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**Recognition of a Specific Self-Peptide: Self-MHC Class II Complex Is Critical for Positive Selection of Thymocytes Expressing the D10 TCR<sup>1</sup> FREE**

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# Recognition of a Specific Self-Peptide: Self-MHC Class II Complex Is Critical for Positive Selection of Thymocytes Expressing the D10 TCR<sup>1</sup>

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We examined the specificity of positive and negative selection by using transgenic mice carrying a variant of the D10 TCR. We demonstrate that a point mutation at position 51 within the CDR2 $\alpha$  segment significantly reduces the avidity of this TCR for its cognate ligand, but does not impact recognition of nonself MHC class II molecules. Although structural studies have suggested that this TCR site interacts with the MHC class II  $\beta$ -chain, the avidity of this TCR for its ligand and the function of the T cell can be reconstituted by a point mutation in the bound antigenic peptide. These data demonstrate that the bound peptide can indirectly alter TCR interactions by influencing MHC structure. Remarkably, reducing the avidity of this TCR for a specific antigenic peptide-MHC ligand has a dramatic impact on thymic selection. Positive selection of thymocytes expressing this TCR is nearly completely blocked, whereas negative selection on allogenic MHC class II molecules remains intact. Therefore, the recognition of self that promotes positive selection of the D10 TCR is highly peptide-specific. *The Journal of Immunology*, 2003, 170: 48–54.

The expression of a functional  $\alpha\beta$  TCR is necessary but not sufficient for the maturation of CD4 T cells (1, 2). The TCR of the developing CD4 T cell must interact with MHC class II molecules loaded with self-peptides that are expressed on the surface of thymic cortical epithelial cells. An appropriate interaction of TCR with MHC class II molecules delivers a signal to the developing thymocyte that allows for survival and differentiation (3). The nature of this MHC:TCR interaction is a question under a considerable amount of debate. Furthermore, the impact of this interaction on the overall specificity of the mature TCR repertoire is uncertain. The peptide specificity of positive selection has been studied by reconstituting MHC class I expression in fetal thymic organ cultures (FTOC)<sup>4</sup> (4–8). In general, these studies have found that peptides the same as, or closely related, to the peptide that the T cells were originally raised against worked best for inducing positive selection. More recently, naturally occurring self-peptide:MHC ligands that induce positive selection of thymocytes bearing a transgenic TCR were identified (9–11). In both systems, however, several peptides with divergent sequences also proved capable of promoting positive selection of a single TCR.

Other studies have made use of mice that were genetically manipulated such that almost all MHC class II molecules were loaded

with the same peptide. This was accomplished either by disrupting the peptide exchange molecule, H-2M (12–15), or by covalently linking a peptide directly to the MHC molecule (16–18). The rationale behind these systems was that if self-peptides define the specificity of the mature TCR repertoire, the repertoire selected in mice expressing only a single type of MHC class II:peptide complex should produce a dramatically restricted TCR repertoire. Initial results obtained from such mice suggested that the reverse was actually true and that a diverse TCR repertoire could be selected. Subsequent work using the H-2M-deficient mice has demonstrated that the TCR repertoire in these mice is actually very restricted (19). Similar findings were made later in mice with a peptide covalently linked to the MHC (20, 21). Complicating the issue even more is recent work that demonstrated that positive selection in these mice might actually occur on peptides other than the dominant peptide (22–24). In perhaps the most elegant work concerning this question, Rudensky and colleagues (25) have recently directly demonstrated that different peptides positively select T cells with different specificities (25). Intriguingly, Janeway and colleagues (18, 26, 27) have speculated that the key feature of a positively selecting peptide is that it does not induce TCR aggregation, perhaps because it can not induce a conformational change in the TCR complex. This change may be similar to the conformational change that occurs in CD3 $\epsilon$  following TCR engagement allowing for the recruitment of the adaptor protein, Nck (28).

Overall, it appears that intrathymic self-peptides must have an impact on the specificity of the mature TCR repertoire. However, the question of specificity of the interaction with the TCR during selection remains open. The FTOC systems clearly suggest that CD8 T cells require self-peptides for selection, but that the recognition can be degenerate. The MHC class II systems also suggest that self-peptides are critical, but specificity of the interaction has not yet been defined. FTOC systems involving MHC class II-reactive T cells have not been informative.

In this study, we have mutated the  $\alpha$ -chain of the D10 TCR such that the recognition of a peptide:MHC complex by this TCR is altered. Importantly, this mutation leaves TCR recognition of nonself MHC molecules intact. This altered TCR  $\alpha$ -chain was then introduced as a transgene together with the wild-type D10  $\beta$ -chain.

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<sup>4</sup> Abbreviations used in this paper: FTOC, fetal thymic organ culture; CA-wt, wild-type conalbumin peptide.

Using this system, we demonstrate that altering peptide specificity, while leaving MHC specificity intact, drastically reduces positive selection of thymocytes bearing this TCR, but leaves negative selection intact.

