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*J Immunol* 1998; 160:634-642; ;
http://www.jimmunol.org/content/160/2/634
The Level of CD4 Surface Protein Influences T Cell Selection in the Thymus

Gregory D. Frank*† and Jane R. Parnes²†

During T cell development thymocytes are subjected to positive and negative selection criteria to ensure that the mature T cell repertoire is MHC restricted, yet self tolerant at the same time. The CD4 and CD8 coreceptors are thought to play a crucial role in this developmental process. To elucidate the role of CD4 in T cell selection, we have produced a mouse strain that expresses CD4 at a reduced level. We used homologous recombination in embryonic stem cells to insert neo into the 3’ untranslated region of CD4. The resulting mice have a reduction in the percentage of CD4⁺ cells in the thymus and a concomitant increase in CD8⁻ cells. In addition, breeding two individual class II-restricted TCR transgenic mice onto the CD4low (low level of CD4) mutant background affects the selection of each TCR differentially. In one case (AND TCR transgenic), significantly fewer CD4⁺ cells with the transgenic TCR develop on the CD4low mutant background, whereas in the other (SC,7 TCR transgenic), selection to the CD4 lineage is only slightly reduced. These data support the differential avidity model of positive and negative selection. With little or no avidity, the cell succumbs to programmed cell death, low to moderate avidity leads to positive selection, and an avidity above a certain threshold, presumably above one that would lead to autoreactivity in the periphery, results in clonal deletion. These data also support the idea that a minimum avidity threshold for selection exists and that CD4 plays a crucial role in determining this avidity.


Mature T cells are divided into almost mutually exclusive subsets of cells that express either CD4 or CD8 (reviewed in Refs. 1 and 2) and have helper or cytotoxic function, respectively. It is during development in the thymus that T cells become MHC restricted and committed to their respective lineages. To ensure that the T cell repertoire is self tolerant yet retains the ability to recognize Ag in the context of self MHC, thymocytes are subjected to positive and negative selection. Positive selection is the process that yields T cells that have TCRs that recognize self MHC molecules (3, 4), while negative selection is the process by which potentially autoreactive thymocytes are deleted in the thymus (5, 6). Because rearrangement of the TCR genes is random, many T cells that are generated at the double-negative (DP, CD4⁺CD8⁻) stage have TCR with little or no affinity for MHC or never successfully rearrange their TCR genes. These cells are subjected to programmed cell death (PCD). This fate, which has also been termed death by neglect, is the default state that ensues without a signal to rescue the developing T cell, i.e., without a signal for positive selection. The term PCD is used here to refer only to those cells that fail positive selection and not those that die by negative selection. The avidity of the interaction between developing T cells and thymic stromal cells determines the fate of that cell. While the affinity of the TCR for its ligand is probably the single most important variable determining avidity, it is not the only factor. Avidity includes the influence of other proteins, such as coreceptors (2) and adhesion molecules, on the stability of the TCR/MHC complexes as well as the density of TCR and its cognate ligand (7). Thus, an emerging T cell with a high avidity interaction would lead to negative selection (also known as clonal deletion), one of moderate avidity would lead to positive selection, and one of low or no avidity would lead to PCD or death by neglect. This model, now widely known as the differential avidity model for T cell selection (8), is supported by recent in vivo (9, 10) and in vitro (11–13) findings.

CD4 is an adhesion molecule that interacts with MHC class II and, by binding to a nonpolymorphic region of class II (14, 15), can also engage in coreceptor signaling (16, 17). Coreceptor signaling is the synergy that results (18) from CD4 and TCR binding the same MHC ligand and the concomitant juxtaposition of Lck with the CD3 signaling complex. Lck is a nonreceptor protein tyrosine kinase that binds to the cytoplasmic domain of CD4 (19). CD4 plays a crucial role in the development of class II-restricted Th cells in great part due to its ability to boost the avidity of the interaction between thymocyte and stromal cell during selection. Neonatal injection of anti-CD4 mAb blocked the development of CD4 single positive (SP) thymocytes (20) as it did in fetuses of pregnant mice treated until birth (21). CD4 can also function independently of its association with Lck, albeit less efficiently than CD4 with Lck. This was revealed in experiments with mice expressing a CD4 transgene (tg) that lacks the cytoplasmic tail, on a CD4 knockout (CD4⁻) background (22). Strikingly, there was a direct correlation between the proportion of CD4-lineage cells and the level of cell surface expression of the tailless CD4 molecule. The greater the overexpression of the tg, the more CD4 T cells. Thus, a tailless CD4 expressed at physiologic levels results in a...
reduction in CD4 T cells, but this inability to coreceptor signal can be compensated for by gammad overexpression. Interestingly, mice that lack CD4 altogether have a small population of double-negative (DN), i.e., CD8− peripheral T cells with helper function (23, 24). Interpreted in the context of the differential avidity model, coreceptors increase the avidity of T cells engaged in selection, and their associated protein tyrosine kinases actively take part in the TCR signaling pathway. CD4 is not essential for the development of class II-restricted Th cells, but greatly enhances this process.

