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Efficient positive selection of a broad repertoire of T cells is dependent on the presentation of a diverse array of endogenous peptides on MHC molecules in the thymus. It is unclear, however, whether the development of individual TCR specificities is influenced by the abundance of their selecting ligands. To examine this, we analyzed positive selection in a transgenic mouse carrying a TCR specific for the human CLIP:I-Ab class II complex. We found that these mice exhibit significantly reduced CD4⁺ T cell development compared with two other transgenic mice carrying TCRs selected on I-A². Moreover, many of the selected cells in these mice express endogenous and transgenic receptors as a consequence of dual TCRα expression. Dramatic enhancement of the selection efficiency is observed, however, when fewer transgenic cells populate the thymus in mixed bone marrow chimeras. These results suggest that positive selection is limited by the availability of selecting peptides in the thymus. This becomes apparent when large numbers of thymocytes compete for such peptides in TCR transgenic animals. Under such conditions, thymocytes appear to undergo further TCRα gene rearrangement to produce a receptor that may be selected more efficiently by other thymic self-peptides. The Journal of Immunology, 2000, 164: 6252–6259.

T hymocytes proceeding along the αβ T cell lineage must pass through several developmental checkpoints before they can mature into functional T cells. To ensure the generation a diverse repertoire of T lymphocytes that can effectively respond to a myriad of potential Ags, developing thymocytes undergo random gene rearrangement at the TCR loci to produce a unique receptor for each cell. Productive rearrangement of the TCR β-chain gene on one chromosome prevents further gene rearrangements at the second TCRβ allele and allows the initiation of TCRα gene rearrangement (1). Allelic exclusion is not as tightly controlled at the TCRα locus, however, resulting in some T cells that express two α-chains paired with a common β-chain on the cell surface (2–6). Expression of a second TCR generally occurs when the first receptor fails to interact with thymic self-MHC molecules (7–11), which is required for cells to be positively selected to mature (12).

Numerous studies have shown that positive selection of T cells is critically dependent on the makeup of the endogenous peptide repertoire expressed on thymic MHC molecules (reviewed in Ref. 13 and 14). Initial in vitro studies of positive selection of class I MHC-restricted CD8⁺ T cells showed that only specific peptides can promote the development of TCR transgenic T cells in class I-deficient fetal thymic organ cultures (15–18). A similar requirement for peptide specificity was shown for the selection of class II MHC-restricted CD4⁺ T cells in studies analyzing thymocyte development in mice expressing a severely reduced endogenous peptide repertoire. H-2M⁺ mice, for example, express predominantly the invariant chain proteolytic fragment, CLIP, on class II molecules due to the inability to remove this peptide and allow antigenic peptide binding (19–24). Although a large population of CD4⁺ T cells can still be selected in these mice, the selected repertoire is incomplete and less diverse in the types of TCR Vα-Jα joins used (25–27). Moreover, our previous studies suggest that a large number of the CD4⁺ T cells may be selected by the few endogenous non-CLIP peptides displayed on class II molecules in H-2 M⁺ mice (28). Finally, mice engineered to express a single I-A² binding peptide, Eo52_55, on most class II molecules exhibit normal selection of CD4⁺ T cells; however, when crossed onto an H-2M⁺ background, CD4⁺ T cell development in these mice is severely diminished (29). Thus, positive selection in this system occurs only when other endogenous peptides can be loaded onto the small percentage of class II molecules not occupied by the Eo peptide.

These studies suggest that positive selection of a normal T cell repertoire requires a diverse array of endogenous peptides, including those that are expressed in relatively low abundance in the thymus. However, it is unclear whether the selection of T cells by self-peptides that are expressed at lower levels by thymic cortical epithelial cells is as efficient as selection mediated by more abundant peptides. Several studies suggest that this might not be the case, since they show that the number of thymocytes induced to undergo positive selection can be strongly influenced by the level of expression of MHC molecules (30–33). To explore this issue further, we have examined the development of class II-restricted CD4⁺ T cells in three different TCR transgenic mice carrying receptors selected on I-A². Two of these mice express a TCR specific for the human CLIP:I-Ab class II complex. We found that these mice exhibit significantly reduced CD4⁺ T cell development compared with two other transgenic mice carrying TCRs selected on I-A². Moreover, many of the selected cells in these mice express endogenous and transgenic receptors as a consequence of dual TCRα expression. Dramatic enhancement of the selection efficiency is observed, however, when fewer transgenic cells populate the thymus in mixed bone marrow chimeras. These results suggest that positive selection is limited by the availability of selecting peptides in the thymus. This becomes apparent when large numbers of thymocytes compete for such peptides in TCR transgenic animals. Under such conditions, thymocytes appear to undergo further TCRα gene rearrangement to produce a receptor that may be selected more efficiently by other thymic self-peptides. The Journal of Immunology, 2000, 164: 6252–6259.