## Materials and Methods

### TCR-transfected cell lines

As previously described (29), expression constructs containing the cDNA for D10 TCR or for the L51S TCR were transfected into the 4G4 cell line (originally obtained from E. Palmer, Basel Institute, Basel, Switzerland). G418-resistant clones were tested by response to anti-CD3 Ab (2C11) and by FACS analysis with Abs against V $\alpha$ 2, V $\beta$ 8, and CD4. Independently derived clones were tested with similar results. Transfectants used for these studies responded similarly to stimulation by an Ab against CD3 (2C11) and had similar levels of V $\alpha$ 2, V $\beta$ 8, and CD4 by FACS.

### Peptides

Peptides were synthesized at the W. M. Keck Biotechnology Resource Laboratory (Yale Medical School, New Haven, CT). Peptides were characterized by RP-HPLC, amino acid analysis, and mass spectroscopy. Their sequences were as follows: CA-wt, HRGAIEWEGIESG; CA-R2G, HGGAIIEWEGIESG.

### Measuring T cell responses with the CTLL-2 cell line

Transfected T cells ( $5 \times 10^4$ ),  $3 \times 10^5$  of the appropriate APC and peptide were incubated in a volume of 200  $\mu$ l in 96-well, flat-bottom plates overnight. The supernatant (100  $\mu$ l) was then removed and placed into a fresh 96-well plate. These plates were quickly frozen and thawed to kill any transferred cells. IL-2 production by the transfectants was measured by the use of the IL-2-dependent cell line, CTLL-2, as previously described (30). In assays to determine the response of these transfectants to nonself MHC molecules,  $5 \times 10^4$  transfectants were incubated overnight with titrated numbers of mitomycin C-treated splenocytes from either C57BL/6 or B10.D2 mice. The presence of IL-2 in the supernatants was detected by the use of the CTLL-2 assay.

### Ab blocking of T cell responses

Titred amounts of purified anti-V $\beta$ 8 mAb (F23.1; BD PharMingen, San Diego, CA) were added to  $5 \times 10^4$  TCR-transfected cells in flat-bottom 96-well plates. After approximately a 15-min preincubation,  $3 \times 10^5$  of the appropriate APC and peptide were added to the wells. Assays involving blocking of alloreactivity to nonself MHC were done similarly, except that  $5 \times 10^5$  T cell-depleted, mitomycin-C-treated APCs were added to each well as stimulators. Each experiment was done in triplicate and experiments were repeated at least three times. Concentrations of F23.1 above 10  $\mu$ g/ml caused activation of the TCR transfectants. Production of IL-2 was determined with CTLL-2 cells, as described above.

### Production and breeding of transgenic mice

The TCR  $\alpha$  transgene previously used to make the D10 TCR transgenic (31) was modified by site-specific mutagenesis as reported in Blander et al. (32). Expression of this 36-kb transgene is controlled by the endogenous TCR  $\alpha$  locus promoters and enhancers. The transgene was coinjected with the D10 TCR $\beta$  transgene into day 1 F<sub>2</sub> C3H/B6 embryos. Founder mice were screened by PCR of tail DNA (31). A positive founder was backcrossed to B10.BR and then to mice with a disrupted TCR  $\alpha$  gene (33). The offspring were intercrossed and mice were obtained that carried the two, cointegrated transgenes, the mutated TCR  $\alpha$  gene and that were homozygous for the H-2<sup>k</sup> MHC haplotype. These mice were maintained by breeding TCR transgene-positive, TCR  $\alpha^{-/-}$  mice to transgene-negative TCR  $\alpha^{-/-}$  littermates.

Transgenic animals were bred under specific pathogen-free conditions in the Immunology Mouse Unit (Yale University School of Medicine) and in the Research Animal Resource Center facilities at Memorial Sloan-Kettering. Other mice used in these studies were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice used in these studies were sacrificed when they were 4-wk-old. Animal use was approved by the Memorial Sloan-Kettering Cancer Center Internal Animal Care and Use Committee.

### FACS analysis

Single cell suspensions of thymocytes or lymphocytes were made by dissociation of the tissues with glass slides. Cells were counted in the presence of trypan blue. Cells ( $1-5 \times 10^6$ ) were incubated on ice with the appropriate Abs for 30 min, washed, and incubated with secondary reagents

