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An antagonist peptide mediates positive selection and CD4 lineage commitment of MHC class II–restricted T cells in the absence of CD4

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T cell development in the thymus results from interactions between developing thymocytes and thymic stromal cells, with the outcome dependent on the avidity of the TCR–pMHC interaction; high avidity interactions lead to negative selection, intermediate interactions lead to positive selection, and weak or no interactions lead to death by neglect (1, 2). These interactions are dependent on TCR recognition of either MHC class I or class II molecules associated with self-peptides and with positive selection leading to T cells that contain cytotoxic (CD8) or helper activity (CD4), respectively. The coreceptors, CD4 and CD8, play crucial roles in these interactions during T cell development, as they stabilize TCR interactions with their respective MHC class II or class I molecules (3, 4) and facilitate the association of signaling molecules such as Lck and LAT (5, 6). However, in TCR transgenic mice expressing mutant CD4 or CD8 molecules unable to interact with Lck, positive selection still occurred (7, 8), and conflicting data exists as to whether or not the cytoplasmic tail of coreceptors imparts unique signals directing lineage commitment (9–11). Therefore, the exact roles of the coreceptors remain unclear during positive selection and lineage commitment.

Most studies on the role of peptides in positive selection and lineage commitment have focused on the use of MHC class I–restricted fetal thymic organ cultures (FTOCs), where they showed that antagonist ligands induced positive selection of CD8+ T cells (12, 13), whereas agonists generally induced negative selection but positive selection at lower concentrations (14–19). The role of CD8 during positive selection has also been assessed in FTOCs deficient in CD8, showing that positive selection and CD8 lineage commitment could be restored by altered peptide ligands (APLs) of higher affinity (20, 21).

In contrast, little is known about the specific contribution of the CD4 coreceptor during T cell development. Studies on the role of peptides in positive selection and lineage commitment of MHC class II–restricted T cells have been less decisive, as it was impossible to completely eliminate the presentation of endogenous class II peptides in MHC class II–restricted FTOCs. Yet, these studies demonstrated an array of T cell selection outcomes, from peptide-specific negative selection (22–24), antagonism of positive selection (25), to selection of MHC class II–restricted T cells into the CD8 lineage.
Other approaches addressing the role of peptides on positive selection and lineage commitment of MHC class II-restricted T cells have included either a two-step thymic-reaggregate system (27), adenoviral delivery of invariant chain–peptide fusion proteins to the thymus (28), or in vivo injection of soluble peptides (29). Studies have also used mice expressing a single peptide/MHC class II molecule, either via transgenic expression of MHC class II molecules covalently linked to peptide (30, 31), or mice deficient in H-2M (32–37), with the results arguing against the role of specific peptides in selection of a MHC class II–restricted T cell repertoire. Regardless, none of these studies addressed the role of CD4 during T cell development. Therefore, although it is recognized that CD4 is important during T cell maturation, its specific functions during positive selection and lineage commitment remain unresolved.

However, it is clear that the selection and commitment of DP T cells to either the CD4 or CD8 T cell lineage is an asymmetrical process, with different signaling requirements dictate this decision, with one major difference being that CD4 associates with more Lck than does CD8 (5, 38, 39). This modified version of the instructive model of lineage commitment, termed the “strength-of-signal model,” is widely accepted as it proposes that CD4 lineage choice is dictated by TCR signals of either stronger signal intensity (10, 40–46) or longer/lasting signal duration (27, 47–49). This is in contrast with the stochastic/selecion model, which proposes that lineage decision is a random process, with further survival dependent on having the correct TCR and coreceptor choice (50–52). Initial studies using chimeric molecules consisting of the extracellular domain of CD8 and the cytoplasmic domain of CD4 have implicated the cytoplasmic tail of CD4 as responsible for CD4 lineage commitment (10, 53). However, another paper using similar chimeric molecules has argued against this, suggesting instead that the CD4 tail only determines the number, not the lineage direction of positively selected thymocytes (11). Additional experiments obtained from mice deficient in CD4, which showed either a substantial presence of CD4+CD8− T cells (7, 54) or a skewing toward the CD8 T cell lineage (40) along with studies disrupting CD4–MHC class II interactions (55–57) or CD4-p56Lck (7) yielded disparate results, depending on