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3 Abbreviations used in this paper: CLIP, class II-associated invariant chain peptide; hCLIP, human CLIP; mCLIP, murine CLIP; Ii, invariant chain; Ii⁻, Ii deficient; H-2 M⁺, H-2 M deficient; BrdU, 5-bromo-2-deoxyuridine; RAG, recombinase-activating gene; RAG2⁻, RAG deficient; SP, single positive; DP, double positive.
mice, TαA and OT-II, have been previously described (28, 34), whereas the third, TClI, is a novel strain recently generated in our laboratory. Since TCR transgenic mice provide a system where the thymus is saturated with cells specific for the same selecting ligands, we could examine whether the relative availability of selecting peptides for each TCR played an important role in development. We found that positive selection of thymocytes bearing the TClI TCR was significantly less efficient than the selection of CD4+ T cells observed in the other TCR transgenic mice. This was apparently due to thymocyte competition for selecting peptides expressed in limited abundance in the thymus. To compensate for this, thymocytes expressed an additional receptor through secondary TCRα gene rearrangement, allowing positive selection by other, more readily available thymic peptide:MHC complexes.

Materials and Methods

Animals
Six- to 8-wk-old female C57BL/6J (B6, H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the animal facility at the University of Washington. Recombinase activating gene-2 (RAG2)-deficient mice (H-2b) were purchased from Taconic Farms (Germantown, NY). Invariant chain-deficient (Ii-/-) mice (H-2b) were provided by Elizabeth Bikoff (Harvard University, Boston, MA). H-2 M-deficient (H-2 M+/-) mice (H-2b) were provided by Luc Van Kaer (Vanderbilt University, Nashville, TN). OT-II/TCRα and OT-II/TCRβ TCR transgenic mice were provided by Harriet Sur (The Scripps Research Institute, La Jolla, CA). TαA TCR transgenic mice were generated as previously described (28) and were bred to RAG2+/- mice for some experiments. Mutant and transgenic mice were bred and maintained under specific pathogen-free conditions at the animal facility at the University of Washington (Seattle, WA).

Generation of TClI TCR transgenic mice
The hCLIP-I-Aβ-specific T cell hybridoma 51-11.5 was generated by immunizing H-2b mice with hCLIP peptide (Iiα-Iioα) in CFA and subsequently fusing activated lymph node cells with the TCRαβ-negative BW5147 fusion partner (35). RT-PCR with a panel of TCR Vα-specific primers (provided by Pam Fink and Joan Goverman, University of Washington) paired with a Cα primer and Vβ-Cβ primers indicated that this hybridoma uses Vα18 (P14A.1)-Iio5 and Vβ6-Dβ1-Jβ2.2. PCR primers (Vα18 9, 5′-TTC CCC GGG AAG AAT GCA CAG CCT CCT AGG GTT 3′; Jα44 9, 5′-CTT TGC GGC GGC TGT ACT TTG TGA TCA C-3′; Vβ6 9, 5′-AAA GAA ACT CGA GCC AAA CTA TGA ACA AGT GG-3′; Jβ2.2 9, 5′-TCC CGC GGT ACC CCTG CTG CTT ACC CAC CAC T-3′) located 5′ and 3′ of the relevant variable region leader and J region splice donor sites were designed to amplify the rearranged TCR variable region sequences from 5′-11.5 genomic DNA and to introduce appropriate restriction sites for insertion into the XbaI-SacII or XhoI-SacII cloning sites of the pTCα and pTCβ genomic cassettes (36), respectively (provided by Diane Mathis). The expression and functionality of the TCR encoded by the resulting genomic constructs were confirmed by their transfection into a TCR-loss variant of the DO11.10 T cell transgenic line. Constructions of the TCRβ transgenic mice involved injection of only the TCRβ construct. TCI TCRαβ transgenic mice were additionally crossed onto the RAG2+/- background.

Radiation bone marrow chimeras
Bone marrow cells collected from the femurs and tibias of TCI RAG2+/- or TCI-RAG2+/- TCR transgenic or wild-type mice were washed in supplemented RPMI 1640 and depleted of T cells by treatment with a mixture of anti-CD4, anti-CD8 mAbs, and rabbit complement (Accurate Chemical & Scientific Corp., Westbury, NY). Cells were then washed several times in serum-free RPMI and 5 × 10^9 cells were injected into the tail vein of lethally irradiated (950 rad) recipient chimeras. Chimeras were given antibiotic-containing water and were healthy when analyzed 6–10 wk after bone marrow transfer.