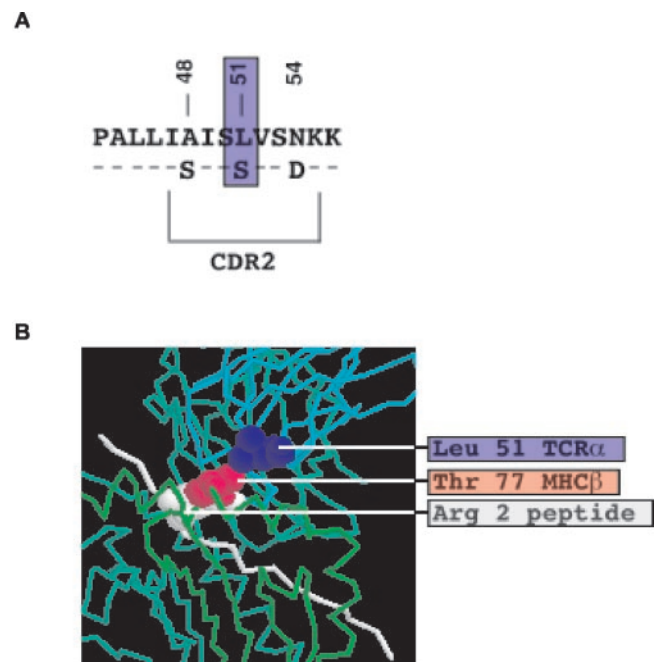
when necessary. Analysis was done on a BD Biosciences FACSscan or LSR (Mountain View, CA). Abs used were anti-CD4 Quantum Red (Sigma-Aldrich, St. Louis, MO), anti-CD8 PE (Life Technologies, Grand Island, NY), and biotinylated 3D3 (34). Transfectants were screened with anti-CD4 Quantum Red (Sigma-Aldrich), anti-V $\alpha$ 2 PE (BD PharMingen), and anti-V $\beta$ 8 FITC (BD PharMingen), or anti-CD4 and biotinylated 3D3.

## Results

### CDR2 $\alpha$ of the D10 TCR influences interaction with the antigenic peptide

Two T cells (D10.G4.1 and AK8) that were derived by immunization of AKR (I-A<sup>k</sup>) mice with the protein conalbumin have been described (30). The  $\beta$ -chains in these T cells used V $\beta$ 8.2 and are identical to each other. The  $\alpha$ -chains are very similar, in that both are from the V $\alpha$ 2 family, however, they differ in both the CDR3 $\alpha$  segment and at several amino acids within the V $\alpha$  gene since different subtypes of V $\alpha$ 2 are used. The similarity in V gene usage results in some differences in the specificity of the two TCR. The most notable difference between these two TCR is in the recognition of nonself MHC class II molecules. D10 responds to I-A<sup>b,v,p,q,d</sup>, while AK8 lacks reactivity to any nonself MHC molecule.

Most of the seven amino acid differences in the V $\alpha$  gene segments between D10 and AK8 occur within the CDR1 and CDR2 regions (30). As shown in Fig. 1 A three differences are found within, or close to, CDR2 $\alpha$ . Analysis of the D10 TCR:CA-peptide: I-A<sup>k</sup> structure (35) suggests that the leucine at position 51 of the D10  $\alpha$ -chain interacts the  $\beta$ -chain of I-A<sup>k</sup>. We examined the responses of cells transfected with either the wild-type D10 TCR or the mutated L51S TCR. These transfected cell lines are similar to those used in earlier work (29, 30). The cells used for these experiments responded similarly to stimulation with the anti-CD3



**FIGURE 1.** A, Comparison of the CDR2 $\alpha$  segments of two similar TCRs (30) that both recognize the CA-wt peptide bound to I-A<sup>k</sup>. The D10 TCR (top sequence) has a leucine at position 51 while the AK8 TCR has a serine. (B) Examination of the D10 TCR:CA-wt:I-A<sup>k</sup> crystal structure (35) reveals that the leucine at position 51 (blue) of the D10 TCR  $\alpha$ -chain interacts with a threonine at position 77 (red) the MHC  $\beta$ -chain. This threonine interacts with the arginine at position 2 of the CA-wt peptide (white). The figure is oriented with the TCR  $\alpha$ - and  $\beta$ -chains at the top and the MHC  $\alpha$ - and  $\beta$ -chains at the bottom. The backbone of the CA-wt peptide is shown in white.

mAb, 2C11 (Fig. 2A). The response of the L51S TCR-transfected cells to the wild-type conalbumin peptide (CA-wt) presented by the I-A<sup>k</sup>-bearing CH27 B cell line, however, was ~2 logs lower than the response of the wild-type D10 TCR-transfected cells (Fig. 2B). The use of a peptide altered at position 2 from an arginine to a glycine (peptide CA-R2G), however, increases the response of L51S back to levels equivalent to the response of D10 cells to the wild-type peptide (Fig. 2B). It should be noted that the CA-R2G peptide also enhances the response of D10 T cells (29). These data were previously interpreted to define position 51 within CDR2 $\alpha$  of the D10 TCR as a point of interaction between this MHC class II-restricted TCR and position 2 of the antigenic peptide (29).

