR-α^-H2-M^-Ii^- mice were used for thymectomy or as thymic transplant recipients. Surgical procedures were performed as described previously (19). Mice were handled accordingly with the institutional guidance.

Protein Sequence Analysis. Protein sequence database at the National Center for Biotechnology Information was searched using BLAST, Psi-BLAST, and FASTA search algorithms with the protein motif (AF)XX(AT)(VLFI)AXX(AN) as a query.

Results

Characterization of Peptide Agonists for the PCC-specific Transgenic αβ TCR. To examine the role of peptides during positive selection in vivo, we generated transgenic mice expressing class II–restricted TCR specific for analogues of a PCC(43–58) peptide presented by the A^b molecule (16). This receptor recognizes analogues of PCC(43–58) in which aspartic acid in position 50 is replaced by amino acids with neutral/hydrophobic (PCC50V, PCC50V54A, PCC46A49A50V54A, PCC50L, PCC50F) side chains (Fig. 1). The TCR transgenic mice were backcrossed to C57BL6 TCR-α^- knockout mice so that almost all T cells expressing transgenic TCR become $CD4^+$ T cells (Fig. 2, A and B). Initially we examined the capacity of agonist peptides to induce negative selection of transgenic

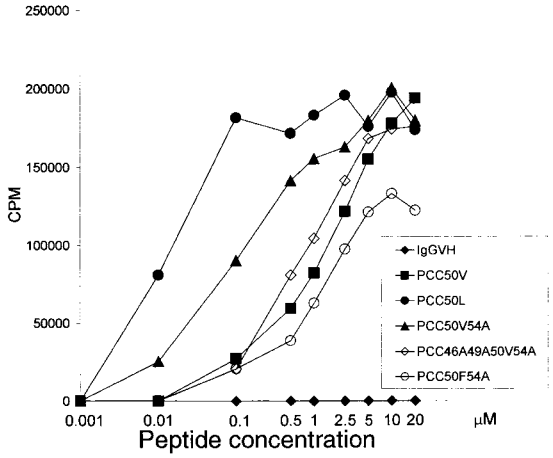

Figure 1. Comparison of the agonist potency of the PCC50V, PCC50L, PCC50V54A, PCC46A49A50V54A, and PCC50F54A peptides. Total lymph node cells from TCR^{Tg}TCR-α^- mice were stimulated with increasing concentrations of the agonist peptides. Unrelated peptide IgGVH(59–74) was used as a control. Irradiated splenocytes from mice expressing wild-type A^b were used as APCs.

Abbreviations used in this paper: FTOC, fetal thymic organ culture; PCC, pigeon cytochrome C; TCR^{Tg}, TCR transgenic mice.

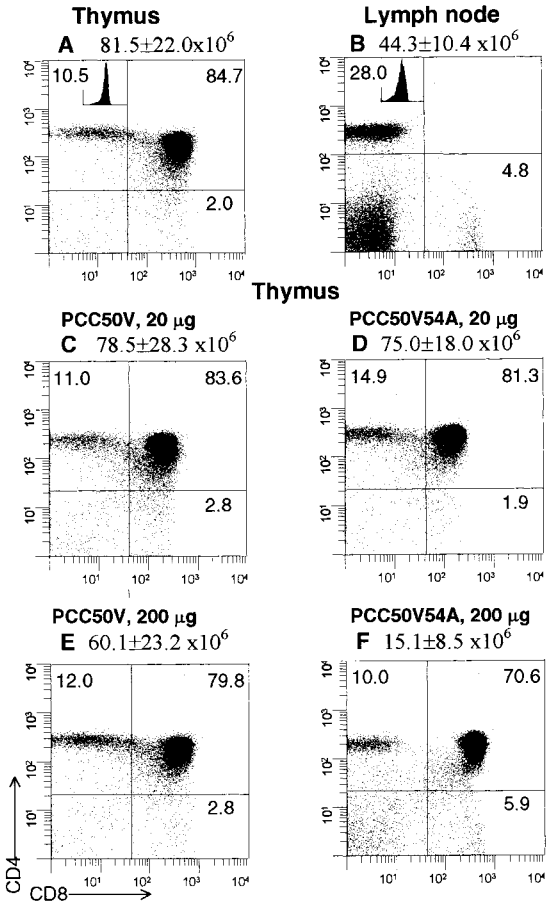

Figure 2. (A and B) Development of transgenic cells in TCR-α^- C57BL6 mice. TCR^{Tg}TCR-α^- thymocytes were efficiently selected in the thymus expressing wild-type A^b molecules (A) and repopulate peripheral lymphoid organs (B). The insets in (A) and (B) represent the expression of transgenic Vβ8 chain on CD4$^+$ thymocytes and lymph node cells. (C–F) The capacity of agonist peptides to induce negative selection of transgenic thymocytes correlates with their agonist potency. Intraperitoneal injection of a moderate agonist peptide PCC50V did not induce negative selection of transgenic thymocytes at a dose of 20 μg and induced only limited deletion at 200 μg/mouse (C and E). Injection of the strong agonist peptide PCC50V54A at 20 μg did not induce negative selection but a dose of 200 μg/mouse-induced profound deletion of transgenic thymocytes (D and F). Thymocytes and lymph node cells were stained with anti-CD4 and anti-CD8 antibodies and analyzed by flow cytometry. Peptides were injected intraperitoneally at 20 or 200 μg per mouse and mice were killed after 24 h. The total number of recovered cells is presented above each panel. At least three mice were used in each experiment.