Abs and peptides
The following mAbs directed to mouse cell surface Ags were purchased from PharMingen (San Diego, CA): anti-CD4-PE (RM4-5), anti-CD4-peridinin chlorophyll protein (RM4-5), anti-CD8α-FITC (53-6.7), anti-CD8a-allophycocyanin (53-6.7), anti-TCR-β-FITC (H5-579), anti-TCR-α-biotin (H5-579), anti-CD3e-biotin (B20.1), and anti-CD8a-biotin (B20.1). Anti-Vα11-biotin (R85-1), anti-CD4-9.1-FITC (30C7), anti-TCR-β-FITC (H551), and anti-CD3-145.2C11). Biotin-coupled anti-TCR Vα Abs (Vβ2-B2.06, Vβ3-K25, Vβ5-K4.T1, Vβ5-M9R-4, Vβ6-R4-7, Vβ7-TR130, Vβ8-F23.1, Vβ9-MR10-2, Vβ10-B21.5, Vβ11-R3-15, Vβ14-14.2) were provided by Ann Pullen. Abs used for cell depletions were GK1.5 (anti-CD4, American Type Culture Collection, Manassas, VA) and 53-6.72 (anti-CD8, American Type Culture Collection). Anti-597, anti-deoxyuridine I-Aβ (anti-597)FITC Ab was obtained from Becton Dickinson (Mountain View, CA). All peptides were synthesized with a Syn-ergy 432 (Applied Biosystems, Foster City, CA) automated peptide synthesizer using F-moc chemistry and analyzed by reverse phase HPLC. The purity of peptides used was >90%.

Flow cytometry and BrdU labeling
In three-color analyses, approximately 5 × 10^5 cells were incubated on ice for 30 min with FITC-, PE-, and biotin-conjugated Abs; washed in PBS containing 1% FCS (Life Technologies, Grand Island, NY) and 0.1% sodium azide; and incubated with streptavidin-Tricolor (Caltag, San Francisco, CA) for an additional 30 min on ice. In four-color analyses, approximately 5 × 10^5 cells were incubated on ice for 30 min with PE-, peridinin chlorophyll protein-, allophycocyanin-, and biotin-conjugated Abs; washed in PBS containing 1% FCS (Life Technologies) and 0.1% sodium azide; and incubated with fluorescein-avidin D (Vector, Burlingame, CA) for an additional 30 min on ice. Labeled cells were washed and analyzed on a FACScan or FACScalibur flow cytometer (Becton Dickinson) using CellQuest and ReproMac software. For continuous BrdU labeling, BrdU (Sigma, St. Louis, MO) was dissolved in PBS at 0.8 mg/ml and given fresh daily to mice for a period of 1–4 days, after which mice were sacrificed, and their thymi were isolated for flow cytometric analysis.

T cell proliferation assays
For measurement of proliferative responses of peripheral T cells from TCR transgenic mice, 4 × 10^5 pooled lymph node cells were cultured per well with titrated amounts of CLIP peptide in flat-bottom 96-well plates. T cell proliferation was quantitated after 48 h of culture by pulsing cells with 1 μCi [3H]thymidine/well for 20 h before harvesting cells onto glass-fiber filters and determining incorporated radioactivity using a BETAPlate liquid scintillation counter (Wallac, Turku, Finland). Results shown are expressed as mean counts per minute of duplicate cultures. In some experiments, T cells were first sorted on a FACSStar Plus and plated at approximately 1 × 10^5 to 4 × 10^5/well with approximately 5 × 10^5 irradiated syngeneic B6 splenocytes plus peptide or Con A. Cells were pulsed with 1 μCi of [3H]thymidine/well after 48 h of culture as described above. For analysis of endogenous TCRβ expression among TCI T cells after cognate peptide stimulation, Ly-9.1+ TClI splenocytes were mixed with wild-type Ly-9.1+ B6 splenocytes at a 1:5 ratio for a total of 2 × 10^6 cells/well in a 24-well plate and pulsed with either 0.2 μg/ml hCLIP or 5 μg/ml Con A. After 7 days, cells were analyzed by flow cytometry. All cell cultures were maintained in RPMI 1640 supplemented with 200 μM l-glutamine, 10 mM HEPES, 5 × 10^-3 M 2-ME, antibiotics (penicillin-streptomycin), and 5% FCS at 37°C, 5% CO2.

Results

Diminished CD4+ T cell development in human CLIP-I-Aβ-specific TCI TCR transgenic mice
To determine whether the efficiency of positive selection is similar for TCRs specific for the same restriction element, we examined CD4+ T cell development in three different lines of TCR transgenic mice harboring receptors selected on I-Aβ. The TαA TCR (Vβ6-Vα2.2) recognizes the E052–68 epitope derived from the class II I-Eα-chain, while the OT-II TCR (Vβ5-Vα2) is specific for the OVA 323–339 peptide from chicken OVA, both in the context of I-Aβ (28, 34). We have also created a novel TCR transgenic mouse, TClI, which carries a receptor specific for the human invariant chain-derived CLIP peptide (hCLIP) in the context of I-Aβ. TCI mice express a Vα18-Jβ45 TCRα chain paired with a Vβ6-Dβ1-Jβ2.2 TCRβ-chain cloned from the hCLIP-I-Aβ-specific TCR.
cell hybridoma 51-11.5 previously described (35). Similar to the TEa mice, expression of the transgene is driven by the natural TCR promoter/enhancer elements engineered in the pTα and pTβ TCR expression cassettes into which the TCli TCR was cloned (36). T cells from the TCli mice exhibit uniformly high Vβ6 expression and strong proliferative responses to even small amounts of hCLIP (Fig. 1B and data not shown).