![Figure 1](https://journals.org/jem/figure/10.1083/jem.200409047/fig1A.png)

**Figure 1.** N3.L2 Rag−/−CD4−/− mice are severely impaired in positive selection with few T cells in the periphery. (A) Lymph node cells from N3.L2 Rag−/−CD4−/− and N3.L2 Rag−/−CD4−/− mice were analyzed for CD4 and CD8 expression (left). Cells were gated for either CD4+CD8− cells or CD4−CD8− cells and analyzed for 3L2 TCR clonotype expression (TCR+; right). (B) The number of CD4+CD8− TCR+ (black bars), CD4−CD8− TCR+ (white bars), and CD4−CD8− TCR− (black bars, “CD4” positive) cells per lymph node from N3.L2 Rag−/−CD4−/− and N3.L2 Rag−/−CD4−/− mice is depicted. Error bars, standard error of the mean. (C) Thymocytes from N3.L2 Rag−/−CD4−/− and N3.L2 Rag−/−CD4−/− mice were analyzed for CD4, CD8 (left), and TCR clonotype expression (right). Histogram gates and numbers mark the percentage of total thymocytes that were high for TCR clonotype expression (right). (D) TCRhi thymocytes from C were gated and analyzed for CD69 expression.
the system used. What exactly is the specific contribution of the CD4 coreceptor during positive selection and lineage commitment? Does CD4 play different roles during these two processes? Does CD4 deliver a unique signal for CD4 lineage commitment?

To understand the specific role of the CD4 coreceptor in T cell ontogeny, we studied MHC class II–restricted T cell maturation in the absence of CD4 in vivo. We used N3.L2 TCR transgenic mice deficient in RAG and CD4, which have profound defects in positive selection, with a paucity of T cells detected in the periphery. Our laboratory has also previously characterized a unique transgenic system in which targeted expression of APLs in MHC class II–expressing cells mediated specific positive and negative selection of 3.L2 T cells in vivo. With this approach, we have defined a kinetic threshold of positive and negative selection based on the half-life of the TCR–pMHC interaction, with long half-lives leading to negative selection, and shorter half-lives leading to either enhanced positive selection or no effect (58–60). Therefore, we asked whether the absence of CD4 would impact this threshold of positive and negative selection, and subsequently, on CD4 lineage commitment. Our results show that positive selection could be restored in the presence of antagonist ligands, with functional T cells of the CD4 lineage appearing in the periphery. In mice expressing other APLs, we show that the kinetic threshold for positive and negative selection was lowered in the absence of CD4. However, the absence of CD4 did not affect lineage commitment, as there was no skewing toward the CD8 lineage with weaker ligands. Thus, the present paper clarifies the role of CD4 during positive selection and argues against the need of the CD4 coreceptor to deliver a unique instructional signal for CD4 lineage commitment.

RESULTS

Few T cells develop in N3.L2 TCR transgenic mice deficient in Rag and CD4

For these studies, we used N3.L2 TCR transgenic mice that expressed receptors (Vα18/Vβ8.3) specific for the hemoglobin peptide (Hb(64–76)) presented by I-Eκ. To ensure that we focused on T cells that expressed only the N3.L2 TCR and not TCRs from endogenous rearrangement of other α and β loci, we crossed these mice with mice deficient in the Rag1 gene (61). As expected, N3.L2 T cells from these mice (N3.L2 Rag−/−CD4+/−) were skewed toward the CD4 lineage, with no CD8 T cells detected in the periphery (Fig. 1, A and B).