T cells. We found that the efficiency of negative selection correlated with the potency of individual agonist peptides. However, an injection of 20 μg of any tested agonist peptide, in particular a moderate and a strong agonist (PCC50V and PCC50V54A, respectively) did not induce negative selection of transgenic thymocytes as assessed by thymus cellularity, annexin V staining, and TUNNEL assay (Fig. 2, C and D, and data not shown). Strong agonists PCC50L and PCC50V54A (Fig. 2 F) induced profound negative selection when injected at 200 μg per mouse, while moderate agonist PCC50V induced only marginal deletion of CD4$^+$CD8$^+$ cells (Fig. 2 E).

Peptide Agonists Induce Positive Selection of Transgenic CD4$^+$ T Cells. To examine the potential effect of agonist peptides on in vivo positive selection of transgenic thymocytes, we followed the ontogeny of these cells in a nonselecting thymic environment where A^b molecules are devoid of selecting peptides. For that purpose, we crossed TCR^{Tg}TCR-α^- mice to Ii$^-$ and H2-M$^-$ mice to obtain TCR^{Tg} on a triple knockout background (TCR^{Tg}TCR-α^-H2-M$^-$Ii$^-$). The development of the majority of CD4$^+$ thymocytes is severely impaired in mice lacking H2-M and Ii molecules, two molecular chaperones that participate in peptide loading to MHC class II molecules (20–22). As expected, the thymic development of the transgenic T cells was arrested at the stage of CD4$^+$CD8$^+$ thymocytes and only very few transgenic CD4$^+$ T cells were detected in the periphery (Fig. 3 A). The lack of the natural positively selecting A^b–peptide complex(es) resulted in a block in thymocyte development and increased thymic cellularity in TCR^{Tg}TCR-α^-H2-M$^-$Ii$^-$ mice. After these observations, we attempted to restore positive selection in TCR^{Tg}TCR-α^-H2-M$^-$Ii$^-$mice by providing exogenous peptides. A number of irrelevant A^b-binding peptides (IgGVH[59–74], OVA[323–339], Eα[52–68]), and analogues of PCC (50A, 50N, 50E, 52Q) without agonist properties had no effect on thymic selection (data not shown). As shown in Fig. 3, B–D intraperitoneal injection of a nondeleting dose of PCC50V54A agonist peptide restored selection of CD4$^+$ single positive thymocytes. Simultaneously, a number of CD4$^+$CD8$^+$ thymocytes upregulated CD69 and bcl-2 expression (Fig. 3, E and F). Positive selection of CD4$^+$ thymocytes was sustained for 14 d after a single injection of the selecting peptide ligand (Fig. 3 D). Contrary to the recent report that agonist peptides induce selection of regulatory CD4$^+$CD25$^+$, cells in our model newly selected CD4$^+$ thymocytes were CD25$^-$ (data not shown, and reference 23). The cellularity of the thymus and the number of apoptotic cells detected by TUNNEL assay and annexin V staining were the same in controls and in mice injected with 20 μg of agonist peptide PCC50V54A (data not shown). Four other analogues of the PCC peptide, PCC50V, PCC50L, PCC46A49A50V54A, and PCC50F54A, injected at the same dose (20 μg), also restored positive selection of transgenic thymocytes (data not shown). These results prove that positive selection of CD4$^+$ T cells can be induced in vivo by different agonist ligands. An injection of soluble agonist into TCR^{Tg}TCR-$\alpha^-\rightarrow$H2-M$^-$Ii$^-$ radiation chimeras also resulted in positive selection of transgenic CD4$^+$ T cells, despite the expression of wild-type A^b–peptide complexes on bone marrow–derived thymic stromal cells (data not shown).

Positively Selected CD4$^+$ Thymocytes Repopulate Peripheral Lymphoid Organs and Respond to Antigens. To determine whether positively selected CD4$^+$ T cells leave the thymus as functional, mature T cells, we analyzed lymph node cells 2 wk after peptide administration. As shown in Fig. 3 D, TCR^{Tg}TCR-α^-H2-M$^-$Ii$^-$mice injected with peptide had $\sim$20% CD4$^+$T cells in the lymph nodes when compared with 1–2% CD4$^+$ T cells found in control animals (Fig. 3