When we analyzed the development of transgenic T cells in the thymus, we were surprised to find that positive selection of CD4+ T cells was significantly reduced in TCli mice compared with that of the other two transgenic lines (Figs. 1 and 2). Thymic cellularity was similar among the three transgenics, and flow cytometric analysis of thymocytes from all three lines showed the increased ratio of CD4+ to CD8+ T cells expected for class II-restricted TCR transgenic mice. However, the skewing toward CD4 single-positive (SP) T cells in the thymus of TCli mice was consistently much less pronounced than that in the TEa and OT-II mice, which have a much higher proportion of cells in the CD4 SP compartment (Figs. 1 and 2).

To further assess the selection efficiency of the TCli αβ TCR, we crossed the transgenons onto RAG2−/− mice to prevent rearrangement of endogenous TCRα-chain genes that could contribute to the expression of nontransgenic receptors. Interestingly, although T cell development was still skewed toward the CD4+8− compartment, there was a further reduction (>50%) in the percentage of CD4 SP thymocytes in TCliRAG2−/− mice compared with that in RAG2+ transgenic littermates (Fig. 1A). The majority of TCliRAG2+ thymocytes accumulated at the CD4+8− double-positive (DP) stage, and the thymic cellularity was about 2-fold higher than that seen in TCliRAG2+ mice. The drop in the percentage of CD4 SP cells was considerable compared with that in the other TCR transgenic mice when endogenous TCRα-chain expression was abrogated. Flow cytometric analysis showed that the percentage of CD4 SP cells decreased only modestly in TEaRAG2+ and OT-II/TCRα− strains relative to that in their RAG2+ or TCRα− littermates, with a large percentage of cells still able to transit efficiently from the DP to CD4 SP stage of development (Fig. 2). It is also noteworthy that for a class I-restricted TCR transgenic mouse, OT-I, on the H-2b background, we observed no decrease in CD8+ T cell selection in the absence of RAG (not shown).

The dramatic reduction in CD4+ T cell selection in TCliRAG2+ mice implied that a large proportion of the CD4 SP T cells selected in the RAG-sufficient transgenics express endogenous TCRα-chains paired with the transgenic β-chain. To confirm this, we assessed surface expression of other TCRα-chains in TCliRAG2+ mice and could readily detect CD4+ T cells expressing Vα2. -3.2, -8, and -11 (Fig. 1C). In contrast, we found very few cells bearing background.

FIGURE 1. TCli TCR transgenic mice exhibit weak positive selection of CD4+ T cells and express many endogenous TCRα-chains. A, Thymus CD4/CD8 flow cytometric profiles for wild-type littersmates, TCliRAG2+/−, and TCliRAG2− mice. The percentages of cells in each quadrant are indicated. B, Proliferative response of lymph node cells from wild-type (▲) vs TCliRAG2−/− mice (■) to hCLIP. C, Expression of endogenous TCRα-chains in wild-type B6, TCli, and TEa mice. The percentages of lymph node CD4+ T cells positive for the indicated Vβ or Vα by FACS analysis are indicated. Both TEa and TCli TCRs use Vβ6, but the TCli TCR uses Vα18, while the TEa transgenic TCR uses Vα2.

FIGURE 2. TEa and OT-II TCR transgenic mice show efficient positive selection of CD4+ T cells. A, CD4/CD8 FACS profiles for Eα25–32/I-Aβ− specific TEaRAG2+ and TEaRAG2− thymocytes. The percentages of cells falling in each quadrant are indicated. B, CD4/CD8 FACS profiles for OVA323–339/I-Aβ− specific OT-II/TCRα+ and OT-II/TCRα− thymocytes. The percentages of cells falling in each quadrant are indicated.
endogenous TCR Vα3.2, -8, and -11 among CD4+ T cells of TEa and OT-II mice (Fig. 1C and data not shown).

Expression of both endogenous and transgenic TCRα-chains on the majority of TCIi T cells

Several studies have shown that TCRαβ-expressing thymocytes that fail to be positively selected can undergo further TCRα gene rearrangement to produce a selectable TCR, thereby expressing two TCRα-chains on the cell surface (7, 8, 10, 37). Since the TCIi TCR is selected relatively poorly, we reasoned that the prevalent endogenous α-chain usage might reflect dual TCRα expression as a means of improving selection efficiency. Alternatively, poor expression of the transgenic TCRα-chain could result in large numbers of T cells that express only endogenous TCRα-chains. To exclude the latter possibility, we assessed whether the endogenous TCRα expression observed in TCIiRAG2+ mice reflected prominent dual TCRα expression on transgenic T cells.