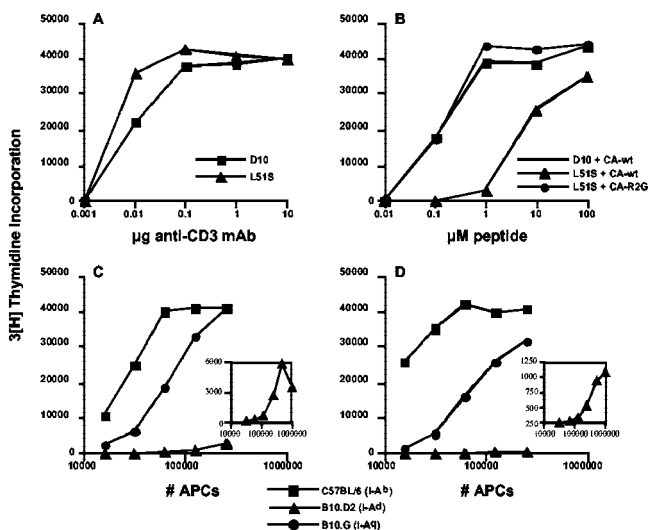
As shown in Fig. 1B, the structure of the D10 TCR bound to its ligand suggests that the interaction between the leucine at position 51 of the TCR with position 2 of the peptide is actually indirect. The structure shows an interaction of position 51 of the TCR with a highly conserved threonine at position 77 of the MHC  $\beta$ -chain. In turn, this threonine interacts with the arginine at position 2 of the conalbumin peptide. Intriguingly, a similar interaction has been defined by Gasciogne and colleagues (36–38) as critical in CD4 vs CD8 lineage commitment.

One of the interesting aspects of the D10 TCR is its unusual quality of responding to several nonself MHC class II molecules (39). Indeed, the D10 TCR mediates recognition of nonself MHC by naive T cells as well as negative selection of thymocytes in D10-transgenic mice (40). As shown in Fig. 2C, the D10 TCR-transfected cell lines respond strongly to splenocytes expressing I-A<sup>b</sup>, less well to I-A<sup>q</sup>-expressing splenocytes, and weakly to I-A<sup>d</sup>-expressing splenocytes. This pattern of response is consistent with previous studies with the D10.G4.1 T cell clone (39, 41). The

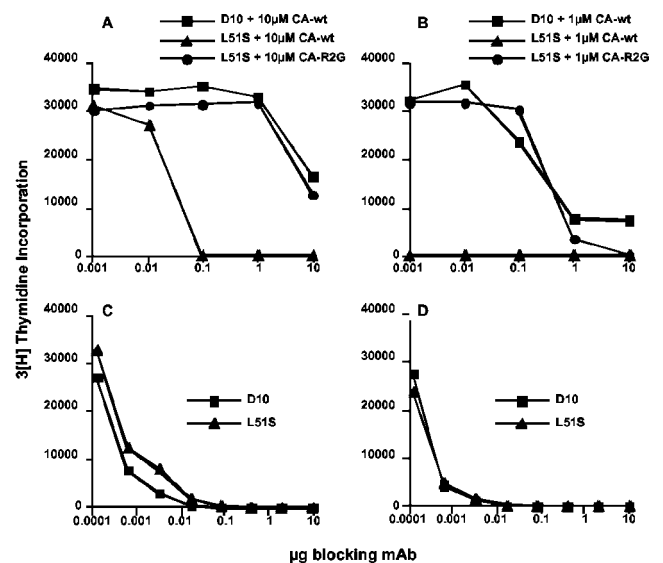
L51S TCR-transfected cells maintained a similar response pattern to these three MHC class II alleles (Fig. 2D). The response of L51S TCR-transfected cells to I-A<sup>d</sup> was reduced but always well above background (Fig. 2, C and D, insets). Therefore, although a TCR-MHC interaction has been altered resulting in altered peptide recognition, responses to nonself MHC are retained.

*The L51S mutation reduces TCR avidity for I-A<sup>k</sup>:CA-wt, but not for I-A<sup>k</sup>:CA-R2G or nonself, MHC alleles*

We next examined the relative avidities of the wild-type D10 TCR and the mutated L51S TCR for the various ligands tested above. The anti-TCR V $\beta$ 8 Ab F23.1 can be used to block proliferation of V $\beta$ 8-expressing T cells (42). Because the D10 TCR uses the V $\beta$ 8.2 gene segment, we tested whether its response to I-A<sup>k</sup> APCs loaded with the CA-wt peptide could be blocked with F23.1 in a dose-dependent manner. The response of D10 T cells to 10  $\mu$ M CA-wt peptide can be significantly reduced by the addition of 10  $\mu$ g/ml F23.1 (Fig. 3A). It should be noted that this concentration of Ab does not activate these T cell transfectants (data not shown). In contrast, as little as 0.1  $\mu$ g/ml Ab completely inhibited the response of the L51S transfectants. This suggests that the L51S TCR must be of significantly lower avidity for this peptide:MHC ligand because 100-fold less Ab is sufficient to block the response. Remarkably, the blocking Ab titration curve for the response of L51S TCR-transfected cells to 10  $\mu$ M mutated CA-R2G peptide is nearly identical to that for the response of D10 TCR cells to CA-wt peptide (Fig. 3A). When less CA-wt peptide is used (1  $\mu$ M; Fig. 3B) 0.001  $\mu$ g/ml F23.1 is sufficient to completely block the response of the L51S TCR-transfected T cell line. Nearly 1000 times as much Ab is needed to block the response of the wild-type D10