To study the role of CD4 in positive and negative selection, we set out to replace the exons that encode the transmembrane and cytoplasmic domains of CD4 with those of CD8. To do this, we used homologous recombination (25, 26) in embryonic stem (ES) cells. However, instead of the intended integration, we inserted the neomycin resistance gene (neo) into the 3′ untranslated region (UTR) of CD4. The resulting mice (CD4low), after blastocyst injection and breeding for homozygosity of the mutant CD4 gene, have a reduced level of CD4 protein on the surface of their T cells throughout development. In the CD4low mutant mice we found a decrease in the proportion of CD4 cells in the thymus with a concomitant increase in CD8 cells and a significant skewing of the TCR repertoire with respect to several V region gene products. To study the fate of individual TCRs with reduced CD4, we bred two different class II-restricted Tg tcg onto the CD4low mutant background. In these TCR tg/CD4low mutant mice we saw a reduction in the percentage of T cells being selected into the CD4 compartment. Our data are consistent with, and interpretable in the context of, the differential avidity model of T cell selection.

Materials and Methods

Construction of targeting vector

The 10.5-kb 3′ EcoRI fragment of mouse genomic CD4 (27) containing exons 5 to 10 in the pBluescript vector (Stratagene, La Jolla, CA) was used to generate the targeting vector. The 3.5-kb BglII fragment was excised and inserted into a modified Bluescript vector that contained no polylinker and only a BglII site for cloning. The following manipulations were performed on this BglII cassette. The ScaI-PvuII fragment (exons 8 and 9 and part of exon 10, representing the region coding for the transmembrane and cytoplasmic domains) was removed, and the SacI-BglII/blunt fragment of genomic mouse CD8 (representing exons 3 and 4 and part of exon 5) was cloned in its place. The SphI site in the 3′ UTR (exon 10) was converted into an XhoI site with linkers. The 1.3-kb EcoRI-BamHI fragment of neo under control of the PGK promoter (28) was cloned into this site with XhoI linkers. The BglII cassette was then inserted back into the BglII site of the 10.5-kb EcoRI fragment of genomic CD4. The Clon-NotDblunt (polynucleotide sites) fragment of this construct was cloned into the ClaI-HindIII blunt site of pHSV-10 (TV5k; Fig 1B). Twenty micrograms of this final targeting vector was linearized with ClaI for transfection. All DNA manipulations were performed as previously described (29) with enzymes from New England Biolabs (Beverly, MA).

Generation of CD4low mutant mice

The linearized targeting vector (20 μg) was electroporated at 275 V and 200 μF, with a BTX 300 electroporator (BTX, Inc., San Diego, CA) into ES-D3 C-12, a subclone of D3 ES cells (30). Cells were subjected to G418 (Life Technologies, Gaithersburg, MD; 400 μg/ml) and 1-[2-deoxy-2-fluoro-β-D-arabinofuranosyl]-5-iodouracil (Fluorouridine) (10 days). Single-colony wells were expanded for DNA isolation and Southern blot analysis. Chimeric mice were generated by microinjection of the targeted ES cell into the blastocoeal cavity of 3.5-day-old C57BL/6 blastocysts (31). Chimeric males were mated with C57BL/6 females, and agouti-colored offspring were screened for germ-line transmission by Southern blot analysis.

Generation of inbred strains

Heterozygous offspring were backcrossed onto C57BL/6 or B10.BR mice for a minimum of six generations. At each generation, tail DNA was isolated, and Southern blot analysis was performed to track the mutant gene. For experimental mice, heterozygous mice were intercrossed to generate wild-type, mutant, and heterozygous offspring. Wild-type and mutant offspring from the same litter (littermates) were used for experiments. For double-mutant mice, CD4low homozygous mutant mice (CD4low mutant) were crossed to βm-deficient mice (βm−/−). Double-heterozygous littermates were intercrossed to obtain CD4low/−/−/βm−/− mice, which were then intercrossed to get CD4low/−/−/βm−/− and CD4WT/−/−/βm−/− littermates for experimentation. The SC7.C7 TCR tg mice were generated by Dr. Mark M. Davis (Stanford University School of Medicine), were maintained on the B10BR background. The AND TCR tg mice, a gift from Dr. Stephen M. Hedrick (University of California, San Diego, CA) (32) were maintained on the C57BL/6 background. For experiments with tg/CD4low mutant mice, tg/CD4low/−/− mice were intercrossed with tg/CD4low/−/−/βm−/− mice to obtain tg/CD4low/−/−/βm−/− mutant, and tg/CD4WT/−/−/βm−/− littermates. All mice used for breeding were obtained from The Jackson Laboratory (Bar Harbor, ME). TCR tg mice were identified by cell surface staining with fluorochrome-labeled Abs and flow cytometry, while the CD4 gene was tracked by Southern blotting of tail DNA.

