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In the Normal Repertoire of CD4+ T Cells, a Single Class II MHC/Peptide Complex Positively Selects TCRs with Various Antigen Specificities

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In the thymus, immature T cells are positively and negatively selected by multiple interactions between their Ag receptors (TCRs) and self MHC/class II peptide complexes expressed on thymic stromal cells. Here we show that in the milieu of negative selection on physiological self MHC/class II peptide complexes (A\(^b\)wt), a single class II peptide complex A\(^b\)Ep\(_{32-68}\) positively selects a number of TCRs with various Ag specificities. This TCR repertoire is semidiverse and not biased toward Ep-like Ags. Our finding implies that the degeneracy of positive selection for peptide ligands exceeds peptide-specific negative selection and is essential to increase the efficiency and diversity of the repertoire so that T cells with the same Ag specificity can be selected by different self MHC/peptide complexes. The Journal of Immunology, 1999, 162: 95–105.

In the thymus, the Ag receptors (TCR) expressed on immature double-positive CD4\(^+\)CD8\(^+\) thymocytes are required to weakly recognize self MHC molecules bound with endogenously derived peptides (1–4). This early engagement between the TCRs present on thymocytes and the self MHC/class II peptide complexes, expressed on thymic stromal cells, is called thymic positive selection. In the thymus, immature T cells are positively and negatively selected by multiple interactions between their Ag receptors (TCRs) and self MHC/class II peptide complexes expressed on thymic stromal cells. Here we show that in the milieu of negative selection on physiological self MHC/class II peptide complexes (A\(^b\)wt), a single class II peptide complex A\(^b\)Ep\(_{32-68}\) positively selects a number of TCRs with various Ag specificities. This TCR repertoire is semidiverse and not biased toward Ep-like Ags. Our finding implies that the degeneracy of positive selection for peptide ligands exceeds peptide-specific negative selection and is essential to increase the efficiency and diversity of the repertoire so that T cells with the same Ag specificity can be selected by different self MHC/peptide complexes. The Journal of Immunology, 1999, 162: 95–105.

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techniques. Two separate models, one based on mice that express single class II MHC molecules (Ab wt) bound with peptide (Ep) via the flexible linker (AβEp⁻¹ mice) and the second based on mice that lack DM molecules that dislocate CLIP (class II-associated invariant chain peptide) from class II MHC (DM-deficient mice (DM⁻)), showed that about 15–50% of the normal number of CD4⁺ T cells are selected in these animals, respectively (22–25).

The repertoire of TCRs on CD4⁺ T cells selected by single (AβEp⁻¹) or dominant (DM⁻) class II MHC/peptide mice used various TCR Vβ segments, which suggested that TCRs may encode various Ag specificities. However, it was also revealed that approximately two-thirds of CD4⁺ T cells from AβEp⁻¹ mice respond to wild-type Aβ/peptide complexes in vitro, which may lead to their negative selection in normal mice in vivo (22). Therefore, <5% of the normal number of CD4⁺ T cells can be positively selected on a single class II MHC/peptide complex and potentially can be included in the normal repertoire. Ag specificities encoded by TCRs positively selected by one, but negatively selected by multiple, class II MHC/peptide complexes remain to be determined.

The finding that one class II MHC/peptide complex can positively select various TCRs was further confirmed by experiments where an intrathymic injection of a recombinant adenovirus led to the expression of an Eβ⁺ molecule bound with neoepitope exclusively on the thymic epithelial cells (26). The repertoire of TCRs on CD4⁺ T cells selected by Eβ/neopeptipe was heterogeneous. Undoubtedly, then, some class II MHC/peptide complexes can select a number of different TCRs, but not every TCR. For example, several transgenic TCRs found to be selected on wild-type Aβ/self peptide complexes were not positively selected in either DM⁻ mice or AβEp⁻¹ mice (27, 28). Thus, although the repertoires of CD4⁺ T cells selected in thymi expressing single or dominant class II MHC/peptide complexes are more diverse than previously predicted, these repertoires are limited in volume and probably in the number of encoded Ag specificities.

All tested models investigating thymic selection of either CD4⁺ or CD8⁺ T cells agreed that the wild-type repertoire of TCRs requires a diverse set of self derived peptides to be involved in thymic selection. It is still questionable how many different Ag specificities are encoded by the set of TCRs selected by the defined single MHC/peptide complex and are potentially present in the normal repertoire. Recent studies have shown that, in contrast to previous expectations, the selecting and antigenic peptides recognized by the same TCR do not have to be related in primary amino acid sequence (26, 29, 30). Moreover, analysis of the repertoire of CD4⁺ T cells selected in DM⁻ radiation chimeras reconstituted with wild-type bone marrow suggested that these CD4⁺ T cells can respond to allogenic MHC molecules and other proteins (28). The interpretation of this last result, however, was complicated by a finding indicating that the residual self peptides present in DM⁻ mice may significantly contribute to positive selection of CD4⁺ T cell in these mice (27). Hence, to determine the physiological outcome of positive selection on a single class II MHC peptide complex, we have studied the Ag specificities of CD4⁺ T cells positively selected in vivo by a single AβEp complex and negatively selected by wild-type Aβ/peptide complexes. We found that negative selection on the normal spectrum of self class II MHC/peptide complexes deletes more than half the CD4⁺ T cells positively selected by AβEp. The remaining repertoire of CD4⁺ T cells is semidiverse and is not significantly biased in CD4⁺ T cells specific to antigenic peptides with primary amino acid sequences close to Ep. Analysis of the DNA sequences of the TCRs selected on the AβEp complex and specific for different antigenic peptides revealed that this repertoire consists of a number of α- and β-chains, some of which are similar. This result indicates that in the normal repertoire, positive selection on a single class II MHC/peptide complex contributes a limited set of TCRs with a broader than expected repertoire of antigen specificities.

