Alterations in CD4 dependence accompany T cell development and differentiation

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Abstract

Several studies have indicated that the necessity for co-receptor engagement during T cell activation depends on the avidity of the TCR-MHC interaction under investigation. Using thymocytes, naive T cells and a long-term T cell line isolated from 2B4 TCR transgenic mice, we have examined the role of the CD4 co-receptor on cells expressing the identical TCR at multiple stages of T cell maturation. When anti-CD4 Fab fragments were used to block CD4-MHC class II interactions, we found decreasing CD4 dependence as T cells matured. As a second approach to examining the role of the CD4 co-receptor, we generated I-Ek mutants defective in CD4 interactions. In the course of this study, we identified a new potential site for CD4 interaction in the fa domain of I-Ek. The new fa mutation and a mutation in the previously described CD4 binding site in the fc domain both interfere with stimulation of 2B4 thymocytes, but not mature T cells. Together these data demonstrate that the role of the CD4 co-receptor depends on the state of maturation of the T cell.

Introduction

CD4 and CD8 are well established as T cell co-receptors, capable of enhancing T cell and thymocyte responses by augmenting cell-cell adhesion and transmitting cytoplasmic signals (for review, see 1). It is less clear what determines whether or not a T cell will depend on these co-receptor contributions for activation in a particular circumstance. Several previous studies have established that the avidity of a TCR-MHC interaction can determine the co-receptor dependence of a T cell response. As a result, it has been proposed that the weaker the TCR interaction, the greater the requirement would be for co-localization of CD4 or CD8 to increase cell-cell adhesion and enhance recruitment of the signaling molecule p56lk into the TCR complex. For example, it has been shown for cytotoxic T lymphocytes that CD8 dependence is inversely correlated with sensitivity to antigen density on antigen-presenting cells (APCs) (2,3). In addition, studies with helper and cytotoxic T cells have documented the requirement for co-receptor during activation events induced by recognition of low affinity antigens (4,5). Experiments utilizing CD4- and CD8-deficient mice have further supported this idea, demonstrating that the requirement for co-receptor during negative selection in the thymus varies with the presumed affinity of the TCR for its MHC-peptide ligand (6-8).

Other observations have suggested that co-receptor dependence may be influenced by variables other than TCR avidity (9). For instance, many studies documenting changes in the activation requirements of naive versus primed T cells (10,11) are consistent with the possibility that p56lk participation in T cell activation may be more important for naive T cells than for primed (memory) T cells. Additionally, for cytotoxic T cells, it has been shown quite clearly that CD8 is more important during the priming of naive cells than in the lysis of target cells (2,12). However, no systematic analysis comparing the co-receptor requirements of thymocytes and mature cells expressing a single defined TCR has yet been done.

We have addressed this issue using the well-studied 2B4 TCR, specific for moth or pigeon cytochrome c plus I-Ek (13,14). This TCR, originally isolated from a T helper hybridoma, has been expressed in transgenic mice and shown to be positively selected specifically into the CD4 lineage (15). In addition, the fine recognition specificity of this...
TCR for both I-E<sup>k</sup> and cytochrome c antigen has been extensively characterized (16, 17). Finally, the 2B4 TCR is one of the few TCRs whose affinity for its MHC–peptide ligand has been measured (18). Interestingly, the 2B4 hybridoma is CD4<sup>-</sup>; thus, these cells are considered to be ‘CD4 independent’, based on the fact that activation with cytochrome c plus H-<sup>2k</sup> APCs occurs in the complete absence of the CD4 co-receptor. Together, these observations present an enigma: in a transgenic mouse, thymocytes expressing the 2B4 TCR are apparently influenced by CD4 interactions with MHC class II (I-E<sup>k</sup>) during their differentiation into CD4<sup>+</sup> T cells, yet the activation of the 2B4 hybridoma is completely independent of a CD4 co-receptor signal.