Abs and Ig flow cytometry

Anti-CD8α (clone 53.67.2, American Type Culture Collection, Rockville, MD) and anti-Vα8.1, 8.2, 8.3 (clone F23.1) were purified from culture supernatant by protein G-Sepharose (Pharmacia, Uppsala, Sweden) chromatography and conjugated to FITC (Molecular Probes, Eugene, OR) or biotin (Pierce Chemical Co., Rockford, IL). The following Ab were purchased from PharMingen (San Diego, CA): PE-conjugated anti-CD4 (clone RM4–5), biotinylated anti-CD5 (clone 53–7.3), biotinylated anti-CD3ε (clone 145–2C11), biotinylated anti-CD69 (clone H1.2F3), biotinylated anti-CD24 (HSA; clone M1/69), FITC-conjugated anti-Vβ3 (clone JF53), FITC-conjugated anti-Vβ8.1, 8.2 (clone MR5–2), biotinylated anti-Vα2 (clone B20.1), and biotinylated anti-Vε11 (clone RR8–1). The following Ab were used as culture supernatant: anti-Vα3.2 (clone RR3–18; a gift from Dr. Osami Kanagawa, Washington University, St. Louis, MO) detected with FITC-conjugated mouse anti-rat Fe (Jackson ImmunoResearch Laboratories, West Grove, PA) and anti-Vβ14 (clone 14.2; a gift from Dr. David Raulert, University of California, Berkeley, CA) detected with FITC-conjugated goat anti-rat IgM (Jackson ImmunoResearch Laboratories). Anti-Vα3, a rabbit antisemur, was a gift from Drs. Bee-Cheng Sim and Nicholas R. J. Gascoigne (The Scripps Research Institute, La Jolla, CA) and was detected with FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories). Ab were used at predetermined optimal concentrations. Spleens and thymi were prepared for flow cytometry by making a single-cell suspension with the sintered ends of two glass slides. One million cells were stained and washed in PBS/2% FCS/0.05% azide. Three-color flow cytometry was performed with a FACScan cytometer (Becton Dickinson, San Jose, CA), and data were analyzed using FACS-DESK software as displayed as density plots or as 5% probability contour plots. FITC-labeled, PE-labeled, and biotinylated mAb, with PerCP (BD, San Jose, CA) secondary reagent, were used throughout. PBL from transgenic mice were isolated using Lymphocyte-M (Cedarlane Laboratories Ltd., Hornby, Canada) and stained as described above.

Southern blotting

Tail biopsy was incubated with proteinase K (Life Technologies) at 56°C overnight in serum separator tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The genomic DNA was extracted with phenol/chloroform, then with chloroform, in the same tube. DNA was precipitated in microfuge tubes with sodium acetate and isopropanol, then washed with ethanol. Five micrograms of DNA was digested overnight at 37°C with EcoRI (New England BioLabs, Beverly, MA). Gel electrophoresis was performed as previously described (29). Transfer to a nylon membrane, probe labeling, hybridization, washing, and detection were performed with the Genius System (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. The probe was a 630-bp EcoRI fragment of genomic CD4 from the 3′ flanking region immediately outside the targeting vector (Fig 1C).

Northern blotting

Total RNA was isolated from thymus using RNAzol (Tel-Test, Inc., Friendswood, TX) according to the manufacturer’s instructions. mRNA was then purified using Oligotex (Qiagen, Chatsworth, CA). Twenty micrograms of total RNA or 0.2 μg of mRNA were electrophoresed, transferred to a nylon membrane (Boehringer Mannheim), hybridized, and washed as previously described (33). The EcoRI-BamHI fragment of neo or the 414-bp SacI-Kiw1 (exons 2–5) fragment of the mouse CD4 cDNA was labeled with 32P by random hexamer priming and used as a hybridization probe.
Immunoprecipitation and immunoblot analysis