Materials and Methods

Mice

Mice expressing the single A Ep complex were produced at the National Jewish Center (Denver, CO) as described previously (22). These mice and mice deficient for class II MHC or invariant chain as well as AβEp⁻¹ β⁻ mice were further bred in the animal care facility at the Medical College of Georgia (Augusta, GA). Mice expressing single AβPCC43–58 complex were bred at the transgenic facility of the Medical College of Georgia (Augusta, GA). C57BL/10 (Aβ wt) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Chimeric mice were generated by irradiation of 6- to 12-wk-old animals (950 rad) followed by immediate i.v. reconstitution with 5 × 10⁶ fetal liver cells from donor fetuses (day 15 gestational age). Chimeric mice were used for experiments no earlier than 8 wk after reconstitution.

Cell staining

Cells were stained for CD4, CD8, and different Vα and Vβ segments of TCR as previously described (31). The fluorescein- and phycoerythrin-labeled Abs were prepared in our laboratory or purchased from Phar-Mingen (San Diego, CA). Cells were suspended in staining buffer (BSS, 0.1% sodium azide, and 2% FBS) and were incubated for 30 min at 4°C with the Abs of interest in the presence of 10% normal mouse serum and 10% culture supernatant of anti-Fc receptor Ab 2.4G2 (32) to block non-specific binding. Cells were then washed three times with staining buffer and analyzed using a FACS Calibur instrument (Becton Dickinson, San Jose, CA).

Quantitative analysis of naive T cells

Cell suspensions were prepared from the spleen and the pool of inguinal, axillary, and mesenteric lymph nodes. The number of naive T cells was evaluated as previously described (33). In brief, the number of CD4⁺CD44⁺CD45RB⁺⁺ T cells in each organ was calculated from the number of cells recovered using immunofluorescence analysis. The total number was obtained by adding the number of CD4⁺CD44⁺CD45RB⁺⁺ T cells from spleen and twice that from lymph nodes.

Production of T cell hybridomas

The Aβ wt→AβEp⁻¹ chimeras were primed s.c. at the base of the tail with different synthetic peptides in CFA; 7 days later the draining lymph nodes were harvested. Lymph node cells were cultured for 3 days in culture medium in the presence of the antigenic peptide (50 μg/ml). On day 3 dead cells were removed by gradient centrifugation on Lymphocyte Separation Media (Cellgro, Herndon, VA). The recovered live cells were expanded in the presence of IL-2 for 3 days and converted into T cell hybridomas as previously described (34). Hybridomas were analyzed for the expression of CD4 and TCR. Double-positive hybridomas were screened for peptide-specific response using an HT-2 assay (35). Briefly, 1 × 10⁵ hybridoma cells were incubated with 5 × 10⁵ splenocytes from Aβ wt mice with or without peptide. After 24 h, the amount of secreted IL-2 was measured using the detecting cell line HT-2. The proliferation of HT-2 cells in response to IL-2 was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assay (36).

Analysis of Vα and Vβ segments used by T cell hybridomas

The repertoire of Vβ and Vα segments was established by flow cytometric analysis of the hybridomas after staining with currently available anti-mouse Vα and Vβ reagents. RNA was isolated from hybridomas using the Ultraspec RNA isolation kit (Biotex, Houston, TX) and was converted to cDNA using the RT system (Promega, Madison, WI). TCR α- and β-chains were amplified from the cDNA by PCR using constant region and Vα/Vβ-specific primers (37). PCR products were gel purified and sequenced by automated fluorescent sequencing on an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Foster City, CA).

Proliferation assays

The response of CD4⁺ T cells selected in Aβ wt→AβEp⁻¹ chimeras was analyzed by proliferation assay. Tested peptides were injected into individual Aβ wt→AβEp⁻¹ chimeras or wild-type mice in the base of the tail in the presence of CFA (Sigma). On day 7 the lymph nodes from these
mice were harvested, and non-CD4+ T cells were eliminated by complement-mediated lysis as follows. RBC were removed by incubation with buffered ammonium chloride solution, and cells were resuspended in BSS to a final concentration of 5 × 10^6 cells/ml and incubated for 30 min at 4°C in the presence of mAbs: YTS 169.4 (anti-CD8) and 25-9-3S (anti-I-A^b) at a concentration of 1–10 μg/ml. Cells were washed twice with BSS, resuspended to a final concentration of 10^5 cells/ml and incubated for 45 min at 37°C with complement. After two washings, cell viability was estimated by trypan blue exclusion. The purity of the CD4+ population was confirmed by FACS analysis.