Remarkably, when these mice were additionally crossed with mice deficient in CD4 (7) (N3.L2 Rag−/−CD4−/−), few T cells developed (Fig. 1). There was a paucity of CD4−/−CD8− T cells in the lymph nodes of N3.L2 Rag−/−CD4−/− mice (∼1.5 × 104 cells/lymph node), a nearly fivefold decrease in the number of N3.L2 T cells compared with wild-type mice (∼7.2 × 104 cells/lymph node; Fig. 1 A). Curiously, we detected only slight skewing toward the CD8 lineage in N3.L2 Rag−/−CD4−/− mice, as few CD8+ T cells were detected in the periphery (Fig. 1, A and B), less drastic than the skewing observed in other TCR transgenic mice deficient in CD4 (40). In the thymus, we detected fewer TCRhi cells in N3.L2 Rag−/−CD4−/− mice compared with wild-type mice (Fig. 1 C), in accordance with the few T cells detected in the periphery (Fig. 1 B). We also detected a dramatic decrease in the number and percentage of TCRhiCD69+ cells in N3.L2 Rag−/−CD4−/− mice compared with wild-type mice (Fig. 1 D). Together, our results present a unique monoclonal TCR transgenic system in which the absence of CD4 leads to both severe impairment of positive selection and very little skewing toward the CD8 lineage, thus allowing us to determine whether we can replace the function of CD4 and induce T cell maturation using APLs.

Antagonist ligands can restore positive selection of T cells in the absence of CD4

We reasoned that if CD4 functioned to enhance the overall avidity of the TCR–pMHC interaction, then stronger TCR ligands that normally deleted wild-type T cells should be able
to induce positive selection of N3.I2 T cells in the absence of CD4. We took advantage of a series of well-defined APLs for the 3.I2 TCR (58, 59) that were based on mutations of the primary P5 TCR contact residue, asparagine, at position 72 of the Hb[64–76] peptide (Fig. 2 A). These ligands have been classified as either an agonist, (Hb[64–76]), weak agonist (I72), strong antagonist (I72), weak antagonist (A72), or null ligand (Q72, E72) based on their potencies for T cell activation (58), and correlate with the longevity of the TCR–pMHC interaction (Fig. 2 A and reference 59). More importantly, we have previously created transgenic mice in which all class II–positive cells express functional APL activity in vivo, with agonists and antagonists mediating negative selection, and null ligands expressing enhanced positive selection or no effect in vivo (60, 62). Therefore, we sought to determine whether any of these APLs could increase thymocyte avidity sufficiently in the absence of CD4 and restore positive selection of N3.I2 Rag<sup>−/−</sup>CD4<sup>−/−</sup> T cells in vivo.

For our initial experiments, we chose an APL of intermediate TCR ligand strength, an antagonist (I72), which has previously been shown to delete 3.I2 CD4<sup>+</sup> T cells (60). We made radiation chimeras with bone marrow cells from CD4<sup>+</sup> B6.K mice and sorted CD4<sup>−/−</sup> or CD4<sup>+</sup> mice (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041574/DC1). In addition, we were able to iden-