Thymocytes were surfaced labeled with sulfo-NHS-biotin (Pierce) (34). Total cell lysates were made by incubating cells in lysis buffer containing 1.0% Triton X-100 (Sigma) and protease inhibitors (Boehringer Mannheim) on ice for 30 min. Nuclei and cytoskeletal components were removed by microcentrifugation at maximum speed for 15 min in the cold room. Lysates were frozen at −80°C or used immediately. Immunoprecipitation with anti-CD4 (CT CD4, Caltag) or anti-CD8α/β (BD Pharmingen) mAb preincubated with protein G-Sepharose (Pharmacia; 5 µl of protein G slurry) was performed at 4°C using a rotary mixer. Lysates were precleared with nonspecific isotype-matched control Ab, preincubated with protein G-Sepharose, for 1 h at 4°C. The samples were washed five times, resuspended in loading buffer containing 1% 2-ME, and electrophoresed on a 10% SDS-polyacrylamide gel. The gels were electroblotted onto nitrocellulose (Schleicher and Schuell, Keene, NH). Western blotting was performed with peroxidase-conjugated streptavidin (Jackson Immunoresearch Laboratories) in PBS/0.1% Tween (Sigma)/5% nonfat dry milk powder (Carnation, Los Angeles, CA) followed by ECL (Amersham, Arlington Heights, IL) and exposure to Hyperfilm (Amersham).

cDNA library construction, screening, and sequencing

Total RNA was isolated from CD4low mutant thymi as described above. mRNA was purified using the Poly(A) Quick mRNA Isolation Kit from Stratagene (La Jolla, CA). The library was constructed using the ZAP-cDNA Gigapack II Gold Cloning Kit from Stratagene and screened according to the manufacturer’s instructions using the same CD4 probe as that used for Northern analysis. Six individual clones were isolated, then sequenced in both directions using the dideoxy chain termination method. The numbering for the cDNA sequence shown is taken from the mouse CD4 sequence in GenBank (accession no. X04836).

Results

Generation of CD4low mutant mice

CD4low mice were generated by standard techniques using the 129/sv embryonic cell line D3 (31). A targeting vector was constructed to introduce an expressible neo into the 3′ UTR of CD4 by homologous recombination. The targeting vector is shown in Figure 1B. Genomic CD4 spanning intron 5 through the 3′ flanking region was used to generate the targeting vector. The neo gene was inserted in the 3′ UTR at the Sphl site using Xhol linkers. This neo gene was driven by the PGK promoter (28) and used the poly(A) signal of the CD4 gene for expression after integration. The HSV- tk gene driven by the PGK promoter was inserted at the 3′ end of the targeting vector to allow negative selection (35) against random integrants by plating on FIAU. The targeting vector was linearized with ClaI and electroporated into ES-D3 cells (30). These cells were subjected to both positive (G418) and negative (FIAU) selection. G418-resistant clones were analyzed by Southern blot (Fig. 2) and used for blastocyst injection. Although we originally intended to introduce exons encoding the transmembrane region and cytoplasmic domain of CD8 into the CD4 locus (Fig. 1B, gray exon boxes), homologous recombination occurred such that only neo was introduced. The remainder of the CD4 gene remained intact. Two clones were selected for microinjection into blastocysts and gave germ-line transmission. One line was interbred to generate homozygous mice. The mutation was backcrossed onto two different inbred mouse strains, C57BL/6 and B10.BR, for at least six generations. The mice were viable and healthy and displayed no overt abnormalities. In-depth characterization of the CD4 gene and flanking regions by Southern blot analysis was performed using a variety of restriction digests and probes (data not shown). This analysis revealed that a single copy of neo was introduced, and the sites flanking this integration were intact. Based on tail DNA analyses of hundreds of progeny generated by intercrossing heterozygous mice, the ratio of birth of wild-type (wt), heterozygous, and mutant (CD4low) mice was approximately 1:2:1.

Result of targeting the 3′ UTR

Placing neo into the 3′ UTR of CD4 resulted in a lower level of CD4 surface protein on all T cells that normally express CD4. This is revealed by flow cytometric analysis in Figure 3. The difference is most obvious in the DP population in the thymus, where the level of CD4 in the mutant is 50% that in the wt. In the SP thymocyte population and on splenic T cells, the level of CD4 in the
mutant is 80% that in the wt. Immunoprecipitation of CD4 from thymocytes (Fig. 4) revealed about a twofold difference in the amount of protein from mutant vs. wt cells. Whole thymocyte suspensions were surfaced labeled with biotin, and total cell lysates from equal numbers of cells were subjected to immunoprecipitation with anti-CD4 mAb and protein G-Sepharose. The immunoprecipitations were resolved by PAGE, and the biotinylated proteins were detected by ECL and x-ray film exposure. Densitometry (data not shown) revealed a twofold difference in CD4 protein from mutant vs. wt cells when corrected for immunoprecipitation of the control protein, CD8 (Fig. 4). To try to understand the cause of the lower level of CD4 protein in mutant cells, we performed Northern blot analysis of RNA from mutant and wt thymi (data not shown). Not so unexpectedly, we found two species of CD4 RNA in the mutant mouse. The predominant species is larger than wt CD4. Its size suggests that it represents complete read-through of the CD4 gene including neo. This conclusion is substantiated by the finding that parallel blots hybridized with a neo probe revealed the same size band (data not shown). A minor species on the CD4-hybridized blot is smaller and results from utilization of an alternate, yet inefficient, polyadenylation signal. This was verified by the isolation and sequencing of clones from a cDNA library constructed from mutant thymus RNA (data not shown). The sequence of this poly(A) signal is AATGAA. Sheets et al. (36) have shown that a motif with this sequence can direct polyadenylation, albeit to an extent far less than the canonical AATAAA.