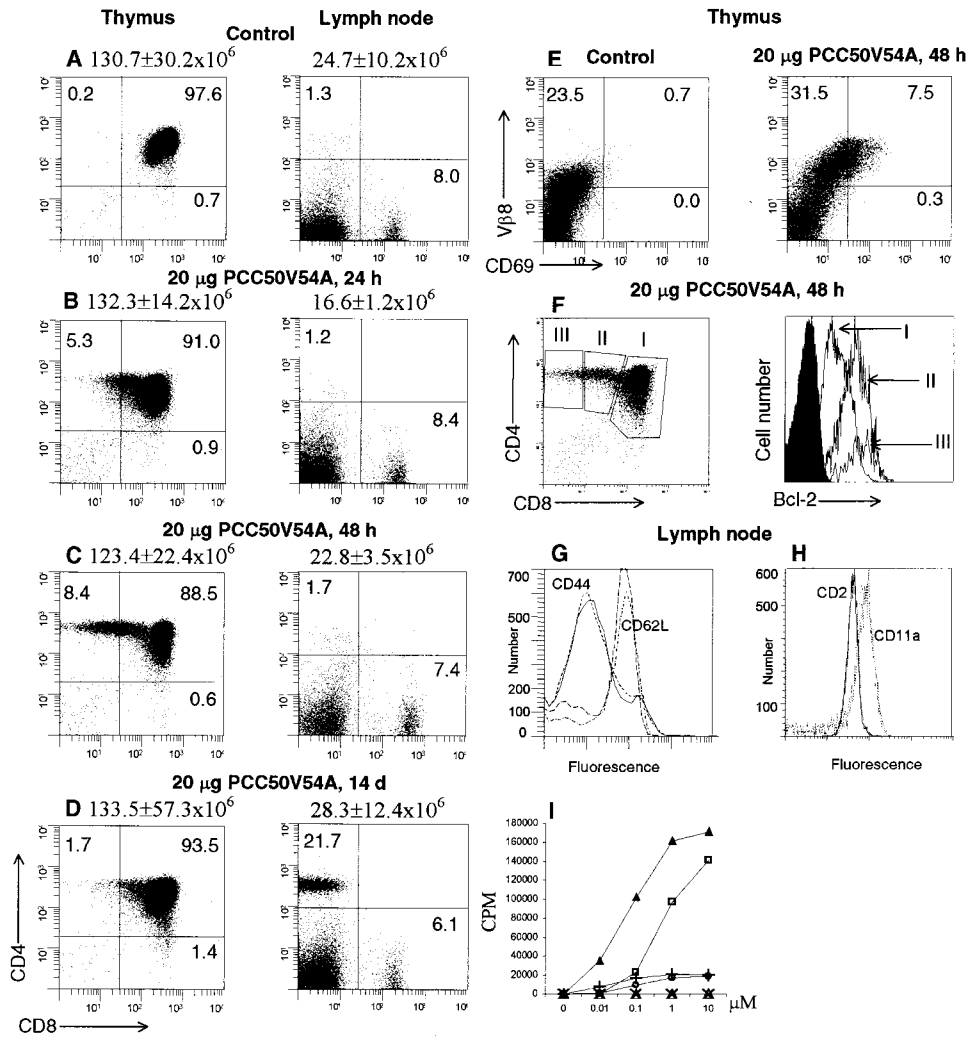

Figure 3. (A–D) The development of CD4+ T cells in TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ control mice (A) and mice injected with the selecting peptide PCC50V54A (B–D). Control TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice were injected with IgGVH peptide (A). Experimental mice were injected with 20 μg of the PCC50V54A peptide and analyzed after 24 h (B), 48 h (C), and 14 d (D). Thymocytes (left) and lymph node cells (right) were stained with anti-CD4 and anti-CD8 antibodies and analyzed by flow cytometry. Absolute numbers for thymocytes and lymph node cells are shown above each panel. (E) Injection of the positively selecting peptide upregulated TCR and CD69 expression on thymocytes. Control mouse (left) and mouse injected with the selecting peptide (right) were analyzed by flow cytometry 48 h after peptide administration. (F) bcl-2 was upregulated in positively selected thymocytes. TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mouse was injected intraperitoneally with 20 μg of PCC50V54A peptide and thymocytes were analyzed after 48 h. Left panel shows thymocyte staining with CD4 and CD8 antibodies and three gates, based on the expression of these two surface markers. Right panel shows bcl-2 expression on gated thymocyte populations I–III. Filled histogram shows staining with isotype-matched control antibody. (G) Peripheral lymph node cells that differentiated in mice injected with the selecting peptide had naive phenotype. Histograms compare expression of CD44 and CD62L on gated CD4+ cells from TCR^{Tg}TCR-α$^-$ and injected TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice. CD44 expression is depicted by (unbroken line) and (broken line) and CD62L expression by (—— ——) and (-·-) for cells isolated from TCR^{Tg}TCR-α$^-$ and injected TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$mice, respectively. (H) The expression of CD2 (LFA-2) and CD11a (LFA-1) was the same on peptide selected CD4+ T cells as on cells selected in wild-type TCR transgenic mice. Histograms compare expression of CD2 and CD11a on gated CD4+ cells from TCR^{Tg}TCR-α$^-$ and injected TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice. CD2 expression is depicted by (unbroken line) and (broken line) and CD11a expression by (—— ——) and (-·-) for cells isolated from TCR^{Tg}TCR-α$^-$ and injected TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice, respectively. (I) Peripheral lymph node cells that developed in TCR^{Tg}TCR-α$^-$ (▲, □, X) and PCC50V54A injected TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ (+, ○, △) mice respond in vitro to the selecting peptide PCC50V54A (▲, +) or another agonist peptide PCC50V(□, ○). IgGVH (X, △) peptide was used as a negative control.

C). Newly selected CD4+ T cells had normal levels of TCR and CD4, and the phenotype of naive T cells (CD44$^-$CD62L$^+$; Fig. 3 G). Also the levels of adhesion molecules CD2 (LFA-2) and CD11c (LFA-1) were the same on CD4+ lymphocytes from transgenic and peptide-injected TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice (Fig. 3 H). Peptide-selected cells responded to the agonist peptide, though with much lower potency than CD4+ transgenic cells isolated from mice expressing wild-type A^b (Fig. 3 I).