Isolated CD4+ T cells were used in proliferation assays. CD4+ T cells were cultured for 6 days with irradiated splenocytes (3000 rad) in the presence of the specific antigen or irrelevant peptides. On days 3 and 5, 20 IU of IL-2 were added to each well. The proliferative response was measured using the MTT assay as previously described (36).

**Results**

**The majority of CD4+ T cells positively selected on single A^bEp complex are deleted in vivo by wild-type A^b due to the lack of tolerance to many self-derived peptides**

In a previous study we analyzed the repertoire of CD4+ T cells selected in thymus expressing single class II MHC covalently bound with single peptide, Ep_p2 - 68 (30). Our investigation was complicated by a high incidence of reactivity to wild-type A^bwt peptide complexes in CD4+ T cells selected in A^bEpII- mice. We interpreted this property of CD4+ T cells selected in A^bEpII- mice to indicate a lack of tolerance to a diverse set of self-derived peptides presented by wild-type A^b molecules. Alternatively, it was suggested that an imprint of positive selection by a single A^bEp complex makes this repertoire of CD4+ T cells so restricted that the reported reactivity to A^bwt APCs reflects the recognition of one or a few, rather than many, A^b/self peptide complex(es) (38). It was also possible that the A^b molecule itself folds differently when bound with any peptide other than Ep, and this can activate a large number of CD4+ T cells selected in A^bEpII- mice.

To determine whether a diverse set of peptides bound by wild-type A^b molecules produced the phenomenon described above, we tested the proliferative responses of CD4+ T cells selected in A^bEpII- mice to A^b occupied with a different range of peptides. Purified CD4+ T cells from A^bEpII- mice were incubated with irradiated spleen APCs expressing similar levels of A^b molecules bound by many peptides (from A^bEpII- mice), some peptides (from A^bwtII- mice), or one peptide (A^bPCCc43-58II- mice) distinct from Ep. After 5 days, the proliferation responses to these different APCs displaying A^b peptide complexes with various degrees of peptide occupancy were estimated using the MTT incorporation assay. A representative experiment (one of three) is shown in Fig. 1. We found that APCs that express A^b bound to many self peptides induced the strongest proliferative response. The APCs derived from A^bwtII- mice were weaker stimulators of A^bEp-selected CD4+ T cells, and virtually no proliferative response to APCs from A^bPCCcII- mice was recorded. This result indicated that the range of peptide diversity correlates with the magnitude of the CD4+ T cell proliferative response. However, whether few or many self-derived peptides are recognized by CD4+ T cells selected by the A^bEp complex remained to be determined, since it is unknown to what extent presentation of self peptides recognized by these CD4+ T cells depends on the presence of invariant chain. To reconcile this controversial issue it was requisite to determine the Ag specificities of CD4+ T cells selected in A^bwt→A^bEpII- radiation chimeras, where CD4+ T cells were positively selected on one and negatively selected on many A^b/self peptide complexes (see next section).

Initially, however, it was necessary to ascertain that many of A^bEp-selected CD4+ T cells are extensively negatively selected in vivo when exposed to wild-type A^b/self peptide complexes. To prove our in vitro estimates (30), we made different radiation chimeras. The A^bEpII- mice were lethally irradiated and reconstituted with 5 × 10^6 autologous or wild-type fetal liver cells. After an 8-wk period to allow for full reconstitution, we analyzed the number of CD4+ T cells in the thymus and periphery. As shown in Fig. 2, approximately 3% of thymocytes and 12% of peripheral T cells were CD4+ single positive in A^bEpII-→A^bEpII- chimeras. In contrast, the number of CD4+ T cells was severely reduced in A^bwt→A^bEpII- chimeras. In both thymus and lymph nodes, the reduction of CD4+ T cells was approximately 50%, indicating ongoing negative selection on wild-type A^b/self peptide complexes. This number is similar to the number of A^b/self peptide-reactive cells in A^bEpII- mice estimated in vitro as well as the range of deletion observed in A^bwt→DM- chimeras (28, 39). Despite a prominent negative selection, around 3–5% of CD4+ T cells found in wild-type mice were present in the periphery in A^bwt→A^bEpII- chimeras. We assumed that this population includes CD4+ T cells positively selected on the A^bEp complex and therefore focused on the complexity and Ag specificities of its TCRs.