**FIGURE 2.** A point mutation in CDR2 $\alpha$  of the D10 TCR results in a reduced response to wild-type peptide that can be compensated for by a peptide altered in position 2. *A*, Transfected cell lines bearing the L51S TCR or the D10 TCR respond similarly when stimulated with the anti-CD3 mAb, 2C11. *B*, L51S TCR cells respond less well to CA-wt than cells transfected with the D10 TCR. Response of the L51S TCR cells is restored when the CA-R2G (position 2 of CA-wt changed from R to G) peptide is used. Mitomycin C-treated splenocytes from C57BL/6 (I-A<sup>b</sup>) mice, B10.G (I-A<sup>q</sup>), or B10.D2 (I-A<sup>d</sup>) mice were used as stimulators of the (C) D10 TCR and (D) L51S TCR-transfected cell lines. *Insets* in C and D show the responses to I-A<sup>d</sup> at greater APC numbers and also are graphed with a different y-axis. T cell response was measured by IL-2 production as detected by the growth of CTLL-2 cells. Stimulation with syngenic APCs (from either B10.BR mice or the CH27 cell line) resulted in no detectable levels of IL-2 production (not shown).



**FIGURE 3.** The relative avidity of the L51S TCR to the wild-type D10 TCR as measured by blocking with an anti-TCR Ab. The response of L51S TCR-expressing cells to (A) 10  $\mu$ M CA-wt or (B) 1  $\mu$ M CA-wt peptide presented by I-A<sup>k</sup> is blocked by much lower concentrations of an anti-V $\beta$ 8 Ab (F23.1) than is the response of D10 TCR-expressing cells under the same conditions. The avidity of the L51S TCR for the CA-R2G:I-A<sup>k</sup> complex (A and B) is similar to the avidity of the D10 TCR for CA-wt:I-A<sup>k</sup>. Alloreactive responses of the two T cells to splenocytes from (C) C57BL/6 (I-A<sup>b</sup>) or (D) B10.G (I-A<sup>q</sup>) were blocked by similar amounts of F23.1. Responses of both T cells to splenocytes from B10.D2 (I-A<sup>d</sup>) were completely blocked at all doses of F23.1 (data not shown). No IL-2 production was detected when the T cells were incubated with syngenic APCs plus the F23.1 Ab (data not shown).

TCR transfectants. Again, however, blocking the stimulation of the L51S TCR cells with CA-R2G peptide requires much more Ab (Fig. 3B). These data show that the L51S TCR is of much lower avidity for the CA-wt:I-A<sup>k</sup> ligand as compared to the D10 TCR, but that this can be compensated for by mutating the peptide at the second position.

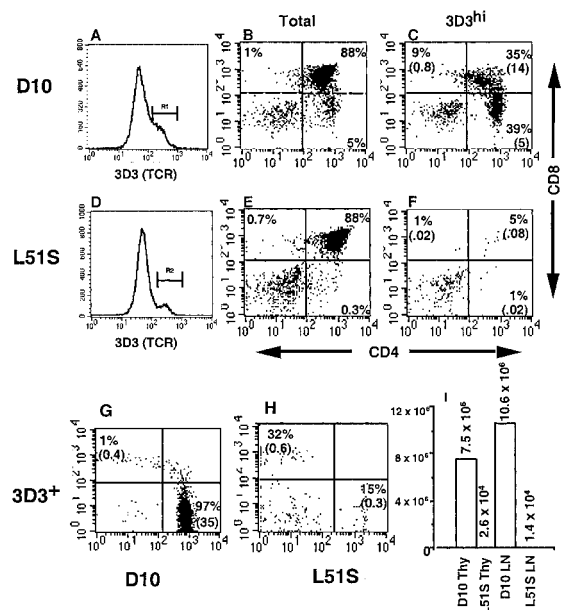
The similarity of the responses of the L51S and D10 TCR-transfected cell lines to APCs expressing nonself MHC class II suggested that these two TCRs have similar affinities for this ligand. As above, we compared the relative avidities of these TCRs by adding titrated amounts of the blocking F23.1 Ab to T cell assays. The transfected T cell lines were cultured with  $5 \times 10^5$  T cell-depleted splenocytes that expressed either I-A<sup>b</sup> or I-A<sup>q</sup>. The response to I-A<sup>b</sup> by both the D10 and L51S cells was completely blocked by 0.1  $\mu\text{g/ml}$  mAb (Fig. 3C). The lower avidity of the D10 TCR for I-A<sup>b</sup> and I-A<sup>q</sup> as compared I-A<sup>k</sup>:CA-wt is consistent with previous work (39). Overall the dose-response curves for the two T cells were nearly identical in this assay system.

Both D10- and L51S-transfected cell lines respond less well to I-A<sup>q</sup> than to I-A<sup>b</sup> (Fig. 2, C and D). Accordingly, somewhat less ( $\sim 0.01 \mu\text{g/ml}$ ) mAb was needed to completely block the T cell responses when I-A<sup>q</sup> stimulators were used. Again, however, the dose-response curves for the two cell lines were nearly identical. Responses of both the D10 and L51S cells to the weak I-A<sup>d</sup> stimulators were completely blocked at all Ab concentrations tested and therefore these data were not informative for comparing the relative affinities of the D10 TCR to the L51S TCR (data not shown). Overall, these data demonstrate that the L51S point mutation reduces the avidity of TCR for the I-A<sup>k</sup>:CA-wt ligand, but does not detectably alter the avidity for I-A<sup>k</sup>:R2G or for the nonself MHC alleles I-A<sup>b</sup> and I-A<sup>q</sup>. Importantly, the L51S mutation can be compensated for by a mutation in the antigenic peptide.