Figure 3. Phenotypic analysis of peripheral T cells from CD4<sup>−/−</sup>→I72 chimeras. (A) Lymph node cells from CD4<sup>−/−</sup>→I72 mice were gated on CD4<sup>+</sup>CD8<sup>−</sup> (left) or CD4<sup>−</sup>CD8<sup>+</sup> (right) expression and analyzed for TCR clonotype expression. Histogram gates and numbers denote the percentage of cells that were positive for TCR clonotype expression. Data are representative of at least three similar experiments, with two to four mice per group. (B) Total RNA from an equivalent number of sorted CD4<sup>+</sup> or CD8<sup>+</sup> T cells from B6.K mice and sorted CD4<sup>−</sup>CD8<sup>−</sup> TCR<sup>+</sup> thymocytes, splenocytes, or lymph node cells was extracted, reverse transcribed, and PCR-amplified for exons 1–5 upstream of the Neo insertion site (63), reasoning that CD4<sup>−</sup>CD8<sup>−</sup> TCR<sup>+</sup> T cells in these chimeras should express this truncated CD4 mRNA indicative of their commitment to the CD4 lineage. As expected, negative selection was detected in CD4<sup>−/−</sup>→I72 chimeras, evident by the paucity of TCR<sup>hi</sup> thymocytes (Fig. 2 B) and absence of peripheral clonotype positive (denoted as TCR<sup>+</sup>) T cells (Fig. 3 D). In contrast, positive selection was restored in CD4<sup>−/−</sup>→I72 chimeras, as we detected a dramatic increase in TCR<sup>hi</sup> thymocytes (Fig. 2 C), up-regulation of CD69 expression (Fig. 2 C), and increase in the percentage (Fig. 3 A) and number (see Fig. 5 B) of CD4<sup>+</sup>CD8<sup>+</sup> TCR<sup>+</sup> T cells in the periphery. These results suggest that increasing thymocyte avidity with antagonist ligands can replace CD4 coreceptor function and restore positive selection of N3.I2 Rag<sup>−/−</sup>CD4<sup>−/−</sup> T cells in vivo.

I72-selected CD4<sup>−/−</sup> T cells commit to the CD4 lineage

Positive selection of I72-selected CD4<sup>−/−</sup> T cells resulted in a predominantly CD4<sup>+</sup>CD8<sup>−</sup> T cell population in the lymph nodes, with few cells skewed toward the CD8 lineage (Fig. 3 A). Because these I72-selected CD4<sup>+</sup>CD8<sup>−</sup> T cells cannot express surface CD4, a marker for commitment to the CD4 lineage, it was possible that these cells were not of the CD4 lineage and belonged to an alternative lineage. To address this, we attempted to detect CD4 mRNA using primers specific to exons 1–5 upstream of the Neo insertion site (63), but by RT-PCR, we detected CD4 message in CD4<sup>−</sup>CD8<sup>−</sup> TCR<sup>+</sup> T cells in vivo. These results are consistent with the requirement of CD4 for positive selection of 3.I2 T cells in vivo. Therefore, we sought to determine whether any of these APLs could increase thymocyte avidity sufficiently in the absence of CD4 and restore positive selection of N3.I2 Rag<sup>−/−</sup>CD4<sup>−/−</sup> T cells in vivo.

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ify DN thymocytes (CD4−CD8−TCR−) that did not express truncated CD4 message in N3.L2 CD4−deficient mice, ruling out the possibility that premature expression of the TCR transgene in DN thymocytes was responsible for CD4 T cell development (Fig. S1). Moreover, the CD4−CD8−TCR+ T cells from CD4−/−→I72 chimeras were negative for CD3ε (Fig. 3 A), B220, NK1.1, γδ TCR (not depicted), and CD25 (Fig. 3 C), ruling out the possibility that these cells belonged to other T cell lineages such as the CD3ε+ intraepithelial, DN B220+, NKT, γδ, or T regulatory cell lineages, respectively. Together, these results show that the CD4−CD8−TCR+ T cells selected by antagonist ligands were CD4 lineage T cells.

It is also possible that the phenotype observed in CD4−/−→I72 mice represents enhanced homeostatic proliferation of few selected T cells, rather than efficient positive selection by antagonist ligands. We believe this to be unlikely, as the I72-selected CD4−/− T cells were predominantly of the naive phenotype, expressing CD44b and CD62Lh, and not activation markers such as CD25 (Fig. 3 C) or CD69 (not depicted), suggesting that these cells were quiescent and not actively proliferating in the periphery. Also, in control chimeras expressing no APLs (CD4+/−→B6.K, CD4−/−→B6.K; Fig. 3 D), positive selection was similar to wild-type N3.L2 Rag−/−CD4−/− and N3.L2 Rag−/−CD4−/− mice (Fig. 1 A), suggesting that bone marrow chimeras faithfully recapitulate positive selection seen in wild-type mice. In fact, positive selection was similar between CD4−/−→I72 and CD4+/−→B6.K mice (see Fig. 5 A), with more CD4−CD8−TCR+ peripheral T cells detected in the former than the latter (see Fig. 5 B). Thus, these results further substantiate our observation that antagonists can replace CD4 and mediate efficient positive selection of CD4 lineage T cells in vivo.