We also tested whether the difference in the expression levels of A^bEp complex vs wild-type A^b accounts for the massive deletion of CD4+ T cells observed in A^bwt→A^bEpII- chimeras. It was shown that the level of class II MHC in A^bEpII- mice is 5–10 times lower than that in wild-type mice due to the low expression of the transgenic A^βEp construct (22). To investigate that issue, mice expressing the single A^bEp complex were reconstituted with fetal liver from mice expressing the same A^βEp transgene in the presence of an invariant chain (A^bEpII-). The A^bEpII- mice express virtually the same amount of A^b molecules on the cell surface as A^bEpII- mice, but in the presence of invariant chain the covalent Ep is extensively cleaved and replaced with endogenous peptides (22). As also shown in Fig. 2, A^bEpII→A^bEpII- radiation chimeras appeared to have only slightly more CD4+ T cells in the thymus and periphery than A^bEpII- chimeras reconstituted with wild-type fetal liver. This result suggested that it is primarily the peptide content of class II MHC molecules rather than the difference in MHC expression per se that is responsible for the
deletion of CD4+ T cells in A^b wt → A^b EpIi− chimeras. Furthermore, CD4+ T cells from A^b EpIi− → A^b EpIi− chimeras were tolerant to A^b wt, as tested by the in vitro response of the panel of T cell hybridomas (data not shown). This last result suggested that the low level of transgenic A^b molecules does not significantly hamper negative selection and that the lack of tolerance to self peptides is what makes A^b Ep-selected CD4+ thymocytes susceptible to deletion by wild-type A^b/peptide complexes.

CD4+ T cells positively selected on A^b Ep and tolerant to A^b wt have a naive phenotype and bear a heterogeneous set of αβ TCRs.

We next analyzed the surface phenotype of CD4+ T cells selected in A^b wt → A^b EpIi− chimeras. The purpose of this investigation was to examine whether exposure of A^b Ep-selected CD4+ T cells to many self class II MHC/peptide complexes alters the expression of some surface molecules or reshapes their repertoire of αβ TCRs. In particular, we have analyzed the expression of T cell activation markers such as CD44, CD69, and CD45RB, inspired by a recent report that the majority of peripheral CD4+ T cells from A^b wt → DM− chimeras have an activated phenotype. Moreover, these CD4+ T cells were found to be incapable of responding to in vitro stimulation with mAb against TCR (28). Accordingly, we found that a number of CD4+ T cells from A^b EpIi− mice and A^b wt → A^b EpIi− chimeras also express the phenotypic profile of activated T cells (Fig. 3). We have sorted CD4+ CD45RBlow and CD4+ CD45RBhigh T cells from A^b wt → A^b EpIi− chimeras and found that only the last subpopulation of CD4+ T cells proliferates when cultured on plates coated with anti-TCR mAb (data not shown). Furthermore, we analyzed activation markers expressed on CD4+ T cells from newly established A^b EpIi− βm− mice. In contrast to the original A^b EpIi− mice, the A^b EpIi− βm− mice had majority of CD4+ T cells CD44low/CD69low/CD45RBhigh (Fig. 3). We concluded that the repertoire of CD4+ T cells in A^b EpIi− mice and A^b wt → A^b EpIi− chimeras includes a number of CD4+ T cells selected on nonclassical MHC elements, and these T cells often express activation markers as previously described (40). In contrast, most of CD4+ T cells positively selected on the A^b Ep complex are similar to naive CD4+ T cells in wild-type mice and can normally respond to challenge with Ags (see below).
We have also determined whether introduction of extensive negative selection in A<sup>wt</sup>→A<sup>Ep</sup> cells changed the usage of different TCR V<sub>β</sub> segments in the CD4<sup>+</sup> T cell repertoire. For this purpose, the CD4<sup>+</sup> T cells selected in A<sup>wt</sup>→A<sup>Ep</sup> cells were stained with a set of mAbs specific for various TCR V<sub>β</sub> segments and compared with the V<sub>β</sub> repertoire of CD4<sup>+</sup> T cells selected in A<sup>Ep</sup>I<sub>II</sub>→A<sup>Ep</sup>I<sub>II</sub> and A<sup>wt</sup>→A<sup>wt</sup> chimeras. As shown in Fig. 4A, introduction of the negative selection on self peptides A<sup>Ep</sup>I<sub>II</sub>→A<sup>Ep</sup>I<sub>II</sub> chimeras reduced the numbers of V<sub>β</sub><sub>1</sub>, V<sub>β</sub><sub>12</sub>, and V<sub>β</sub><sub>14</sub>-bearing CD4<sup>+</sup> T cells and increased the frequency of V<sub>β</sub>5 and V<sub>β</sub>9. Furthermore, we have screened the TCR V<sub>β</sub> repertoires expressed by populations of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from the same set of the radiation chimeras (Fig. 4B). This staining revealed that the repertoire of CD4<sup>+</sup>CD45RB<sup>high</sup> cells from A<sup>wt</sup>→A<sup>Ep</sup>I<sub>II</sub> chimeras had reduced numbers of V<sub>β</sub>4 and V<sub>β</sub>14 and increased numbers of V<sub>β</sub>5, V<sub>β</sub>7, V<sub>β</sub>8, and V<sub>β</sub>9 CD4<sup>+</sup> T cells.