Since the selecting peptide is an agonist, one could argue that the observed phenomenon results from the expansion of a small number of peripheral transgenic CD4+ T cells rather than from induced positive selection (24). To test this possibility, two types of experiments were performed. In the first experiment, the TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice were thymectomized and then injected with the PCC50V54A peptide. 2 wk after injection, the mice were killed and the number of peripheral CD4+ T cells was counted. As shown in Fig. 4 A, the number of CD4 transgenic T cells was very low and did not increase in comparison with control mice that were thymectomized, but did not receive the PCC50V54A peptide. In the second experiment, TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ neonates were injected with the PCC50V54A peptide and after 24 h thymi from injected and control neonates were transplanted under the kidney capsules of H2-M$^-$Ii$^-$TCR-α$^-$ mice, which are devoid of T cells (25). After 10 d, recipient mice were killed and the presence of transgenic CD4+ T cells in the transplanted thymus and host lymph nodes was determined by FACS® analysis. As shown in Fig. 4 B, transgenic CD4+

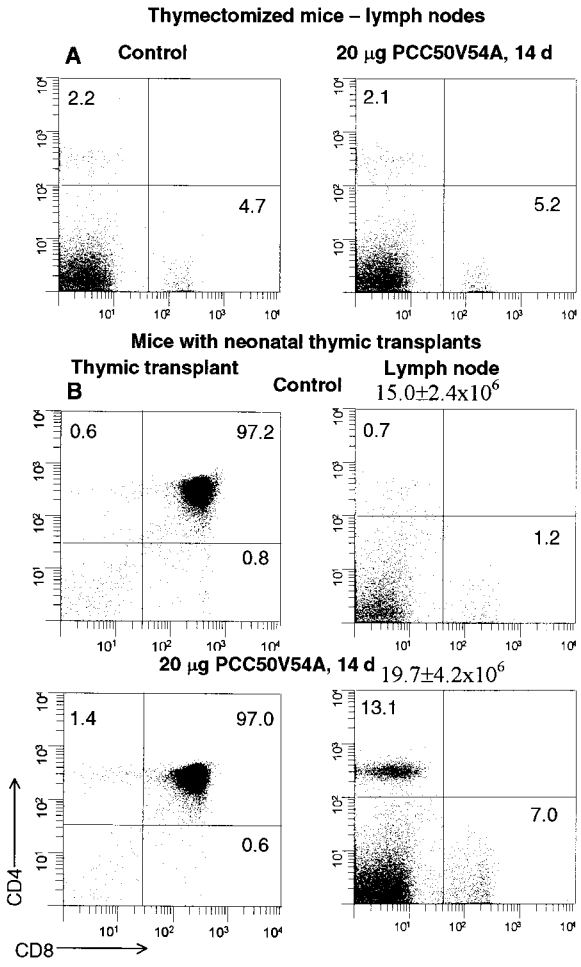

Figure 4. $CD4^+$ cells that appear in injected $TCR^{Tg}TCR\text{-}\alpha^-H2\text{-}M^-Ii^-$ mice were selected in the thymus. (A) $TCR^{Tg}TCR\text{-}\alpha^-H2\text{-}M^-Ii^-$ mice were thymectomized and some mice were injected with the selecting peptide. After 14 d the mice were killed and lymph node cells analyzed by flow cytometry. Both control (not injected, left) and injected mice (right) had very few $CD4^+$ peripheral T cells. (B) $TCR^{Tg}TCR\text{-}\alpha^-H2\text{-}M^-Ii^-$ thymocytes are selected by the PCC50V54A peptide in thymic graft recipients and repopulate peripheral lymph nodes. Thymi from $TCR^{Tg}TCR\text{-}\alpha^-H2\text{-}M^-Ii^-$ 2-d-old neonates (top) or the same neonates injected with 2 μg of the selecting peptide PCC50V54A (bottom) were transplanted under kidney capsule of the $TCR\text{-}\alpha^-H2\text{-}M^-Ii^-$ recipient mice. After 14 d, recipient mice were killed and cells isolated from transplanted thymic tissue (left) and recipient lymph nodes (right) were stained with anti-CD4 and anti-CD8 antibodies. The absolute number of lymph node cells in control and experimental recipient animals is shown.

T cells were found only in mice grafted with thymi from neonates injected with the selecting peptide. Therefore, we conclude that $CD4^+$ T cells appear in the peripheral lymph nodes of $TCR^{Tg}TCR\text{-}\alpha^-H2\text{-}M^-Ii^-$ injected with the agonist peptide as a result of positive selection by this peptide.

Antagonist Peptides Do Not Induce Positive or Negative Selection but Inhibit Positive Selection Induced by Agonist Selecting Ligands. It has been shown that the peptide component of the TCR/MHC/peptide complex influences the strength of interaction between a thymocyte and thymic stromal cell and hence determines the fate of the thymocyte (26). Minor alterations in critical amino acid residues that are exposed towards the TCR may have a profound effect on the outcome of recognition of MHC–peptide complex by the T cell and change the activation properties of a peptide ligand from agonist to an antagonist (27). Peptide ligands positively selecting $CD8^+$ thymocytes were initially described as having antagonist properties. However, later experiments showed that $CD8^+$ thymocytes expressing transgenic TCR were selected by peptides with different activation potencies (28, 29). Similar experiments investigating thymocytes expressing MHC class II–restricted TCRs in fetal thymic organ cultures (FTOCs), showed that antagonist peptides either inhibit thymic positive selection or induce negative selection (30, 31). We have investigated what is the capacity of antagonist peptides to influence thymic selection using our in vivo model. As shown in Table I, we have initially found that analogues of PCC(43–58) with p5 position occupied by E or N have an antagonistic effect on our transgenic $CD4^+$ T cells. Biological properties of agonist and antagonist peptides were preserved in both wild-type and $H2\text{-}M^-Ii^-$ mice (Fig. 5, A–C). The responses of transgenic $CD4^+$ T cells to agonist peptide PCC50V54A were reduced by >50% in the presence of each of these analogues, which is the criteria used to identify a given peptide as antagonist (27). These antagonist variants of PCC peptide inhibited the responses of transgenic $CD4^+$ T cells on APCs derived from wild-type or $H2\text{-}M^-Ii^-$ mice (Fig. 5, B and C). Subsequently, various doses of PCC50E and PCC50N were tested for their ability to affect thymic selection in $TCR^{Tg}TCR\text{-}\alpha^-H2\text{-}M^-Ii^-$ mice. Regardless of a wide range of administered doses (up to 50 μg), injection of the antagonist peptides did not result in increased number of $CD4^+$ transgenic T cells in the thymus nor induced deletion of $CD4^+CD8^+$ thymocytes (Fig. 6 A). In conclusion, contrary to the "weak affinity" peptide ligands that mediate positive selection of $CD8^+$ T cells, we have found that only agonist not antagonist peptides mediate in vivo positive selection of $CD4^+$ T cells.