Reduced CD4 protein alters CD4/CD8 thymic subsets

We were interested to see what effect this reduced level of CD4 surface protein would have on the development of T cells. Two-color flow cytometry revealed an increase in the CD8 compartment and a decrease in the CD4 compartment of thymocytes stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 (Fig. 5A). The number of cells in the mutant thymus was equivalent to that in the wt thymus. The reduction in the CD4 compartment was consistent and averaged 19.1% over 12 pairs of mice.
tested. This reduction in CD4⁺ cells and increase in CD8⁺ cells persisted in peripheral T cells, although to a lesser extent than in thymocytes, as indicated by a similar staining of splenocytes (Fig. 5B). This pattern in splenocytes was also consistent over 12 pairs of mice tested (data not shown). Most notable in the thymus was a consistent increase in the CD8 compartment, which averaged 67.1% over 12 pairs of mice tested.

To characterize this phenotypic change in greater depth, we performed three-color staining of thymocytes with a panel of Abs in conjunction with CD4/CD8 staining. This analysis revealed that the increase in CD8⁺ cells in the thymus of mutant mice is predominantly (>70%) due to cells that are CD3low/med, CD5low, CD69low, and HSAhigh (Table I). This phenotype is indicative of CD8⁺ cells in transition from DN to DP as they are immature in character. Our interpretation of these results is that CD4 mRNA has been destabilized by insertion of neo into the 3' UTR, and this has retarded the surface expression of CD4 protein during this DN to DP transition. The mutant mice have a greater percentage of DN cells, which also supports this interpretation.

To ensure that most of the increase in CD8⁺ cells was due to immature CD8⁺ transitional intermediates, we crossed the CD4 mutant gene onto the β2 m0 background. The β2 m0 mice lack MHC class I surface expression and, hence, are unable to positively select CD8 T cells. In the double-mutant mice (CD4low /β2 m0; data not shown), we saw an increase in CD8⁺ cells similar to that seen in the CD4low/β2 m+ mice, and as in the CD4low/β2 m+ mice, these cells were all CD69low, HSAhigh, CD5low, and CD3low/med (data not shown). Further supporting the idea that these cells are immature intermediates, they did not appear in the periphery of the double-mutant mice (data not shown).

Table 1. The percentage of CD8⁺ cells in gated populations for wild-type (wt) and CD4low (mutant) littermates

<table>
<thead>
<tr>
<th>Gated Population</th>
<th>% CD8⁺CD4⁻ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open Gate</td>
<td>wt</td>
</tr>
<tr>
<td>CD3low</td>
<td>3.19</td>
</tr>
<tr>
<td>CD3high</td>
<td>2.20</td>
</tr>
<tr>
<td>CD3</td>
<td>3.12</td>
</tr>
<tr>
<td>Open Gate</td>
<td>Mutant</td>
</tr>
<tr>
<td>CD3low</td>
<td>0.99</td>
</tr>
<tr>
<td>CD3high</td>
<td>1.75</td>
</tr>
<tr>
<td>CD3</td>
<td>1.37</td>
</tr>
<tr>
<td>Open Gate</td>
<td>Difference</td>
</tr>
<tr>
<td>CD69low</td>
<td>2.09</td>
</tr>
<tr>
<td>CD69high</td>
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<td>1.15</td>
</tr>
<tr>
<td>HSA</td>
<td>3.19</td>
</tr>
<tr>
<td>HSAlow</td>
<td>2.04</td>
</tr>
</tbody>
</table>

a Thymocytes were stained with PE-anti-CD4, FITC-anti-CD8, and a biotinylated third color Ab, revealed by PerCP.
b The difference in the percentage of CD8⁺ cells in the open gated populations is due to normal variation between three color stains in the same experiment.
c The Difference column is calculated as in the following example:
(CD8⁺CD4⁺ CD3low Mutant - CD8⁺CD4⁺ CD3low wt) = (CD8⁺CD4⁺ CD3low Mutant - CD8⁺CD4⁺ CD3low/med wt).