We concluded that negative selection on wild-type A<sup>Ep</sup>/peptide complexes affects different TCRs selected by A<sup>Ep</sup> complex, indicating that the remaining CD4<sup>+</sup> T cells may encode multiple, rather than few, Ag specificities, an assumption that found further support in the following set of experiments.

**CD4<sup>+</sup> T cells selected by single class II MHC peptide and tolerant to wild-type peptides preserved a broad repertoire of Ag specificities**

One of the important questions concerning the formation of the natural repertoire of T cells is whether the selecting peptide ligands leave an imprint on the selected repertoire of TCRs such that the encoded Ag specificities are largely similar to those of the peptides that induced their positive selection (38). Likewise, it has been postulated that a diversity of self peptides is critical for positive selection in the presence of negative selection on many self MHC/peptide complexes to select TCRs with various Ag specificities (29). Mice that express single A<sup>Ep</sup> complex and have been reconstituted with wild-type fetal liver cells provide a model to investigate the Ag specificities of TCRs positively selected by the defined MHC/peptide ligand and tolerant to a normal set of self-derived peptides. The CD4<sup>+</sup> T cell repertoire in such chimeras also represents the potential contribution of positive selection on a single class II MHC/peptide complex in the normal repertoire of CD4<sup>+</sup> T cells.

Subsequently, we performed proliferation assays to test whether a repertoire of CD4<sup>+</sup> T cells selected on a single A<sup>Ep</sup> complex and tolerant to self peptides is skewed toward preferential interaction with Ags that are related in primary amino acid sequence to the selecting Ep (Fig. 5). For this purpose, A<sup>wt</sup>→A<sup>Ep</sup>I<sub>II</sub> chimeric mice were separately primed with several antigenic peptides. The CD4<sup>+</sup> T cells were isolated from these immunized chimeras as described and incubated with the same antigenic peptides presented by the irradiated spleen APCs from wild-type mice. On days 3 and 5, IL-2 was added to further expand cells that specifically responded to the added peptide, and the proliferative response was measured on day 7 as previously described (41).

In five separate experiments, four different soluble peptides have been screened. The amino acid sequences of these peptides, with TCR contact residues indicated in boldface, are listed in Table I. Two of them are single amino acid analogues of Ep<sub>52–68</sub> and were chosen to represent antigenic peptides that are similar in primary amino acid sequence to the selecting Ep. These two Ep analogues were frequently recognized by CD4<sup>+</sup> T cells from A<sup>Ep</sup>I<sub>II</sub> mice, and changed amino acids in position 58 or 63 have been mapped as TCR contact residues (30). Two other peptides different from Ep were also included in this test. One of these (EpKKK) was designed on the backbone of Ep with three TCR-facing residues. The fourth antigenic peptide tested, PCC<sub>3,5</sub>, was chosen from a set of peptides known to bind to A<sup>Ep</sup> molecules.

As shown in Fig. 5, the repertoire of CD4<sup>+</sup> T cells selected on A<sup>Ep</sup> complex and tolerant to wild-type A<sup>Ep</sup> complexes did not respond to the tested Ep analogues significantly better than to the rest of the tested peptides. For example, one of the Ep analogues with single substitution in TCR contact residues did not trigger an
enhanced CD4+ T cell response. Moreover, in proliferation assays the PCC43-58-derived peptide was a very good stimulator of CD4+ T cells selected in Ab wt→AβEpIi+ chimeras, and this peptide differs significantly from Ep.

In conclusion, the finding that peptides very different from the selecting Ep induced proliferation of CD4+ T cells selected in Ab wt→AβEpIi+ chimeras showed that this repertoire, although quantitatively restricted, preserves the capacity to respond to various Ep-related and unrelated Ags. This result contradicts the presumption that the original, quite diverse spectrum of CD4+ T cells found in AβEpIi+ mice will encode few Ep-like Ag specificities after negative selection in vivo on wild-type Aβ/peptide complexes (29, 38).

Repertoires of TCRs specific for the same Ag and selected in Ab wt→AβEpIi+ chimeras or wild-type mice can be similar, but the former repertoire is more restricted

To further investigate the composition of TCRs selected by AβEp complex and tolerant to self-derived peptides, we again primed the Aβwt→AβEpIi+ chimeric mice with six different soluble peptides in the presence of CFA (their amino acid sequences are listed in Table I). The goal of these experiments was to isolate a set of Ag-specific TCRs that developed in these chimeras and to determine their primary sequences. At the same time we primed wild-type mice with identical antigenic peptides so that the wild-type repertoire of TCRs specific for the same antigenic peptides was generated. CD4+ T cells from Ag-primed chimeras and wild-type mice were converted into T cell hybridomas, followed by screening for CD4/TCR expression and Ag specificity. As expected, a large number of CD4+ T cell hybridomas specific for each tested peptide was produced from wild-type mice. The number of CD4+ T cells in Aβwt→AβEpIi+ chimeras is only 3–5% of that seen in the wild-type mice, although in two subsequent immunizations for each tested peptide, we generated a total of 25 CD4+ T cell hybridomas specific for different antigenic peptide. This result correlated with the proliferation assay data and confirmed that the repertoire of CD4+ T cells selected by single AβEp ligand and tolerant to wild-type Aβ/peptide complexes preserves TCRs with various Ag specificities. Interestingly, peptide PCC43-58, which was the most unrelated in primary sequence to Ep, was found to be recognized by five different TCRs that originated from Aβwt→AβEpIi+ chimeras. Moreover, one CD4+ T cell hybridoma specific for EpKKK and one for IgGvH39-74 peptide were also isolated (see below).