Table I. *Peptides Recognized by TCR Transgenic Lymphocytes and Their Biological Activities*

Peptide	Agonist	Antagonist
PCC(43–58)	+/−	−
PCC50V54A	+	−
PCC50V	+	−
PCC50L	+	−
PCC 50F	+	−
PCC46A49A50V54A	+	−
PCC 50E	−	+
PCC 50N	−	+
PCC 52Q	−	−

Peptides recognized by transgenic lymphocytes and their biological activities.

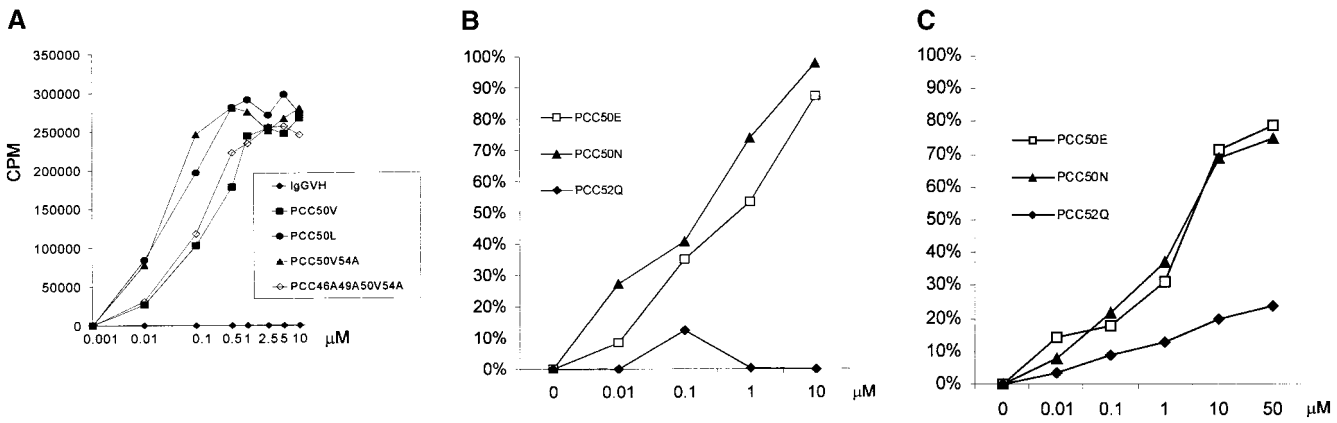

Figure 5. (A–C) Peptide agonist and antagonists preserve their properties presented by APCs from wild-type or H2-M$^-$Ii$^-$ mice. Total lymph node cells from TCR^{Tg}TCR-α$^-$ mice were stimulated with increasing concentrations of the agonist peptides or control peptide −IgGVH(59–74) presented by APCs from H2-M$^-$Ii$^-$ mice (A). Graphs show percentage of inhibition of activation (y axis) with regard to the concentration of the antagonist peptides (x axis, B and C). Wild-type (B) or H2-M$^-$Ii$^-$ (C) APCs were pulsed with 5 μM of agonist peptide PCC50V54A and used to stimulate transgenic T cells. PCC50E, PCC50N antagonist, and PCC52Q neutral peptide were added at increasing concentrations.

A single thymocyte is likely exposed to numerous different peptides bound to MHC and it has been proposed that the sum of signals produced by these interactions determines thymocyte fate. It was shown that antagonist peptides can affect thymocyte selection, however these results were controversial. In one study antagonist peptide inhibited negative selection of thymocytes while in another study the opposite result was reported (30, 31). In addition, an antagonist peptide was also described as being capable of blocking positive selection of CD4$^+$ TCR transgenic thymocytes, although it could not be ruled out that this peptide instead induced late deletion at the stage of single positive cells (32). To examine how the antagonist peptide affects positive selection we coinjected antagonist peptide PCC50E together with the selecting agonist peptide PCC50V54A at the same low dose (3 μg), and evaluated the outcome of selection 4 d later. As shown in Fig. 6, B and C the number of CD4$^+$ thymocytes which were positively selected by PCC50V54A agonist peptide was significantly reduced. This effect was also reproduced for the second antagonist peptide PCC50N. It is unlikely that the reduced number of CD4$^+$ thymocytes in mice injected with agonist/antagonist mixture is a result of late negative selection because a much higher dose of the same antagonists administered alone did not induce negative selection of CD4$^+$CD8$^+$ thymocytes. This result argues that a single thymocyte accumulates signals received by interaction with different MHC–peptide complexes.