Without an anti-clonotypic or anti-Vα18 Ab, we were unable to determine directly whether endogenous and transgenic TCRα-chains were coexpressed on the same cells or expressed on distinct cell populations. To examine this in an alternative fashion, we compared the ability of CD4+ T cells from TCIiRAG2+ and TCIiRAG2- mice to respond to cognate Ag. If nontransgenic and transgenic α-chains were expressed on different populations of cells, then we would expect TCIiRAG2+ cells to give a lower proliferative response to hCLIP than TCIiRAG2+ cells in an assay in which equivalent numbers of CD4+ T cells were analyzed. This would be due to the large proportion of nonresponding, endogenous TCRα-bearing CD4+ T cells in the TCIiRAG2+ mice.

When CD4+ T cells isolated from TCIiRAG2+ and TCIiRAG2- mice were stimulated with peptide-pulsed, irradiated, syngeneic, wild-type B6 splenocytes, a similar degree of proliferation to hCLIP by the two responding populations was observed (Fig. 3A). Comparable responses to hCLIP were also observed in assays where the responder CD4+ T cells from TCIiRAG2+ and TCIiRAG2- mice were titrated from $4 \times 10^3$ to $1 \times 10^3$ cells/well (not shown). This strongly suggested that although many TCIiRAG2+ T cells express endogenous α-chains, these cells coexpress the hCLIP-responsive TCIi TCRαβ.

To show directly that many of the endogenous TCRα-bearing transgenic T cells also express a functional TCIi TCRα-chain on the surface, we sorted Vα2+ CD4+ T cells from TCIiRAG2+ mice and assessed their ability to respond to hCLIP. We found that Vα2+ CD4+ T cells from TCIiRAG2+ mice responded to the cognate peptide almost as strongly as the same number of CD4+ T cells from TCIiRAG2- animals carrying solely the transgenic TCR (Fig. 3B). The slightly lower response is probably due to decreased levels of the TCIi TCR on the cell surface in the presence of an endogenous TCRα-chain that competes for pairing with the TCIiβ-chain. However, one might also argue that pairing of the TCIi TCRβ-chain with an endogenous TCRα-chain could bias cells toward hCLIP reactivity. Indeed, in some experimental systems TCR specificity is dictated in large part by the TCRβ-chain. For example, CD8+ T cells expressing only the β-chain of the chicken OVA peptide-Kb-specific TCR or CD4+ T cells expressing only the β-chain of the TCR specific for the myelin basic protein epitope, Ac1,8-16, presented by I-Ak can mount significant responses to their cognate peptides (38, 39). To exclude this possibility, we also sorted Vα2+ CD4+ T cells from mice that were transgenic for the TCIi TCRβ-chain alone. When these cells were cultured with hCLIP, we were unable to detect any significant proliferative response (Fig. 3C). Thus, Ag-driven proliferation of endogenous TCRα-bearing T cells from TCIiRAG2+ TCRαβ transgenic mice requires coexpression of the TCIi TCRα-chain.

Coexpression of both transgenic and endogenous TCRα-chains on the surface of TCIiRAG2+ T cells was further confirmed by the analysis of TCRα-chain expression among TCIi T cells expanded in vitro with the cognate peptide. TCIiRAG2+ splenocytes bearing the Ly-9.1 marker were mixed with normal Ly-9.1- B6 splenocytes at a 1/5 ratio, stimulated in vitro with either hCLIP or Con A, and analyzed after 7 days. Selective expansion of transgenic CD4+ and Vβ6+ T cells upon stimulation with hCLIP, but not with Con A, indicated an Ag-driven selection for specific T cells (Fig. 4A).

However, analysis of endogenous Vα usage before and after stimulation indicated no significant change in the percentage of transgenic CD4+ T cells expressing endogenous Vα2, -3.2, -8, or -11 after 1 wk in culture with specific peptide (Fig. 4B). These cells would be expected to decrease significantly after antigenic stimulation if they represented a population distinct from the Vβ6+ Vα18 TCIi transgenic T cells. Instead, the results are consistent with endogenous TCRs being coexpressed with the responding TCIi TCR on the same cells. Moreover, these endogenous receptors appear to be neither selected for nor against during the Ag-specific response in the periphery, indicating that they more likely play a role in positive selection in the thymus.
Decreased kinetics of CD4+ T cell development in TCli mice lacking RAG2

Dual TCRα-chain expression among TCliRAG2+ transgenic thymocytes may confer an advantage during positive selection in the thymus by providing developing cells an additional receptor and thus a broader range of thymic peptides that might serve as positively selecting ligands. To show that positive selection occurs more efficiently among transgenic thymocytes that can express endogenous TCRα-chains on the surface, we analyzed the rate of development of mature CD4 SP T cells in TCliRAG2+ vs TCliRAG2° mice, using the thymidine analogue BrdU. Dividing thymocytes that incorporate BrdU can be tracked using an anti-BrdU Ab by flow cytometry. Mice were continuously labeled with BrdU in drinking water for a period of 1–4 days and were analyzed by FACS for the accumulation of labeled CD3high CD4+ thymocytes over time. We found that the accumulation of labeled mature CD4 SP thymocytes was significantly delayed, by approximately 1 day, in TCliRAG2° mice compared with TCliRAG2+ mice (Fig. 5). Thus, the absence of endogenous TCRα-chain expression decreases the rate of CD4+ T cell development in TCli mice.