To compare the complexity of TCR repertoires of CD4+ T cell hybridomas specific for the same antigenic peptide from Aβwt→AβEpIi+ or wild-type mice, we determined their Vβ repertoire using flow cytometry. As expected, the repertoire of TCRs specific for every tested antigenic peptide was significantly more diverse in the hybridomas derived from wild-type mice than in those from Aβwt→AβEpIi+ chimeras. Despite differences in the numbers of CD4+ T cells, both repertoires often shared the most prevalent Vβ segments. For example, either Vβ6 or Vβ14 subpopulations were commonly present among CD4+ T cell hybridomas specific for Ep58K or Ep63K analogues from both types of mice (Fig. 6). In addition, as was previously reported for AβEpIi+ and wild-type mice, four of six hybridomas specific for PCC43-58 were Vβ8+ (30).

We also tested how frequently TCRs expressed on T cell hybridomas derived from Aβwt→AβEpIi+ chimeras cross-react with different Ep analogues. The intent of this experiments was to evaluate the hypothesis that Aα molecules exclusively bound with Ep may positively select CD4+ T cells with a permissive mode of Ag recognition (38). In general, we found that isolated T cell hybridomas are peptide specific and that some can discriminate a single amino acid substitution in antigenic peptide. For example, three different TCRs selected in AβEp exclusively responded to Ep58K, but not to Ep58R or any other tested peptides (Fig. 7). Similarly, of five TCRs specific for the Ep analogue with K and/or R in position 63 (Ep63K, Ep63R), four showed no response to other tested analogues of Ep. Obviously, some of the tested T cell hybridomas shared a common epitope, but no common propensity toward Ep-like Ags could be found. We concluded that TCRs selected in Aβwt→AβEpIi+ chimeras have no prevalent Ep reactivity and are Ag specific, as are normal CD4+ T cells from wild-type mice.

The repertoire of Ag-specific TCRs selected in Aβwt→AβEpIi+ chimeras is diverse, but some TCRs are similar

Since the repertoire of CD4+ T cells positively selected on single AβEp complex and tolerant to Aβwt was found to encode multiple Ag specificities, we further investigated the primary DNA sequences of these TCRs. The aim of this analysis was to determine

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### Table I. Amino acid sequences of peptides used to test Ag specificities of CD4+ T cells selected in Aβwt→AβEpIi+ chimeras

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence*</th>
</tr>
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<tbody>
<tr>
<td>Ep (Eps8-68)</td>
<td>ASFEAQGAALANIAVDKA</td>
</tr>
<tr>
<td>Ep58K</td>
<td>ASFEAQGAALANIAVDKA</td>
</tr>
<tr>
<td>Ep63K</td>
<td>ASFEAQGAALANIAVDKA</td>
</tr>
<tr>
<td>Ep61K</td>
<td>ASFEAQGAALANIAVDKA</td>
</tr>
<tr>
<td>EpKKK</td>
<td>ASFEAQKAALANIAVDKA</td>
</tr>
<tr>
<td>PCC43-58</td>
<td>AEQFSYTDANKKGIT</td>
</tr>
<tr>
<td>IgGvH39-74</td>
<td>NADFFTPAILTVD</td>
</tr>
</tbody>
</table>

* Alignments were established as described elsewhere (30). The peptide amino acids that are predicted to contact TCRs are in bold.
whether the TCRs expressed on CD4+ T cells in Aβwt→AβEpil− chimeras that are capable of responding to a given peptide Ag are related.

The complementarity-determining region (CDR) sequences of 25 Ag-specific TCRs from Aβwt→AβEpil− chimeras were determined (Fig. 8). Two-thirds of these TCRs were specific for the tested Ep analogue. Six other TCRs were specific for PCC43–58, one for IgG59–74, and one for EpKKK.

Although none of the sequences of the isolated TCRs shared common amino acid motifs, some individual TCRs were similar. More importantly, some homology could be found in the amino acid sequences of TCRs specific for different antigenic peptides. For instance, three TCRs, two (no. 87 and 134) specific for PCC43–58 peptide and one (no. 102) specific for Ep58K analogue, used TCR α-chain with the same amino acid sequence coupled with three different TCR β-chains. As shown in Table I, the PCC43–58 peptide and Ep58K analogue are quite different, albeit the p3p5 residues in both peptides may be acceptable for the same TCR β-chain if the proposed common orientation of TCR interchain among all TCC-specific TCRs varied by only one amino acid in both TCR α- and β-chains. In contrast, the length of CDR3 in TCRs specific for the two tested Ep analogues was usually three amino acids in both TCR chains as described below (see Fig. 8).