Identification of a Candidate Natural Peptide that Mediates Positive Selection of TCR^{Tg} Thymocytes. Since the sequence of the exogenous selecting peptide(s) has been determined, we hypothesized that the natural selecting ligand exists that has agonist properties. By testing different PCC analogues we have determined that amino acids A or F in position 46, A or T in position 49, A in position 51, and A or N in position 54 are important for binding to A^b. Moreover, amino acids V, L, F, I in position 50 were important to stimulate TCR^{Tg} lymphocytes. We have used the peptide motif (AF)XX(AT)(VLFI)AXX(AN) to search the nonredundant protein and EST protein databases using different computer algorithms. These searches resulted in identification of a mouse proteins which may encode natural peptides with agonist properties for our TCR. One of these proteins was the neutral ceramidase that contained amino acid motif FXXTLYXXA where only Y does not match the original motif (33). This protein is ubiquitously expressed in many tissues, including epithelial cells. Subsequently, we synthe-

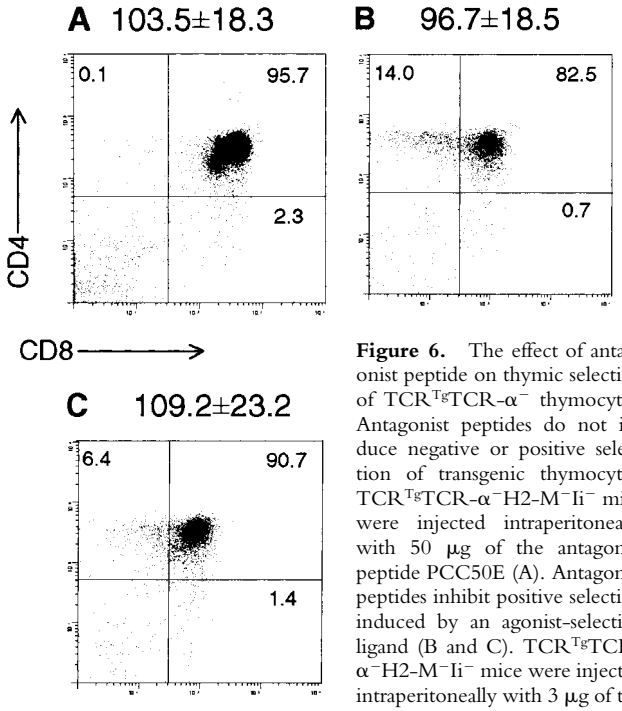

Figure 6. The effect of antagonist peptide on thymic selection of TCR^{Tg}TCR-α$^-$ thymocytes. Antagonist peptides do not induce negative or positive selection of transgenic thymocytes. TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice were injected intraperitoneally with 50 μg of the antagonist peptide PCC50E (A). Antagonist peptides inhibit positive selection induced by an agonist-selecting ligand (B and C). TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice were injected intraperitoneally with 3 μg of the agonist peptide PCC50V54A (B), and mixture of 3 μg of agonist peptide PCC50V54A and 3 μg of antagonist PCC50E (C). Thymocytes were stained with anti CD4 and CD8 antibodies 48 h after peptide injection. Total thymocyte cell numbers are shown above each panel.

sized the peptide AGFFQYTLYILASEG which contained the homologous motif sequence. This peptide when tested in vitro acted as weak agonist eliciting proliferation of naive TCR^{Tg} $CD4^+$ T cells (Fig. 7 A). Injection of 50 μg of neutral ceramidase peptide into TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice resulted in positive selection of transgenic $CD4^+$ thymocytes (Fig. 7 C) and the appearance of $CD4^+$ lymphocytes in the peripheral lymph nodes (Fig. 7 D). These peripheral $CD4^+$ cells weakly proliferated when stimulated with agonist peptide PCC50V (Fig. 7 B). Hence using biocomputing analysis we have been able to identify a candidate natural agonist peptide which after injection into TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice induces positive selection of $CD4^+$ T cells. Our results imply that the natural peptides bound to MHC class II that select thymocytes are recognized by the relevant TCRs with higher affinity than have been previously postulated.