#### Thymocytes bearing the L51S TCR in transgenic mice fail positive selection

To further characterize the L51S TCR, we constructed a transgene carrying this mutated TCR  $\alpha$ -chain. The same DNA construct that was previously used to make the D10 TCR  $\alpha$ -chain transgene was modified to produce the L51S  $\alpha$ -chain transgene (31) and was coinjected with the D10 $\beta$  transgene. A transgenic founder was first backcrossed to B10.BR and then to H-2<sup>k</sup>, TCR  $\alpha^{-/-}$  mice (33) to eliminate endogenous TCR  $\alpha$ -chains. Similar results, however, were obtained on both  $\alpha$ -deficient and -sufficient backgrounds. Mice expressing both the L51S  $\alpha$ -chain and the D10  $\beta$ -chain (L51S TCR  $\alpha^{-/-}$ ) were sacrificed 4 wk after birth and the thymocytes and lymphocytes were analyzed by FACS. Age-matched D10 TCR  $\alpha^{-/-}$  mice were used as controls.

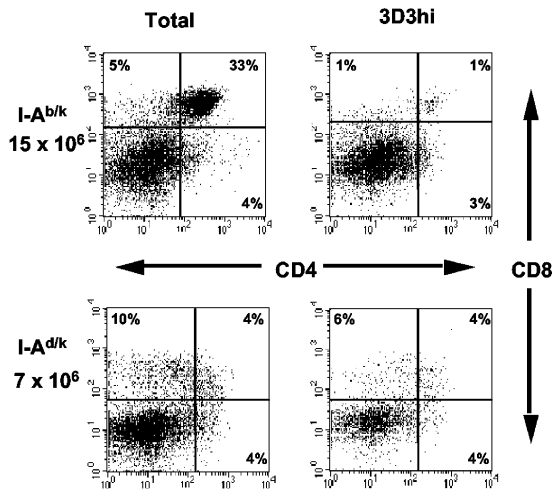
Thymocytes were stained with Abs against CD4, CD8, and the clonotypic anti-TCR Ab, 3D3 (34). We established that the 3D3 clonotypic Ab bound the L51S TCR by staining of the transfectant cell lines (data not shown). FACS analysis of thymuses from D10 TCR  $\alpha^{-/-}$  mice demonstrated that most thymocytes express low levels of the D10 TCR, while some are 3D3<sup>high</sup> (Fig. 4A). The dot plot analysis shows skewing of the thymocytes toward being CD4 single-positive (Fig. 4B). If only 3D3<sup>high</sup>-expressing thymocytes are analyzed (R1 in Fig. 4A), the skewing toward CD4 is more pronounced (Fig. 4C). Similar staining of a thymus from a L51S TCR-transgenic mouse is remarkably different. As in the D10 TCR  $\alpha^{-/-}$  mice, most of the thymocytes express low levels of TCR as determined by 3D3 staining (Fig. 4D). However, the population expressing high levels of 3D3 staining is much smaller. Remarkably, staining with CD4 and CD8 reveals that the thymocytes in L51S TCR  $\alpha^{-/-}$  mice do not progress past the immature



**FIGURE 4.** Thymocytes from L51S TCR  $\alpha^{-/-}$  mice fail positive selection. 3D3 staining of thymocytes from a (A) D10 TCR  $\alpha^{-/-}$  mouse and (D) an L51S TCR  $\alpha^{-/-}$  mouse. Two-color staining of thymocytes from a (B) D10 TCR  $\alpha^{-/-}$  mouse and a (E) L51S TCR  $\alpha^{-/-}$  mouse with Abs against CD4 and CD8. Electronically gating on 3D3<sup>high</sup> thymocytes (R1 and R2 in A and D) highlights the deficiency of positive selection in (F) L51S TCR mice as compared to (C) D10 TCR mice. Mature 3D3<sup>+</sup> T cells in (G) the lymph nodes of D10 TCR-transgenic mice are mostly CD4<sup>+</sup> while (H) few 3D3<sup>+</sup> T cells are found in L51S TCR-transgenic mice lymph nodes. (I) The total number of 3D3<sup>high</sup> CD4SP thymocytes and 3D3<sup>+</sup> CD4<sup>+</sup> T cells found in D10 TCR and L51S TCR mice. The top number in each FACS panel reflects only the percentage of 3D3<sup>high</sup> or 3D3<sup>+</sup> cells within each quadrant. In parentheses is the percentage that the cells within the region represent of the total cell population. Mice were 4-wk-old when sacrificed.