**I72-selected CD4−/− T cells respond to Hb(64–76), and not to APLs**

Another hallmark of successful positive selection and CD4 lineage commitment is the presence of functional MHC class II–restricted T cells in the periphery. We tested the I72-selected CD4−/− T cells for two parameters characteristic of functional wild-type 3.L2 T cells as follows: (a) their ability to proliferate and produce IL-2 in response to Hb(64–76), and (b) their graded responses to APLs (58, 60, 62). As shown in Fig. 4 A, I72-selected CD4−/− T cells responded vigorously to Hb(64–76) in the proliferation assay, confirmed by detection of IL-2 production using the CTLL-2 bioassay (Fig. 4 B). When tested for their response to APLs, I72-selected CD4−/− T cells proliferated weakly to the weak agonist (T72; Fig. 4 A), correlating with low levels of IL-2 production (Fig. 4 B). Not surprisingly, I72-selected CD4−/− T cells did not proliferate or produce IL-2 in response to either the antagonist (I72) or null ligand (Q72). Together, these results demonstrate that the antagonist ligand (I72) promoted positive selection and CD4 lineage commitment of functional MHC class II–restricted N3.L2 CD4−/− T cells.

**Positive selection and lineage commitment of T cells in the presence of other APLs**

We expanded our analysis to include chimeras expressing other APLs in the presence or absence of CD4. Confirming our previous findings, negative selection ensued in CD4+/− chimeras that expressed antagonists, weak agonists, or agonists, with few CD4+CD8−TCRhi SP thymocytes detected in these mice (Fig. 5 A). With the weaker ligand (Q72), we detected an increase in the number of CD4+CD8−TCRhi SP thymocytes, slightly higher than in control CD4+/−→B6.K (no APL) chimeras (Fig. 5 A), consistent with enhanced positive selection described previously (60). These results confirm our previous finds in APL transgenic mice, and indicate that the selection seen was apparently not influenced by the absence of I72–expressing bone marrow–derived cells in the radiation chimeras.

In contrast, a different pattern emerged in chimeras deficient in CD4 as follows: (a) with antagonists (I72), we de-
The central tenet of the strength-of-signal model is that strong TCR signals promote CD4 lineage commitment, whereas weaker ligands would skew development toward the CD8 lineage. Therefore, we wanted to test whether differential lineage commitment would occur using a spectrum of TCR ligands in the absence of CD4. As shown in Fig. 5 B, whereas the agonist ligand (Hb[64–76]) lead to complete negative selection and absence of both CD4 and CD8 lineage T cells in the periphery, the weak agonist (T72) lead to partial positive selection of CD4 lineage (CD4+CD8−TCRhi) T cells. With antagonist ligands (I72), we detected maximal positive selection and CD4 lineage commitment, although a slight increase in CD8 lineage T cells (CD4−CD8−TCRhi) was also observed (Fig. 5 B). However, with the ligand of the lowest affinity in this group (Q72), we detected few CD4−CD8+TCRhi T cells, even though slight positive selection of CD4 lineage cells was detected. In chimeras expressing no APLs (CD4−→B6.K), we detected few CD8 lineage T cells, consistent with the phenotype observed in N3.L2 Rag−/−CD4−/− mice. Together, we have conclusively shown that CD4 lineage commitment can occur in the absence of CD4, thus demonstrating that CD4 does not deliver a unique signal for CD4 lineage commitment.