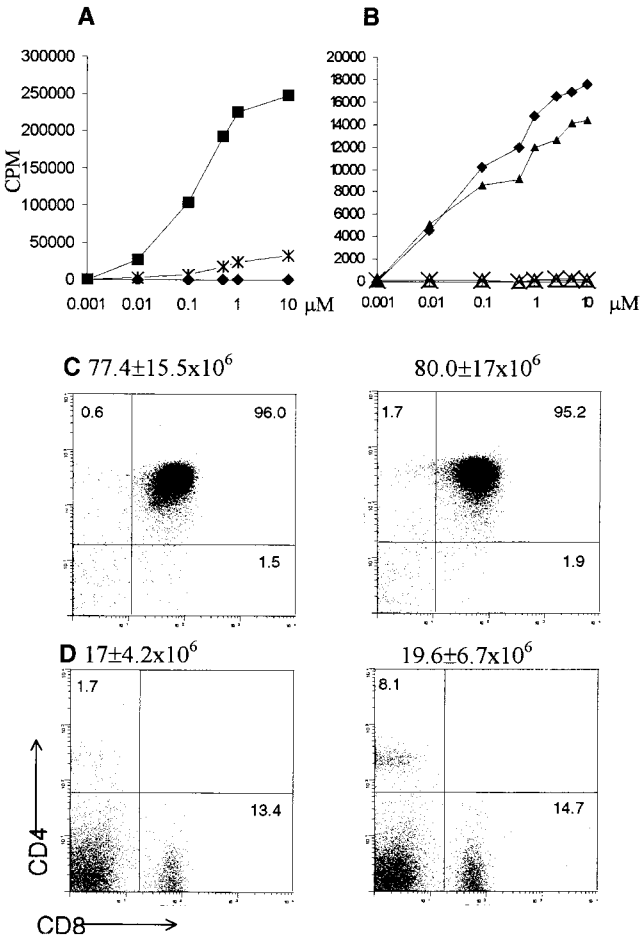

Figure 7. Agonist peptide derived from a mouse natural protein neutral ceramidase has agonist properties and positively selects transgenic thymocytes when injected into TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mouse. (A) Total lymph node cells from TCR^{Tg}TCR-α$^-$ mice were stimulated with increasing concentrations of PCC50V (■) or neutral ceramidase derived peptides (X). Unrelated peptide IgGVH(59–74) (◆) was used as a control. Irradiated splenocytes expressing wild-type A^b were used as APCs. (B) Sorted peripheral lymph node $CD4^+$ cells that developed in TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice injected with PCC50V54A (▲) or neutral ceramidase peptide (◆) respond in vitro to the agonist peptide PCC50V. IgGVH peptide was used as a negative control for the response of $CD4^+$ cells from TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice injected with PCC50V54A (△) or neutral ceramidase peptides (X). Irradiated splenocytes from H2-M$^-$Ii$^-$ mice were used as APCs. (C) Neutral ceramidase peptide mediates positive selection of transgenic $CD4^+$ thymocytes in TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice. TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice were injected intraperitoneally with 50 μg of the IgGVH (left) and neutral ceramidase (right) peptide. The percentage of $CD4^+$ single positive cells was 0.7 ± 0.4 and 1.8 ± 0.6 in control and experimental mice 3 d after peptide injection. (D) Positively selected thymocytes repopulated peripheral lymph nodes. Lymph node cells from TCR^{Tg}TCR-α$^-$H2-M$^-$Ii$^-$ mice were injected with IgGVH (left) and neutral ceramidase (right) peptide and analyzed 12 d later. The percentage of $CD4^+$ positive cells was 2.4 ± 0.8 and 7.6 ± 1.8 in control and experimental mice, respectively.

Discussion

Many studies have analyzed a role of peptides in positive selection of MHC class I– and II–restricted T cells. However, despite great effort these studies have yielded conflicting results on the nature of the positively selecting peptide ligand. Development of $CD8^+$ thymocytes in FTOCs from β2-microglobulin or transporter associated with antigen processing 1 knockout mice showed that positive selection can be peptide specific and that the avidity of the TCR for the MHC–peptide complex determines the fate of immature thymocytes (34, 35). The response of $CD8^+$ thymocytes selected in FTOCs by altered peptide ligands was often compromised (10, 11, 36) and none of the selecting peptides identified in vitro supported positive selection of the respective transgenic receptor in vivo (37, 38). The selection of $CD4^+$ T cells in organ cultures have shown that peptide agonists either delete or select class II–restricted TCR transgenic thymocytes towards the $CD8^+$ lineage (39). Peptide antagonists inhibited the generation of $CD4^+$ thymocytes and promoted the selection of $CD8^+$ cells or induced negative selection (30, 31, 40). Positive selection of $CD4^+$but not $CD8^+$ T cells was achieved using either hybrid monoclonal antibodies or monovalent antibodies against TCR and CD4 (41, 42). It has been reported that agonist ligands can induce differentiation of $CD4^+$ thymocytes in reaggregation cultures or in vivo after the intrathymic injection of the recombinant adenovirus encoding the respective peptide (9, 13, 43). In the latter study agonist, antagonist, and even peptides with unrelated amino acid sequence were capable of selecting TCRs with defined antigenic specificity (9). In contrast, our data strongly suggest that peptides capable of selecting $CD4^+$ T cells may have differing primary sequence, but they are required to possess agonist activity. Antagonist peptides were not able to induce positive or negative selection in wide range of tested concentrations. In addition, antagonists blocked the activity of agonists and inhibited positive selection.

The data reported in this paper suggest that positive selection is peptide specific and, in a natural environment, mediated by agonist peptide ligands. In contrast, analysis of thymocyte development in mice expressing high density of A^b–single peptide complexes implied that selection of multiple TCRs does not require specific peptide (15, 44). Re-

sults obtained in both experimental models are consistent with the avidity model of positive selection which postulates that a T cell might be positively selected by multiple low affinity or few high affinity MHC–peptide complexes (6). The $CD4^+$ T cells found in mice expressing single A^bEp complex had TCRs with undetectable affinity to A^bEp (to avoid negative selection), in contrast to transgenic $CD4^+$ T cells described in this paper that are positively selected by few high affinity (agonist) A^b–peptide complexes. An alternative explanation of T cell selection in mice expressing A^b–single peptide complex postulated that most $CD4^+$ lymphocytes are selected on contaminating, low abundance peptides (45) which is consistent with the reported here positive selection by agonist peptides. The ongoing studies on the complexity of peptides present in mice expressing various covalent MHC class II–peptide complexes in vivo should soon reveal how $CD4^+$ T cells develop in these mice.