CD4<sup>+</sup>CD8<sup>+</sup> stage of development (Fig. 4E). When only the 3D3<sup>high</sup> cells from the L51S TCR  $\alpha^{-/-}$  mice are examined (R2 in Fig. 4D), it can be seen that these cells constitute extremely few CD4 single-positive cells (Fig. 4F). Therefore, positive selection of cells bearing the L51S TCR is severely curtailed. Importantly, the total size (cell number) of the thymuses from these two mice is nearly identical suggesting that the lack of CD4 T cells is not due to de novo negative selection. The actual numbers of CD4 single-positive thymocytes in L51S TCR  $\alpha^{-/-}$  are, however, dramatically reduced as compared to the D10 TCR  $\alpha^{-/-}$  mice (Fig. 4I).

The lack of intrathymic-positive selection in L51S-transgenic mice is evident in the peripheral tissues as well. As shown in Fig. 4G, most peripheral 3D3<sup>+</sup> T lymphocytes in D10 TCR  $\alpha^{-/-}$ -transgenic mice are CD4<sup>+</sup> and represent  $\sim 35\%$  of the lymph node cells. In sharp contrast is the FACS analysis of lymphocytes from an L51S TCR  $\alpha^{-/-}$  mouse, in which very few 3D3<sup>+</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T cells can be found (Fig. 4H). Less than 0.5% of the lymph node cells are 3D3<sup>+</sup> CD4<sup>+</sup> T cells representing a decrease of over 500-fold as compared to D10 TCR  $\alpha^{-/-}$  mice (Fig. 4I). It should also be noted that when the L51S TCR is in "competition" with other endogenous TCRs, (i.e., when the TCR  $\alpha$  locus is wild type), the frequency of 3D3<sup>+</sup> T cells is reduced to nearly zero (data not shown). The frequency of 3D3<sup>+</sup> CD4<sup>+</sup> peripheral T cells increases in older mice (over 14-wk-old) (32), but, intriguingly, unlike T cells in D10 TCR  $\alpha^{-/-}$  mice of the same age, many of these cells have a memory phenotype (CD44<sup>high</sup>L-selectin<sup>low</sup>) suggesting some type of T cell activation or homeostatic expansion



**FIGURE 5.** Thymocytes bearing the L51S TCR are negatively selected in H-2<sup>b</sup> and in H-2<sup>d</sup> MHC haplotype mice. L51S TCR-transgenic mice were backcrossed to either C57BL/6 or B10.D2 to generate transgene-positive I-A<sup>k/b</sup> or I-A<sup>k/d</sup> mice. The thymocytes and lymphocytes from mice were stained with Abs against CD4, CD8, and TCR (3D3). In the dot plots showing the total cells, a tremendous reduction in the percentage of CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) cells was apparent in both mice. Some 3D3<sup>high</sup> cells are present in both mice, however, these cells are mostly negative for both CD4 and CD8 expression as shown in the plots labeled 3D3<sup>high</sup>. The cellularity of the thymuses (left of plots) from these mice was 10–20 times less than that found in transgene-positive I-A<sup>k/k</sup>.

has occurred.<sup>5</sup> Finally, it should also be noted that a substantial population of unusual CD4<sup>+</sup>Vβ8<sup>+</sup>Vα2<sup>-</sup> T cells could be found in these mice (data not shown).

#### Negative selection of L51S TCR-expressing thymocytes

Recognition of nonself MHC molecules expressed in H-2<sup>b</sup> and H-2<sup>d</sup> mice by the L51S-bearing transfectants suggested that L51S-expressing thymocytes would undergo intrathymic-negative selection in mice expressing these MHC alleles. Indeed, this has been shown in mice expressing the D10 TCR (40). Demonstration of negative selection in the L51S TCR-transgenic mice would establish that this TCR is functional. Therefore, we crossed mice carrying the L51S TCR to both C57BL/6 (H-2<sup>b</sup>) and B10.D2 (H-2<sup>d</sup>) mice (Fig. 5). Massive negative selection, comparable to that seen previously using D10 TCR-transgenic mice (40), was observed in both the L51S TCR × BL/6 mice and in the L51S TCR × B10.D2 mice. In both cases, there was approximately a 90% reduction in thymic cellularity. The thymuses from B10.BR mice carrying the L51S TCR transgenes have, on average, 150 × 10<sup>6</sup> cells. In mice crossed to C57BL/6 or B10.D2, this number drops to an average of 15.2 × 10<sup>6</sup> and 7.4 × 10<sup>6</sup>, respectively. As shown in Fig. 5, negative selection is also apparent as a large loss of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes and an increase in the percentage of CD4<sup>-</sup>CD8<sup>-</sup> thymocytes. In both mice, almost all of the TCR<sup>high</sup> thymocytes (detected by staining with 3D3) are CD4<sup>-</sup>CD8<sup>-</sup>. As would be expected, peripheral lymphocytes are essentially devoid of CD4<sup>+</sup>3D3<sup>+</sup> cells (data not shown).