**DISCUSSION**

Many studies have demonstrated that the CD4 and CD8 coreceptors play crucial roles during T cell development, yet it has been difficult to ascertain their specific roles during positive selection and lineage commitment. Most studies have focused exclusively on MHC class II–restricted T cells, showing that CD8 is not crucial for CD8 lineage commitment if selected with low affinity peptides (20, 21). In contrast, few studies have been done to address these same issues for the CD4 coreceptor on the development and maturation of MHC class I–restricted T cells. To resolve this, we asked whether positive selection could be restored in N3.L2 Rag−/−CD4−/− mice, which are severely impaired in positive selection. Our results show the following: (a) positive selection of functional MHC class II–restricted T cells can occur with low affinity peptides in the absence of CD4, and (b) the kinetic threshold for positive and negative selection is lowered in the absence of CD4. Together, our results imply that CD4 does not deliver a unique signal for CD4 lineage commitment.

In this paper, we present evidence that TCR antagonist ligands can restore positive selection of CD4 lineage T cells in the absence of CD4. Remarkably, this same ligand induces negative selection in the presence of CD4, implying that the kinetic threshold for positive and negative selection was lowered by the absence of CD4. We interpret these re-
results to indicate that an increase in the affinity of the TCR ligand can replace the function of CD4 and restore positive selection. If so, perhaps the main function of CD4 during T cell development is to bind and stabilize TCR–pMHC interactions and increase the half-life of TCR–pMHC complexes, as has been proposed previously (64, 65). This model proposes that CD4 can effectively increase the half-life of preformed TCR–pMHC complexes, provided that the TCR–pMHC interaction is of sufficient duration to allow CD4 to be recruited to the immunological synapse, where it can also associate with self-peptide–MHC complexes to form a CD4 “pseudodimer” (66). This interpretation would be compatible with studies in which disruption of CD4–MHC class II interactions blocked CD4 T cell development (56) and disruption of CD4–p56 Lck had no effect provided that the mutant CD4 was overexpressed (7). Therefore, in the absence of CD4, we would predict that the half-life of TCR–pMHC complexes is shorter, and not of sufficient duration to transmit enough signals to achieve the minimum threshold needed for positive selection. In contrast, when compensated with a TCR ligand that has a longer half-life (agonist), sufficient interaction is acquired to cross the minimum threshold for positive selection. With TCR ligands that have an even longer half-life (agonists or weak agonists), negative selection ensues, implying that no advantage is obtained by CD4 in stabilizing and maintaining an already long-lived TCR–pMHC interaction. Collectively, our results imply that during positive selection, CD4 is primarily required for maintaining the avidity of the TCR–pMHC interaction, and is nonessential in providing a qualitative signal for T cell development.

Previous studies have documented that the absence of CD4 leads to the development of MHC class II–restricted T cells in the CD8 lineage (40). This, along with initial studies using CD8/CD4 chimeric molecules, leads to the proposal of the initial strength–of–signal model, which proposes that coreceptors dictate T cell lineage choice (9, 10, 53). Our results challenge this model in the following ways: (a) we detected little skewing toward the CD8 lineage in N3.L2 Rag−/−CD4−/− mice; (b) we detected slight skewing toward the CD8 lineage in CD4−/−→H72 mice; and (c) we were unable to push the cells completely into the CD8 lineage with the weakest TCR ligand used in this paper, Q72. Together, we provide evidence that CD4 does not impart unique signals that determine CD4 lineage commitment. In fact, our studies agree with a previous paper arguing that the CD4 and CD8 tails do not dictate lineage decision (11). Collectively, our results in conjunction with studies citing the nonessential requirement for CD8 in CD8 lineage commitment conclusively rules out the role of coreceptors in lineage decision, and thus challenge the original strength–of–signal model for lineage commitment. Consequently, this would imply that information about MHC class specificity is delivered exclusively by the TCR, and not by the coreceptors. Moreover, our results and others would imply that the primary role of the coreceptors during thymocyte development is for positive selection, where they serve to stabilize their respective TCR–MHC complexes, associate with Lck, and allow for the transmission of the “selecting signal,” of either the right intensity, duration, or both, to the developing thymocyte. However, it would seem that the affinity requirement for positive selection of CD4 SP thymocytes is less stringent than that of CD8 SP thymocytes, as CD8 T cell development is more severely impaired in CD8-deficient mice compared with its CD4 counterpart in CD4-deficient mice. Exactly how the coreceptors contribute to the affinity required for selection into the CD8 or CD4 lineage is still a matter of intense debate and investigation, and may be revealed as more precise measurements of the coreceptors interactions with their respective MHC molecules are obtained.