Thymocyte competition for specific peptides during positive selection of TCli T cells

We hypothesized that the inefficiency of selection of the TCli TCR could be explained by highly selective self-peptide recognition during positive selection and competition for limited selecting peptide:MHC ligand(s) present in the thymus. To test this, we transferred TCli bone marrow into lethally irradiated Ii− and H-2 M° mice, which exhibit much reduced class II peptide diversity (22–24, 28, 40–45). As predicted, TCli→ B6 chimeras showed normal development of transgenic CD4+ T cells, but TCli→Ii− and TCli→H-2 M° chimeras exhibited almost complete abrogation of CD4+ T cell selection (Fig. 6). These results demonstrate the high degree of peptide selectivity involved in positive selection of the TCli TCR.

To assess whether TCli transgenic thymocytes competed for limited amounts of specific selecting peptides, we constructed mixed bone marrow chimeras where Thy-1.1 TCliRAG2+ donor cells were diluted with wild-type Thy-1.1 B6.PL bone marrow cells and injected into lethally irradiated B6 (Thy-1.1+) mice. Upon reduction of the numbers of TCR transgenic precursors seeding the thymus, more efficient development of TCli thymocytes was predicted to occur due to decreased competition for limited selecting peptides. All chimeras that were generated appeared healthy, with thymic cellularity of about 4–7 × 107/animal. When the B6 hosts were reconstituted with 100% TCliRAG2° bone marrow, a relatively small percentage (11%) of TCli transgenic cells was selected into the CD4 SP compartment, similar to what was shown earlier for TCliRAG2° thymocytes (Fig. 7A, compare to Fig. 1A). However, when the percentage of TCli double-negative cells in the thymus was decreased to 20%, we observed a dramatic increase in the efficiency of positive selection of transgenic thymocytes. Here, 65% of the TCli thymocytes that had already matured to the CD4 SP compartment and expressed high levels of Vβ6 (Fig. 7A, inset). In contrast, the proportion of CD4 SP thymocytes that developed from Thy-1.1 wild-type bone marrow did not change with altered bone marrow ratios (Fig. 7B). The slightly increased CD4 SP/DP ratio shown for the wild-type thymocytes appeared to be due to the kinetics of thymocyte repopulation and was not observed at later time points post-transfer. The absolute number of CD4 SP TCli thymocytes was only slightly lower or was not significantly different in the chimeras reconstituted with

FIGURE 5. The rate of CD4+ T cell development is decreased in TCli mice in the absence of endogenous TCR expression. Wild-type B6 (■), TCliRAG2+ (▲), and TCliRAG2° (△) mice were provided fresh drinking water containing 0.8 mg/ml BrdU daily for 1–4 days and subsequently analyzed for the appearance of BrdU-labeled CD3high CD4 SP in the thymus. Shown is the percentage of BrdU+ cells among CD4+ CD3high thymocytes after the indicated number of days of continuous labeling. The data shown are representative of four typical experiments yielding similar results.

FIGURE 4. Endogenous TCRα-bearing TCli CD4+ T cells persist in culture after specific peptide stimulation. RAG2+ TCli splenocytes (Ly-9.1+) were mixed with normal B6 splenocytes (Ly-9.1-) at a 1/4 ratio (20% transgenic, 80% nontransgenic) and cultured for 7 days with either hCLIP or Con A. Cells were then analyzed by flow cytometry. A, Ag-driven expansion of transgenic (Ly-9.1+) CD4+ T cells and Vβ6+ T cells is observed after the culture as shown by the percentage of Ly-9.1CD4+ (left panel) or Ly-9.1Vβ6+ (right panel) splenocytes before stimulation (□), after stimulation with hCLIP (▲), or after stimulation with Con A (△). B, The percentages of CD4+ cells in the transgenic population expressing various TCR Vα-chains before and after stimulation were assessed by flow cytometry. The average values obtained from two to four mice are shown.
fewer TCli bone marrow cells, indicating that selection of transgenic cells was occurring at near-maximal levels. In other irradiated mice reconstituted with intermediate amounts of TCli bone marrow, we observed an intermediate level of selection of TCli CD4+ T cells (data not shown). Thus, the extent of TCli bone marrow chimerism directly relates to the proportion of TCli CD4 SP T cells that mature. This strongly supports the idea that positive selection of the TCli TCR is highly peptide specific, and that the selecting peptide(s) is present in limited abundance in a normal thymus.