#### Discussion

Intrathymic selection of T cells is believed to pick out useful TCR specificities and eliminate potentially harmful specificities. Although the necessity for negative selection is intuitively obvious,

the role of positive selection is less apparent. Nonetheless, it is clear that positive selection has a substantial impact on the specificity of the mature TCR repertoire and, therefore, also has a significant impact on the immune response. Although it is established that the developing T cell requires an interaction between its TCR and the MHC molecules that are expressed on thymic cortical epithelial cells (1, 2), the nature of this interaction has not yet been defined.

Several investigators have attempted to measure the allowable degeneracy of the TCR:peptide contact during positive selection (4–8). These studies have used thymocytes from TCR-transgenic mice and variants of the antigenic peptide for those TCRs to define peptides that would induce positive selection in FTQC. In addition to the original peptide, several different peptides were found to work in these studies. These data suggested that while some restrictions exist, a significant amount of degeneracy is tolerated in the TCR:peptide contact. In the studies presented in this manuscript, we have taken the opposite approach. We have left the peptide repertoire intact, but have altered the ability of a TCR to interact with peptides. Mutation of the CDR2α domain of the TCR from the D10.G4.1 T cell clone resulted in a TCR (L51S) that is less responsive to the antigenic peptide, but retains its reactivity to nonself MHC molecules (29). Importantly, this TCR mutation can be complemented by a mutation in the antigenic peptide.

Mice were generated that carry the transgenes for the L51S TCR to allow for the analysis of positive and negative selection. We reasoned that if the recognition of self-peptide during positive selection is degenerate, the alteration in the peptide specificity of the L51S TCR should be compensated for and positive selection would occur similarly to that seen in mice carrying the wild-type version of the D10 TCR. This would suggest that MHC:TCR interactions primarily govern positive selection or, alternatively, that a different self-peptide could be substituted for the peptide recognized by the D10 TCR. Reduced or eliminated positive selection of thymocytes bearing the L51S TCR would strongly implicate an important role for a specific self-peptide in the positive selection of the D10 TCR.

The L51S mutation to the D10 TCR substantially lowers the avidity of the TCR for the I-A<sup>k</sup>:CA-wt MHC:peptide ligand. As a consequence, the response of L51S TCR-transfected cells to this ligand is substantially reduced. A mutation in the CA-wt peptide (CA-R2G) complements the L51S mutation as measured both by function of the T cell line and avidity of the TCR for the MHC:CA-R2G complex. Analysis of the D10 TCR:peptide:MHC crystal structure (35) suggests that, in contrast to our previous conclusions (29), the complementation of the L51S TCR mutation by this peptide mutation is not due to a direct TCR-peptide interaction. Instead, position 51 of the TCR α-chain appears to interact with a threonine at position 77 of the MHC β-chain. In turn, this amino acid interacts with position 2 of the peptide. Therefore, these data strongly suggest that the bound peptide can alter the surface of the MHC and, thereby, indirectly alter TCR specificity.

Transgenic mice expressing the altered D10 TCR (L51S) had dramatically reduced numbers of CD4 single-positive thymocytes. Therefore, although the L51S mutation can be compensated for by altering the peptide sequence, there is not an intrathymic self-peptide that can substitute for the naturally occurring positively selecting ligand. This implies that positive selection of the D10 TCR is highly peptide-specific. Interestingly, thymocytes bearing the L51S TCR did undergo negative selection when other MHC class II alleles were expressed. Although we provide no direct evidence, it is tempting to speculate that while positive selection of the D10 TCR is exquisitely dependent upon specific interactions with a

<sup>5</sup> J. M. Blander, D. B. Sant'Angelo, and C. A. Janeway, Jr. A pool of memory-like CD4 T cells contains effector memory precursors. *Submitted for publication.*

self-peptide, negative selection of this TCR is more promiscuous. Of course, this may be particular to the D10 TCR.

Our results in this MHC class II system are reminiscent of recent studies using the OT-1 MHC class I TCR-transgenic mice (43). OVA-specific T cells, including OT-1 thymocytes, are positively selected on K<sup>b</sup>, but not on the very closely related mutant K<sup>bm8</sup> MHC class I molecule (44, 45). Because the mutations in K<sup>bm8</sup> primarily impact peptide binding, these data have been interpreted to mean that the failure to select OVA-specific T cells is due to the failure to present a specific self-peptide (45). Stefanski et al. (43) proved that was true by identifying a peptide that when presented by K<sup>bm8</sup> restored positive selection of OT-1 thymocytes.

One rational explanation for the need for positive selection is that MHC allelic variation coupled with the randomness of TCR assembly requires that each TCR be tested for its ability to interact with MHC molecules. However, it is becoming increasingly clear that the genes of the TCR are inherently biased toward MHC recognition (46, 47) and, therefore, selection merely for the ability to interact with MHC does not explain the apparent stringency of the process. Alternatively, as our data suggest, positive selection may skew the TCR repertoire toward recognition of self-peptides. This skewing may be critical because recent data suggest that continued recognition of self-peptides is a requirement for the survival of naive and/or memory T cells (48–51). Furthermore, it has been hypothesized that recognition of self-peptide MHC complexes may be a critical component of the normal T cell response (52). This idea has now received strong experimental support from Davis and colleagues (53).

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