This paper is the first to demonstrate that CD4 is not an absolute requirement for CD4 lineage commitment. Our results also do not favor the stochastic/selection model because we detected primarily only CD4 lineage T cells instead of equal frequencies of MHC class II–restricted T cells in both CD4 and CD8 lineages in any of the CD4-deficient bone marrow chimeras. However, we cannot argue against revised versions of the strength–of–signal model, which are based on quantitative differences in either signal intensity or signal duration (for review and discussion see references 67, 68), except that these signals can be delivered exclusively by the TCR alone, provided that the TCR–pMHC interaction is of sufficient affinity. In fact, our results can be interpreted in light of these quantitative models, as with different TCR ligands, we were able to manipulate the kinetic thresholds of positive and negative selection. However, one caveat remains as to why we could not detect CD8 lineage T cells with the null ligand, Q72. It is possible that other weaker ligands may redirect cells toward the CD8 lineage, with a K off faster than Q72, but still of sufficient affinity for positive selection. It is also possible that the natural intrinsic affinity of the N3.L2 TCR for pMHC is higher than other MHC class II–restricted TCRs, such that even in the absence of CD4, the signal strength is still not low enough to skew toward the CD8 lineage. It is also important to point out that in those studies, skewing toward CD8 lineage T cells in CD4-deficient mice was most evident in mice heterozygous for its selecting MHC (i.e., selection of AND under H-2b/k, or selection of DO10 under H-2d/bk), whereas in anti-HA TCR transgenic CD4-deficient mice, very little skewing toward the CD8 lineage under H-2d/bk was observed (40). Therefore, perhaps for the N3.L2 TCR, lower I-E k levels might lead to redirection toward the CD8 lineage.

A set of studies has suggested that signals specifying CD4 T cell lineage commitment may be distinct from signals that mediate positive selection (69, 70). Our results support this contention, as we show the requirement of CD4 in positive selection, but not in CD4 lineage commitment. Thus, under normal conditions in which mice express the full complement of a normal T cell repertoire and the CD4 coreceptor, it is possible that CD4 functions primarily to stabilize weak/transient TCR–pMHC interactions and maintain a diverse T cell
repertoire, as suggested in previous studies (35, 37, 71, 72). Although it is still unclear what specific signals dictate lineage choice, we have clarified the role of the CD4 coreceptor during T cell development. In conclusion, our findings strongly support the concept that it is the strength of the specific TCR–pMHC interaction that delivers the instructive signal for CD4 lineage commitment, not the CD4 coreceptor.

MATERIALS AND METHODS

Transgenic mice. N3.L2 mice, which express the 3.12 TCR–recognizing Hb(64–76)/I-Ek, have been described previously (73, 74). The original 3.L2 mice (75) contained a single base error in the Jβ segment, leading to a single amino acid difference with the TCR from the 3.L2 T cell hybridoma (73); the N3.L2 mice used herein contain the corrected 3.12 TCR. No difference in T cell reactivity to APLs or T cell development was observed between 3.L2 and N3.L2 mice. In addition, N3.L2 mice crossed onto mHEL expressing mice lack detectable T cells recognizing the mHEL/APL transgenes. All mice were bred and housed under specific pathogen-free conditions in the animal facility at the Washington University Medical Center. All experiments followed protocols that were approved by the Washington University Animal Studies Committee.