Reduced CD4 level affects the selection of class II-restricted TCR
We hypothesized that the reduction in the CD4 SP thymocytes was due to the lower level of CD4 surface protein, and that this lower level reduced the avidity of cells engaged in positive

FIGURE 5. CD4low mutant mice have an altered CD4/CD8 profile in both thymus and spleen. Flow cytometric analysis compared CD4low (mutant) mice with wt littermates. Thymus (A, top panels) and spleen (B, bottom panels) were isolated from 10-wk-old mice and stained with PE-conjugated anti-CD4 (γ-axis) and FITC-conjugated anti-CD8 (x-axis). Data are presented as 5% probability plots. The numbers represent the percentage of cells in the corresponding quadrant.
selection and hence rendered the selection of CD4⁺ class II-restricted T cells less efficient than that in wt littermates. To test this, we mated the CD4<sup>low</sup> mutant mice to two different class II-restricted TCR transgenic mice. One group of mice we tested expressed the 5C.C7 TCR tg. These mice express a TCR that recognizes a peptide of pigeon cytochrome c (PCC) (88–104) in the context of I-E<sub>k</sub> and selects almost exclusively to the CD4 lineage (32, 37). The difference in CD4⁺ SP cells in the thymus of these tg⁺/CD4<sup>low</sup> mutant animals compared with those in tg⁺/CD4 wt animals was modest (Fig. 6). The tg⁺/CD4<sup>low</sup> mutant mice had slightly fewer (12% fewer than tg⁺/CD4 wt; Fig. 6) CD4⁺ SP cells in the thymus. However, when the AND TCR tg was mated onto the CD4<sup>low</sup> mutant background, a much more glaring effect was seen. The AND mice express a TCR tg that recognizes the same peptide of PCC in the context of I-E<sub>k</sub> (32, 38), but because we maintain these mice on the C57BL/6 background the TCR is positively selected by I-Ab. Positive selection can occur on I-E<sub>k</sub> or I-A<sub>b</sub>. This TCR is also almost exclusively selected to the CD4 lineage. As can be seen in Figure 7A, the tg⁺/CD4<sup>low</sup> mutant thymus had a 58% reduction in CD4 SP thymocytes compared with littermates expressing the tg alone (tg⁺/CD4 wt). In four experiments, the average reduction in
CD4 SP thymocytes in the \( t g^+/CD4^{low} \) mutant compared with the \( t g^-/CD4 wt \) was 52% (57, 58, 76, and 16%). In all experiments comparing \( t g^-/CD4 wt \) mice with \( t g^+/CD4^{low} \) mutants using either TCR tg, the thymi in the \( t g^-/CD4 wt \) mice with \( t g^+ \) and express the indicated TCR V gene product, mean for \( n \) experiments.

Table II. Flow cytometric analysis comparing CD4\(^{low}\) (mutant) and wild-type (wt) littermates for usage of various TCR V\(a\) and TCR V\(b\) gene products

<table>
<thead>
<tr>
<th>TCR V Product</th>
<th>Mean % Positive(^a)</th>
<th>Mean % Positive(^b)</th>
<th>( n )</th>
<th>( p )</th>
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<tbody>
<tr>
<td>Va2</td>
<td>1.7500</td>
<td>1.3275</td>
<td>4</td>
<td>0.0236*</td>
</tr>
<tr>
<td>Va3</td>
<td>3.0067</td>
<td>2.2833</td>
<td>3</td>
<td>0.0708</td>
</tr>
<tr>
<td>Va3.1</td>
<td>1.2867</td>
<td>0.9600</td>
<td>3</td>
<td>0.1425</td>
</tr>
<tr>
<td>Va3.2</td>
<td>1.7200</td>
<td>1.3233</td>
<td>3</td>
<td>0.0392*</td>
</tr>
<tr>
<td>Va11</td>
<td>1.2125</td>
<td>1.1175</td>
<td>4</td>
<td>0.2831</td>
</tr>
<tr>
<td>V(b)3</td>
<td>0.9000</td>
<td>0.7925</td>
<td>4</td>
<td>0.1851</td>
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<tr>
<td>V(b)8.1, 8.2</td>
<td>2.6825</td>
<td>2.4225</td>
<td>4</td>
<td>0.1525</td>
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<tr>
<td>V(b)8.1, 8.2, 8.3</td>
<td>2.7325</td>
<td>2.3025</td>
<td>4</td>
<td>0.0077*</td>
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<tr>
<td>V(b)13</td>
<td>2.6100</td>
<td>1.9550</td>
<td>4</td>
<td>0.0033*</td>
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<td>V(b)14</td>
<td>2.3175</td>
<td>1.8625</td>
<td>4</td>
<td>0.1564</td>
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\( * \) Thymocytes were stained for CD4, CD8, and various TCR V\(a\) gene products.