Discussion

In this study we examined the development of TCR transgenic CD4+ T cells specific for the human CLIP peptide bound to I-A^b. Interestingly, positive selection of the TCli TCR is relatively inefficient, resulting in a CD4+ T cell pool where 50% of the cells express a second TCR using an endogenous TCRa paired with the transgenic TCRb-chain. In the absence of RAG2, thymocyte development is markedly reduced in TCli mice such that very little difference in CD4+ T cell selection is observed between transgenic and nontransgenic littermates (Fig. 1A). This is unlikely to be due to increased negative selection of TCli thymocytes in the absence of RAG2. If negative selection were occurring, we would not see the dramatic increase in positive selection of TCli CD4+ T cells when the number of transgenic thymocytes is decreased in our mixed bone marrow chimeras (Fig. 7). Also, TCliRAG2^bCD4^+ T cells that mature and populate the periphery are not anergic and respond to the cognate peptide with a high degree of sensitivity. Thus, positive selection simply occurs much less efficiently in TCliRAG2^+ mice despite transgene-driven expression of a selectable TCR.

It has been realized for some time that positive selection in TCR transgenic animals is not 100% efficient, even though all developing thymocytes express a receptor capable of being positively selected. For example, it has been shown that only about 20% of the DP thymocytes in H-Y TCR transgenic mice mature to the SP stage, and that optimal selection efficiency is obtained only when transgenic cells are reduced to approximately 5% or less of all CD4^+ cells (46). This was explained by a lack of appropriate selecting niches in the thymus to support the development of every transgenic thymocyte (33, 47).

It has been unclear, however, what such selecting niches represent. They may refer to a specialized set of cortical epithelial cells that can be accessed by developing thymocytes, particular microdomains enriched in specific soluble growth or differentiation factors, or specific TCR ligands. However, limited thymocyte-epithelial cell contact or lack of certain microenvironments or factors cannot explain the large differences in selection efficiencies observed among the TCli, TEa, and OT-II TCR transgenic mice or the differences in selection between TCliRAG2^+ and TCliRAG2^b animals.

Our results suggest that the selecting niches for a given TCR are simply the specific self-peptide:MHC complexes that can mediate its positive selection, and their availability largely determines the efficiency of T cell development. Since different self-peptides are expressed at different levels of abundance in the thymus, this may account for the differences in selection observed for different TCR transgenic mice. For the TCli TCR, competition by many transgenic thymocytes for selecting peptides that are expressed in relatively low abundance on thymic class II molecules leads to inefficient positive selection. This would explain how the selection efficiency can be dramatically enhanced by decreasing the proportion of TCli precursors in the thymus. Compared with TCli mice, TEa and OT-II TCR transgenic mice exhibit significantly better selection efficiencies (Fig. 2), most likely because the specific selecting peptides for these two receptors may be more abundant. Since the TEa mice were constructed with the identical TCRa and TCRb expression constructs used for generating the TCli mice.
(28), it is unlikely that differences in transgene expression could account for the different selection efficiencies. TCR-specific differences in selection efficiency have also been reported for three different lines of transgenic mice carrying structurally similar TCRs specific for a cytochrome c peptide bound to I-E\(^+\) (31). These differences were attributed to differences in the selecting niches used by each receptor; some TCRs appeared to require highly specific peptide ligands that became limiting at low doses of the selecting MHC, while other TCRs appeared to be more peptide-promiscuous in their selection requirements. Alternatively, the different TCRs may have different affinities for selecting ligand. Our results further extend the idea set forth by these previous studies that different TCRs can be selected with different efficiencies depending on the abundance of or affinity for the selecting ligand(s).

In our study, however, it is unlikely that TCR affinity for the selecting ligand plays a major role in the results observed. If the weak selection of TCli thymocytes were due to a lower affinity of the TCli TCR for the positively selecting ligand compared with that of the TEa or OT-II TCRs, we would not expect to see the dramatic increase in CD4 SP selection when the number of TCli TCR transgenic precursors is reduced in mixed bone marrow chimeras. Nevertheless, we cannot formally exclude the possibility that the TCli TCR-selecting ligand interaction is of low affinity and that perhaps the selecting niche for which the TCli thymocytes compete is a rare specialized cortical epithelial cell that may provide certain accessory molecules that enhance less avid TCR-peptide-MHC interactions and thereby promote more efficient selection of TCli thymocytes. Since such a subset of thymic epithelial cells has not been previously documented, and the selecting ligands for the TCRs analyzed here are not known, this possibility is difficult to address.