Peptides. The peptides used in this work were synthesized, purified, and analyzed as described previously (58, 76). APLs have been defined previously and are denoted as the one-letter amino acid code of the substituted amino acid at position 72 (Fig. 2 A). The amino acid sequences for the peptides used in this work are as follows: Hb(64–76) GKKVITAFTEGLK; I72 mice were generated by crossing N3.L2 mice with RAG1.AKR mice, which is based on a cross between the (137Cs source; Gammacell 40) and reconstituted with 5–10^5 bone marrow chimeras.

Radiation bone marrow chimeras. To construct bone marrow chimera, 6–12 wk-old recipient mice were lethally irradiated with 1,100 rad (137Cs source; Gammacell 40) and reconstituted with 5–10^5 bone marrow cells that have been depleted of mature T and B cells by treatment with anti-CD3 PE (145-2C11) and anti-B220 PE (RA3-6B2), followed by removal using MACS Microbeads conjugated with anti-PE (Miltenyi Biotec). Reconstituted mice were kept on antibiotic water and analyzed >8 wk later.

Flow cytometry and electronic sorting. The antibodies used in this study were CD4-PE (H129.19), CD8α-FITC (53–6.7), CD69-PE (H1.2F3), CD25-PE (PC61), CD62L-PE (MEL-14), CD44-FITC (IM7), Cab-biotin (mouse anti-3.12 TCR clonotype), G155-178-biotin (mouse anti-TNP, IgG2a isotype control), and streptavidin-tricolor (Caltag Laboratories). Except where noted, all antibodies were purchased from BD Biosciences. Single cell suspensions of thymocytes or lymph node cells were stained with primary antibodies in FACS buffer (PBS with 0.5% BSA and 0.1% NaN3) for 45 min on ice, washed twice with FACS buffer, and where necessary, stained with secondary antibodies for 30 min on ice. Cells were washed and fixed in 2% paraformaldehyde. Samples were analyzed on a FACScan (Becton Dickinson) using CellQuest analysis software. For electronic cell sorting, thymocytes, lymph node cells, or splenocytes were stained with CD4-PE, CD8α-FITC, and/or Cab-biotin (followed by streptavidin-tricolor), and sorted by a FACSCalibur (Becton Dickinson) gated on CD4–CD8–Cab– (for CD4–→I72), CD4+, or CD8+ (B6.K mice) expression.

Detection of CD4 mRNA by RT-PCR. Total cellular RNA from sorted cells as described in the figure legends was extracted with TRIzol following the manufacturer’s protocol (Invitrogen). RNA was reverse transcribed into first-strand cDNA using random hexamers and AMV reverse transcriptase (Promega). PCR amplification was done using primers specific for exons 1–5 of CD4, upstream of the neo insertion site (63) (CD4 forward: 5’-GTCACTCAAGGGAACGCTGTG-3’, CD4 reverse: 5’-AAGGTCTATTGAACACCCACACA-3’) under the following cycling conditions: 38 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C.

T cell proliferation assay and measurement of IL-2 production. Splenocytes from CD4–→i72 mice were harvested, pooled, and depleted of MHC class II–positive cells to avoid peripheral antagonism (62, 77). Depletion of MHC class II–positive cells was done by staining cells with the class II–specific 14–4–4 antibody, followed by removal using MagneBeads conjugated with goat anti-mouse IgG (Cortex Biochem Inc.). For proliferation assays, 3.5 × 10^5 cells/well were incubated with 5 × 10^3 irradiated splenocytes from B6.K mice loaded with increasing amounts of peptide for 48 h, pulsed with 0.4 μCi [3H]thymidine, and harvested 18–24 h later. For measurement of IL-2 production, one fourth of each well was collected 24 h after antigen stimulation and transferred to the IL-2 indicator cell line, CTLL-2, for 24 h, pulsed with 0.4 μCi [3H]thymidine, and harvested 24 h later.

Online supplemental material. Fig. S1 demonstrates that the truncated CD4 message is not detected in CD4–/CD8– TCR+ thymocytes, or in CD8+ T cells from N3.L2 CD4–deficient mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20041574/DC1.

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