Reduced CD4 protein level alters TCR repertoire

To study how the reduced CD4 level affects the TCR repertoire in a more global fashion, three-color staining analysis of thymocytes was undertaken using Abs to CD4, CD8, and a panel of TCR V\(a\) and V\(b\) gene products. We analyzed CD4\(^{low}\) mutant mice that had been backcrossed a minimum of six generations onto either C57BL/6 or B10.BR. The data in Table II show the statistical analysis of the percentage of cells that are CD4\(^+\)CD8\(^-\) and express a particular TCR V region gene product in wild-type vs mutant mice. On the C57BL/6 background, the reduced CD4 protein resulted in statistically significant reduction in four of the TCR V region gene products in the CD4 SP thymocyte population (Va2, Va3,2, V\(b\)8, and V\(b\)13). However, on the B10.BR background, the reduced CD4 level affected only one of the TCR V gene products in a statistically significant fashion (V\(b\)13).

Discussion

The targeting of neo into the 3’ UTR of CD4 (Fig. 1) resulted in mice with a reduced level of CD4 protein (Fig. 3) in all cells. This level is twofold lower in the thymus of mutant mice compared with that in wt littermates (Fig. 4). The reduction in CD4 protein level on the cell surface influenced the proportion of CD4 and CD8 SP cells in the thymus of CD4\(^{low}\) mutant mice (Fig. 5). By reducing the level of CD4 protein on the surface of developing thymocytes, we have rendered the selection of cells to the CD4 lineage less efficient by lowering the overall avidity of the interaction. The increase in the CD8 compartment in CD4\(^{low}\) mutant mice appears to have two explanations. Most of the increase in CD8\(^+\) cells is due to cells with an immature phenotype. These cells are CD3\(^{low}\), CD69\(^hi\), CD5\(^hi\), and HSA\(^hi\). This phenotype is characteristic of cells in transition from DN to DP (39) in certain strains of mice (40). In C57BL/6 mice, cells in transition from DN to DP are skewed toward expressing CD8 before CD4. The most likely explanation for the increase in this immature population in our CD4\(^{low}\) mice is retarded expression of CD4 protein at the transition of DN to DP cells, presumably due to CD4 mRNA instability (41, 42). These immature CD8\(^+\) cells make up the bulk of the increase in the CD8 compartment (75%; Table I). The remainder of the increase (25%) is due to CD8\(^+\) cells that are transitional and mature to mature, i.e., CD3\(^hi\)CD8\(^hi\), CD69\(^hi\), and HSA\(^hi\). Because the thymus from mutant and wt littermates are the same size (data not shown), decreasing the proportion of CD4 cells increases the proportion of CD8 cells. To verify this, we mated the CD4\(^{low}\) mutant mice to the beta\(_m\)\(^m\) mice. In the double-mutant animals an increase in the CD8 compartment persists (data not shown). However, this increase is almost entirely (+90%) composed of CD8\(^+\) immature cells. The almost complete lack of class I surface expression ensures that very few CD8 SP cells will reach full maturity.

In normal mice the level of CD4 is higher in SP thymocytes and spleen than in DP thymocytes. Our mutant mice are no exception. Interestingly, the difference in the level of CD4 between mutant and wt is greater in DP thymocytes (where mutant is 50% of wt) than in SP thymocytes (where mutant is 80% of wt; Fig. 3). We believe that this reflects the process of positive selection and supports a differential avidity model and the idea that the majority of thymocytes fail positive selection (43). Most TCRs, which are generated by the random recombination of gene segments, have little or no affinity for MHC. Cells bearing these receptors die by neglect (43); they are unable to generate a signal through the TCR to rescue them from PCD. Even though we have generated mice with a reduced level of CD4, there is still a normal distribution of the level of CD4 protein on developing thymocytes. Thus, the cells passing positive selection do so with the highest level of CD4. However, this level is still lower, on the average, in the mutant thymocytes than in the wt thymocytes.