This result indicates that a single class II MHC/peptide complex may have more versatility for positive selection of some TCRs. The rest of the TCRs isolated from Aβwt→AβEpil− chimeras were specific for different Ep analogues. These TCRs were sorted into six groups based on their reactivities with different Ep analogues (see Fig. 7). The first group includes TCRs that specifically responded to Ep analogues with K or R present on the C′ terminus of the antigenic Ep analogue. Four of five TCRs specific for these large, charged, side chains in position 8 of Ep used either Vβ6 or Vβ14 segments. We found no other similarities in Vα usage among these TCRs. The fifth TCR (no. 48) in this category responded not only to Ep63K and Ep63R analogues but also to EpKKK. This last TCR was originally generated by priming Aβwt→AβEpil− chimeras with EpKKK, which probably explains its further relationship to the rest of the TCRs in this group.
The second group included three TCRs exclusively specific for a single large charged substitution in position p2 of Ep. Each of these TCRs used different TCR α- and β-chains, and even the lengths of the CDR3 regions between these TCRs are different. However, as mentioned above, two of these TCRs are similar to TCRs specific for other tested antigenic peptides (Fig. 8).

The third group included TCRs that responded to Ep analogues with an unchanged middle sequence of the peptide, in particular the p5 position. Remarkably, two of these TCRs (no. 85 and 141.5) express completely different TCR α-chains coupled with almost identical TCR β-chains (one amino acid difference in CDR3 region). Since the p5 position is likely to contact CDR3 regions on both TCR chains, the similarity between these two TCR β-chains is being further investigated.

The fourth group of TCRs included hybridomas that responded to all the tested Ep analogues that lacked large side chains in the N terminus of the Ep sequence. These TCRs did not respond to Ep analogues with alanine substituted by lysine in the p2p5 positions of Ep. Three TCRs in this group were very different, although one was noticed to be similar to one of the TCRs from group 6.

Two TCRs that could not be placed in any of the five other groups were put into group 6. One of these (no. 16) was exclusively specific for soluble Ep (see Fig. 7). Currently, we are investigating why other Ep analogues were not stimulatory for this TCR. The other TCR in this group (no. 94) responded to every tested Ep analogue with a single amino acid substitution.

In conclusion, Ag-specific TCRs isolated from Aβwt → AβEpII mice revealed that this repertoire remains relatively diverse but far less heterogeneous than the wild-type repertoire of TCRs specific for the same Ags, as immediately evident in a comparison of the TCR Vb repertoires (Fig. 8). Most likely, positive selection on the single AβEp complex combined with extensive negative selection on the normal spectrum of self peptides forces this TCR repertoire to use a more limited spectrum of V, D, and J TCR segments. TCRs specific for antigenic peptides unrelated to Ep seemed to be more restrained than those specific for Ep analogues.

**Discussion**

This report provides the first evidence that in vivo positive selection on a single class II MHC/peptide complex overcomes negative selection on the normal set of self-derived peptides and produces a number of different Ag-specific CD4+ T cells to the periphery. Moreover, TCRs selected in these unusual circumstances, i.e., positive selection by one MHC/peptide ligand and virtually unrestricted negative selection, are not degenerated in their ability to...
specifically respond to Ag presented in the context of the same MHC restriction element. We have found no signs of a dominant specificity directed against one or a few antigenic peptides. Instead, this repertoire is semidiverse, implying that Ag specificities of TCRs positively selected on a single MHC/peptide complex and tolerant to self peptides are quite broad, rather than limited. This result agrees with previous reports that showed that thymic milieus expressing single or dominant class II MHC/peptide complexes propagate TCRs with various Vα/Vβ segments able to respond to Ags unrelated to the selecting peptide. This finding also implies that poor shape complementarity found in crystal structure of TCR engaged by self class I MHC/peptide complexes is likely to be a common mode for in vivo interactions that provoke thymic positive selection of majority of thymocytes (43).

However, it would be misleading to assume that one class II MHC/peptide element can substitute for MHC molecules bound with many self-derived peptides in selecting T cells in the thymus. There are visible limitations, and probably unbalances, in the representation of various Ag specificities in a repertoire selected by a single class II MHC/peptide complex. For instance, some of the Ab Ep selected TCRs that are subject to negative selection on wild-type Ab/peptide complexes, as tested by in vitro response to Ab wt APCs alone, also responded to different antigenic peptides (30). In addition, in Ab wt × Ab EpIi2 mice, CD4+ T cells remaining after negative selection are able to mount specific responses against several very different antigenic peptides. Therefore, the TCR repertoire generated by positive selection on the Ab Ep complex not only exceeds negative selection on the same Ab Ep, but also overcomes negative selection on many other self MHC/peptide complexes and contributes a noticeable number of different CD4+ T cells to the periphery. Since positive selection on the Ab Ep complex promotes 3–5% of the normal number of CD4+ T cells in Ab Ep+ or DM− mice was not recorded in previous in vivo experiments (28, 30, 39).