It has been suggested that CD5 expression levels on mature SP thymocytes reflect the avidity of the positively selecting TCR-peptide interaction (48). We have observed no difference in CD5 expression on TCli and TEa CD4 SP thymocytes (P. Wong, unpublished observations). Instead, we have actually found that expression of this marker on TCR transgenic DP thymocytes is uniformly high and appears to be proportional to the endogenous peptide diversity on thymic class II molecules, but does not correlate with positive selection efficiency (P. Wong et al., manuscript in preparation). Thus, although we cannot rule out the possibility that TCR affinity for selecting ligand can explain the difference in selection efficiencies shown in this study, it is an unlikely explanation. Based on our data, it is more likely that levels of specific selecting peptides play a major role in selection efficiency differences between individual receptors.

Further evidence for the highly selective nature of the peptide recognition events that allow TCli thymocytes to mature is the finding that the TCli TCR is not selected in either the Ii\(^+\) or H-2 M\(^+\) thymic environments where the endogenous peptide repertoire is drastically reduced. It was especially interesting to discover in bone marrow chimeras that even the closely related, endogenous mCLIP peptide expressed abundantly in H-2 M\(^+\) mice was unable to promote the development of the hCLIP-specific TCli T cells (Fig. 6). We confirmed this finding in TCli fetal thymic organ culture studies in which CLIP:1-A\(^b\) complex-specific Abs failed to inhibit positive selection of transgenic T cells (data not shown). This is consistent with recent studies suggesting that the selecting peptides for a given T cell may not necessarily resemble the peptides that activate it in the periphery (49, 50). Since we have previously shown that mCLIP is a fairly well represented self-peptide in a normal thymus (51), its inability to select the TCli TCR is perhaps not surprising given that our data suggest that selection of the TCli TCR is probably mediated by peptides expressed in limiting amounts.

How do developing TCli thymocytes cope with the paucity of selecting peptide ligands? From our results, it appears that when they express a second TCR, the TCli thymocytes have an improved chance at positive selection. Dual TCRα-chain expression has been observed in other TCR transgenic as well as nontransgenic T cells and has been correlated with inefficient positive selection of the initially expressed TCR (2–5, 7, 8, 10, 37). Endogenous TCRβ expression has also been reported in one TCR transgenic mouse exhibiting weak positive selection (32), but this may be transgene specific, and we have not observed this in the TCli mice. In support of ligand availability as a critical factor in positive selection, it was also found in that report that decreased levels of the selecting MHC molecules resulted in inefficient development of transgenic TCR-expressing T cells and increased endogenous TCR usage (32). Our results are consistent with and further extend these previous studies, since we observe less endogenous TCRα expression in TEa and OT-II TCR transgenic mice where CD4 T cell selection is more efficient. In addition, positive selection is not decreased as dramatically in these latter mice in the absence of RAG2, indicating that the majority of CD4 T cells are selected through the transgenic TCR. In contrast, the high frequency of cells bearing endogenous TCRα-chains in TCliRAG2 mice suggests that selection through the TCli TCR is relatively poor. By expressing a second receptor, TCli thymocytes are able to sample other peptide:MHC ligands that may be more readily available than those for the TCli TCR and become positively selected through endogenous TCR-peptide:MHC interactions. Thus, prevalent endogenous TCRα-chain usage among the poorly selected TCli thymocytes as a mechanism for increasing CD4 T cell development is another indication that specific peptide:MHC complex availability is a rate-limiting factor for efficient positive selection.

Since the relative availability of different self-peptides in the thymus plays a critical role in shaping the T cell repertoire, it is interesting to speculate whether peptides expressed in low abundance on thymic cortical epithelial cells have more favorable interactions with cells bearing high affinity TCRs and thereby promote their development. This is again difficult to address, since we do not know what the selecting peptide is for the TCli TCR or what the affinity of the TCli TCR is for its selecting ligand. However, preliminary affinity measurements of the TCli TCR for the hCLIP:1-A\(^b\) complex suggest that this receptor binds to its cognate ligand in the periphery very strongly, with a K\(_d\) of approximately 10\(^9\) M\(^{-1}\) (L. Teyton, C. Cantu III, and P. Wong, unpublished observations). This is about 3 orders of magnitude higher than the affinities reported to date for peptide:MHC/TCR interactions and is similar to the affinity measured for the 30-2 mAb specific for the hCLIP:1-A\(^b\) complex. It will be interesting to determine whether thymic self-peptides present in limiting amounts select for high affinity receptors.

We conclude that peptide specificity is a crucial component of positive selection, but equally important is the accessibility of those peptides to the developing T cell repertoire. Low level expression of the peptides critical for the selection of an individual thymocyte may reduce the efficiency of its development. Inefficient selection through the initially expressed receptor can induce the expression of a second TCR as thymocytes attempt to produce a selectable receptor. Since it is entirely possible that the initially expressed TCR can be positively selected under more abundant ligand conditions, positive selection on the second receptor may allow the development of cells that express two functional self-restricted TCRs in the periphery.
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References


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