The mating of the CD4\(^{low}\) mutant to the two individual TCR tg mice presents us with some interesting data. These experiments open a window into the affinity of the individual TCRs. TCRs that are positively selected and not negatively selected will fall into a certain window of affinity. Because we are limited in the number of mg we use with differing affinity TCRs, it is hard to get a feel for this window or where in this range each individual TCR lies. Several groups have expressed tg coreceptors that increase positive selection (44, 45) or tip the balance on a TCR tg from positive selection to negative selection (9, 10, 46) or vice versa (47, 48). Our system provides a similar, yet more subtle, result. In the AND tg/CD4\(^{low}\) mutant, the positive selection of TCR tg to the CD4 lineage is significantly reduced (58%) compared with that in wt/littermates (Fig. 7). That would place the affinity of this TCR for its ligand near the lower limit, as a reduction in the CD4 level leads to failure of positive selection. The genes encoding this TCR were originally isolated from a T cell clone that recognizes the C-terminal peptide of PCC in the context of I-E\(^k\) (32). Both the AND and 5C.C7 TCRs are very similar, incorporating Va11 and
Vβ3 gene segments. However, they do incorporate different Jα segments and have distinct junctional sequences (49). Despite this, Yelon and Berg (50) recently showed data indicating that AND and 5C.C7 were about as strongly selected as each other on I-Ek, with AND being slightly more efficient than 5C.C7. Since we bred the AND tg onto the C57BL/6 strain, the TCR is being positively selected by the I-Aα molecule because these mice do not express I-E. This positive selection is by cross-reactivity to I-Aα, since PCC-specific helper T cells are restricted by I-E and not I-Aα (37, 51). It seems logical, then, that the TCR is being positively selected with a lower affinity, by cross-reactivity, to I-Aα than it would if it were being selected by I-Ek, its restricting MHC. In agreement with this interpretation, Matechak et al. (52) recently found that H-2b AND TCR mice have small thymy with a reduced compartment of DP thymocytes and an increased proportion of DN thymocytes, while H-2b AND TCR mice have large thymy and mature cells predominantly in the CD4 lineage. Their interpretation is that homozgyous expression of Eκ induces some clonal deletion of AND TCR-expressing cells. By reducing the amount of CD4 available during positive selection, we believe that a significant portion of T cells that would have been positively selected fail to be so, supporting the idea that a minimum avidity threshold exists for positive selection. Having a TCR with some affinity for MHC (AND) may not insure escape from PCD without a minimum overall avidity. Thus, CD4 plays a critical role in determining overall avidity of cells undergoing selection and whether they can escape death by neglect. In the 5C.C7 tg/CD4low mutant, on the other hand, selection to the CD4 lineage is reduced only slightly (Fig. 6). We interpret these data to mean that the affinity of this TCR for its ligand (peptide plus Eκ) is in the upper range of the affinity window. In agreement with these data, Yelon and Berg (50) found that 5C.C7 thymocytes can mature with maximum efficiency even when the level of I-Ek is reduced. The reduced level of CD4 affects selection of the 5C.C7 tg to a lesser degree than that of the AND tg.

We noted that the introduction of either TCR transgene (AND or 5C.C7) onto the CD4low mutant background results in thymy of greater cellularity, a phenomenon that is common in TCR tg mice when thymic selection is compromised. Several possibilities exist. 1) In the case of a relatively high affinity TCR (5C.C7), the selecting MHC may induce deletion as well. By reducing the level of CD4, i.e., weakening the TCR/MHC interaction, this deletion might be attenuated, resulting in increased cellularity. 2) The average lifespan of the DP thymocytes may be increased, resulting in increased cellularity. In the AND line, the cells undergoing selection may be receiving a signal through their TCR that is sufficient to protect them from PCD for a short time but insufficient to positively select them. Thus, they would "stall" in the DP stage for a time, resulting in increased cellularity. The DP stage is one of great overall cellularity. Removal of cells from this pool by positive selection could result in a decrease in overall cellularity. However, in a case of reduced positive selection (CD4low), the average lifespan of DP thymocytes may be lengthened because cells are not transitioning into the SP stage.

Other groups have shown that particular TCR V gene products are preferentially selected into either the CD4 or CD8 lineage (53–57). Here we show that a reduced level of CD4 influences the extent to which particular TCR V region gene products can be selected into the CD4 lineage (Table II). Interestingly, for the panel of Abs we used with specificities for TCR Vα or Vβ domains, we found that four were significantly affected when CD4 was reduced on the C57BL/6 background, while only one was significantly affected in B10.BR mice. This supports the idea that specific TCR V regions are selected by MHC class (I or II) (55) as well as MHC allele.

In conclusion, our data support the idea that CD4 plays a crucial role in determining the avidity of the thymocyte-stromal cell interaction and whether the developing T cell has the avidity necessary to escape PCD. Further studies will be required to place quantitative limits on the window of avidity that exists for positive selection.

Acknowledgments

The authors thank Dr. Mark Moore and Grace Calacano for advice about the ES cell work and generation of the mutant mice; Dr. Christopher J. Wheeler for continued feedback, insight, and creative ideas; Dr. Michelle Tutt Landolfi for Abs and technical advice; Dr. Henry Neuman de Vegvar for computer assistance and technical advice; and Angela L. Lee for reagents and discussion.

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