One of the intriguing phenomenons we have observed is the decreased proliferation of peptide selected $CD4^+$ T cells after TCR stimulation. This effect is not peptide specific since the same cells respond poorly to anti-CD3 antibody stimulation and does not reflect lower expression of the TCR or costimulatory molecules (unpublished data). The discussed feature is also not a characteristic of our experimental system. Cells expressing the same transgenic TCR are very efficiently selected on endogenous peptides present in Ii^- mice and have similarly decreased response to TCR stimulation (data not shown). We are currently investigating if this phenomenon is caused by lower MHC class II expression in H2-M^-Ii^- or Ii^- mice, the lack of the selecting peptide ligand in the periphery, decreased life span, or inherently elevated activation threshold due to the nature of the selecting peptide ligand.

Because αβ TCRs expressed on $CD4^+CD8^+$ thymocytes likely interact with multiple MHC–peptide complexes, the fate of a thymocyte may depend on the net result of all these interactions (32, 46). Experiments described in this report, where coinjected antagonist peptide inhibited positive selection support this hypothesis and raise interesting questions about how the balance of agonist and antagonist ligands impacts T cell development. The doses of selecting agonist and antagonist peptides were chosen to be low to exclude the possibility that the observed phenomenon is due to competition for A^b binding. Instead, the unique signal induced by both agonist and antagonist could result in the inhibition of positive selection. Since the transition from $CD4^+CD8^+$ to $CD4^+$ thymocytes requires 24–48 h experiments where injection of the antagonist peptide is delayed or proceeds the injection of agonist peptide may determine the course of events that lead to inhibition of positive selection by antagonist MHC–peptide ligand.

Recent experiments in reaggregation cultures raised the possibility that bone marrow dendritic cells deliver a strong agonist signal necessary to induce $CD4^+$ T cell differentiation (47). We have investigated the development of transgenic $CD4^+$ T cells in chimeras made by reconstituting H2-M^-Ii^- mice with the bone marrow from TCR^{Tg} TCR-α^- mice (TCR^{Tg}TCR-$\alpha^-\rightarrow$H2-M^-Ii^- chimeras). The development of thymocytes in such chimeras is arrested at the $CD4^+CD8^+$ stage despite the presence of selecting wild-type A^b–self-peptide complexes on bone marrow–derived dendritic cells. Mature $CD4^+$ T cells appeared only after injection of the selecting peptide PCC50V54A (data not shown). This suggest that in our in vivo model, in contrast to the reaggregation cultures (47), radioresistant thymic epithelial cells are required to provide an agonist signal that induces thymocyte differentiation towards $CD4^+$ lineage in vivo.

We have induced positive selection of transgenic thymocytes using a peptide dose that did not delete these cells in wild-type mice. The presentation of injected agonist peptides by wild-type A^b molecules present on bone marrow cells in TCR^{Tg}TCR-$\alpha^-\rightarrow$H2-M^-Ii^- radiation chimeras also did not induce negative selection (data not shown). One could then postulate that agonist peptides differ in their relative capacity to induce positive versus negative selection. Some of these peptides induce negative selection even at a very low dose, but others induce positive selection at a low dose and negative selection only when used at a high dose. Under physiological conditions, it is likely that positively selecting peptides are presented at very low concentration so it seems plausible that these peptides would have agonist activity. Peptide-specific positive selection would favor interaction with a narrow range of more potent agonists while positive selection of more promiscuous TCRs may involve collective interaction with a broader range of peptides sharing lower agonist potency (16, 48). Alternatively, selection of $CD4^+$ T cells was possible in our system because the positively selecting agonist ligand sets the threshold for negative selection, and negative selection requires an interaction with the more potent agonist ligand (49, 50). Signaling studies also support the notion that agonist peptides may be natural ligands for selection of the class II–restricted thymocytes (51, 52), based on the observation that commitment to the CD4 lineage requires a stronger signal via TCR than commitment towards the CD8 lineage. Previously published reports describing natural peptides capable of selecting αβ TCR transgenic T cells used mass spectroscopy analysis to identify peptides eluted from MHC molecules (28, 29). The advantage of this method is that it identifies peptides physically bound to MHC, but because of limited sensitivity of detection this analysis may be biased in favor of the most abundant peptides. Here, we have attempted to use an alternative method to find peptides that may be the natural selecting ligands. We have searched protein database for the presence of a sequence motif that we identified present in agonist selecting peptides. A peptide fragment derived from the identified protein had both agonist properties and positively selected transgenic thymocytes. Although, this method does not guarantee that this particular peptide is presented in the thymus, the expression pattern of neutral ceramidase suggests that this is a good candidate protein to

yield selecting peptides (33, 53). Our approach may become even more useful in the future as more sequences are available in the databases.

Mice transgenic for MHC class II–restricted αβ TCR and lacking H2-M and Ii molecules likely constitute a nonselecting environment for most of MHC class II–restricted transgenic receptors studied so far. We show, for the first time, that in vivo administration of soluble peptide may restore positive selection of $CD4^+$ thymocytes in such mice. This strategy may be used to identify and determine the properties of peptides capable of selecting TCR transgenic $CD4^+$ T cells with different antigen specificities in vivo. Since in this experimental model, the onset of positive selection of a large number of thymocytes is known, it may be particularly useful for analysis of gene expression patterns associated with positive selection and lineage commitment of $CD4^+$ thymocytes within hours after the delivery of the positively selecting signal. Furthermore, in this study we have identified a number of selecting and nonselecting peptides so it will be possible, using soluble recombinant proteins, to determine the relationship between the affinity of the TCR for different peptide–MHC class II complexes and the capacity to induce positive selection in vivo. Our finding challenges the existing dogma that low-affinity peptides are predisposed to induce positive selection while high-affinity ligands only induce negative selection. Instead, for $CD4^+$ T cells the latter not the former peptides seem to be predisposed to induce positive selection.

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