The majority of CD4+ T cells positively selected in vivo on single Ab Ep complexes are negatively selected on Ab molecules occupied with endogenous peptides. The extent of the observed deletion, approximately two-thirds, is the same for repertoires positively selected on two unrelated peptides bound to Ab molecules (Ep or CLIP) expressed at either low or normal levels (Ref. 28 and this report). Here we have shown that this profound deletion reflects that lack of tolerance to self peptides presented in the context of the selecting II MHC. It is unlikely that positive selection by a single MHC/peptide complex narrows such a repertoire toward one or a few Ags. For example, some of the Ab Ep selected TCRs that are subject to negative selection on wild-type Ab/peptide complexes, as tested by in vitro response to Ab wt APCs alone, also responded to different antigenic peptides (30). In addition, in Ab wt × Ab EpIi2 mice, CD4+ T cells remaining after negative selection are able to mount specific responses against several very different antigenic peptides. Therefore, the TCR repertoire generated by positive selection on the Ab Ep complex not only exceeds negative selection on the same Ab Ep, but also overcomes negative selection on many other self MHC/peptide complexes and contributes a noticeable number of different CD4+ T cells to the periphery. Since positive selection on the Ab Ep complex promotes 3–5% of the normal number of CD4+ T cells in Ab Ep+ mice to the periphery, potentially around 100 different self class II MHC/peptide complexes may positively select almost a normal number of CD4+ T cells, a published hypothesis that has not been experimentally tested (44).

<table>
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<th>CDR2</th>
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FIGURE 8. Amino acid sequences of CDR1, CDR2, and CDR3 regions of both Vα and Vβ segments of TCRs of hybridomas from Ab wt→Ab Ep− mice responding to different peptides. Amino acid sequences were deduced from DNA sequencing (in duplicate) of PCR products as described.
Around 50 and 80% of peripheral CD4+ T cells in AβEpIi- and Aβwt→AβEpIi- mice are CD45RBhigh/CD44high, respectively. In contrast, <20% of these CD4+ T cells are found in wild-type or AβEpIi-βm-mice. Moreover, none of the mice tested had many CD45RBlow/CD44high SP CD4+ thymocytes, as previously reported for DM- chimeric mice. These findings suggest that many of these CD4+ T cells may be selected on nonclassical MHC molecules, and they can be abundant in the periphery of mice with restricted positive selection on class II/peptide complexes. Therefore, quantitative and qualitative analysis of the repertoire of TCRs selected by the single or dominant class II MHC/peptide complex will be adequate after this type of CD4+ T cells is excluded. Meanwhile, in AβEpIi-βm-mice, the absolute number of peripheral CD4+ T cells with a naive phenotype almost triples, indicating that the peripheral pool of AβEp selected CD4+ T cells is also controlled by unknown homeostatic mechanisms (see Fig. 3).

Introduction of the negative selection on wild-type Aβ peptide complexes in our chimeric Aβwt→AβEpIi mice also removed an abundance of alloreactive CD4+ T cells present in AβEpIi- mice. We have tested 50 CD4+ TCR+ T cell hybrids from Aβwt→AβEpIi chimeras and found them unresponsive to three different class II MHC molecules (data not shown). This result agrees with our former postulate that negative selection on many self-derived peptides calibrates the MHC restriction such that immature T cells excessively reactive to MHC per se are largely eliminated in the thymus (22).

Analysis of TCRs selected on AβEp and tolerant to wild-type self peptides indicates that this repertoire is relatively heterogeneous, with no dominant pattern of Ag reactivity. The diversity of CDR regions of TCRs specific for Ep-like Ags is higher than the diversity of CDRs of TCRs specific for Ep-unrelated peptides such as PCC. Since covalently bound Ep probably projects one substantial side chain, an isoleucine at position p8 toward TCR, one may argue that peptides with more “bulky” TCR contact residues will positively select a narrower, but more specific, set of TCRs (45). Future experiments involving analysis of Ag specificities and CDR sequences of TCRs selected on MHC coupled with flat or “haairy” peptides should reliably test this speculation.

What, then, are the general rules that govern the positive and negative selection of T cells in the thymus? It seems that positive selection is “promiscuous with borders” such as TCRs with the same Ag specificities are selected by various MHC/peptide ligands. This property of thymic selection may ensure that the specific Ag response does not entirely depend on positive selection by one self MHC/peptide complex. Likewise, for many TCRs there is more than one self MHC/peptide combination able to promote their positive selection. Finally, since the same self peptides are likely to be involved in positive and negative selections, the repertoire that leaves the thymus has scattered, rather than narrow, specificities. Such a general mode of thymic selection, where positive selection is less peptide dependent than negative, enhances its effectiveness and increases the diversity of T cells specific for a particular Ag.

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References