Using 2B4 TCR transgenic mice as a source of 2B4<sup>+</sup> T cells at different stages of development and differentiation, we have examined the requirement for CD4 engagement in TCR-mediated signaling events. We have interfered with CD4–MHC class II interactions via anti-CD4 Fab fragment blocking and MHC class II I-E<sup>k</sup> mutants. With these two approaches, we have tested CD4 dependence during the activation of a long-term IL-2-producing T cell line, during the activation of naive lymph node T cells and during the in vitro stimulation of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. The results of these studies clearly indicate that the CD4 dependence of a TCR-mediated signal decreases with increasing maturity. Therefore, these data offer a potential solution to the enigma of the 2B4 TCR. Of more general relevance, these results suggest a mechanism for the enhanced sensitivity of negative selection in the thymus relative to peripheral T cell activation (19, 20).

**Methods**

**Mice and cell lines**

2B4 transgenic and non-transgenic B10 (H-<sup>2b</sup>) or B10.BR/SgSnJ (H-<sup>2k</sup>) mice (Jackson Laboratories, Bar Harbor, ME) were maintained in a specific pathogen-free facility. Genotypes of transgenic progeny were determined by PCR on genomic DNA which identified the presence of the 2B4 TCR for both I-E<sup>k</sup> antigen has been measured (16, 17). Finally, the 2B4 TCR is one of the few TCRs whose affinity for its MHC–peptide ligand has been measured (18). Interestingly, the 2B4 hybridoma is CD4<sup>-</sup>; thus, these cells are considered to be ‘CD4 independent’, based on the fact that activation with cytochrome c plus H-<sup>2k</sup> APCs occurs in the complete absence of the CD4 co-receptor. Together, these observations present an enigma: in a transgenic mouse, thymocytes expressing the 2B4 TCR are apparently influenced by CD4 interactions with MHC class II (I-E<sup>k</sup>) during their differentiation into CD4<sup>+</sup> T cells, yet the activation of the 2B4 hybridoma is completely independent of a CD4 co-receptor signal.

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**Long-term 2B4 T cell line**

2B4<sup>+</sup> T lymph node T cells were isolated by cell sorting (FACStarPlus; Becton Dickinson, Mountain View, CA; MIT Flow Cytometry Lab, Cambridge, MA). T cells (10<sup>5</sup>) were stimulated in vitro with 10<sup>6</sup> B10.BR (H-<sup>2b</sup>) irradiated spleen cells plus 5 µM moth cytochrome c (MCC) peptide. After 2 days, cultures were expanded into rIL-2 (20 U/ml) plus anti-IL-4 Ab [clone 11B11 (22)]. After an additional 10 days, live T cells were purified on Ficoll gradients and re-stimulated with antigen plus irradiated spleen cells. Control cells stimulated in the absence of moth cytochrome c (MCC) peptide did not proliferate after exogenous cytokine addition. Cells were maintained in culture by repetitive stimulation as above in RPMI medium with 10% FCS and supplements. Cells used in functional assays were from the fifth, sixth or seventh round of stimulation.

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**Thymocyte dulling assay**

Thymuses from 5- to 10-week-old 2B4 H-<sup>2b</sup> mice were dissociated and plated at 10<sup>6</sup> thymocytes/well of a 96-well flat-bottomed plate. H-<sup>2</sup>b transgenic thymuses were used to avoid containing I-E<sup>k</sup>-expressing APCs, and to maximize the numbers of CD4<sup>+</sup>CD8<sup>+</sup> cells in the starting population. Thymocytes were incubated with 3 x 10<sup>5</sup> CHO APCs, in the presence or absence of MCC peptide and/or antibody, in a total volume of 0.2 ml of RPMI with 10% FCS and supplements. Each assay condition was performed in duplicate. Supernatants were then assessed for IL-2 content by titration on HT-2 indicator cells (25). Briefly, 50 µl of each supernatant was subjected to six 2-fold serial dilutions and incubated with 5 x 10<sup>5</sup> HT-2 cells in a total volume of 0.1 ml for 22 h. Then, 0.01 ml of the substrate Alamar Blue (Biosource International, Camarillo, CA) was added to each well. The plates were read 18 h later at a test wavelength of 560 nm and a reference wavelength of 590 nm. Raw data were analyzed as in Hedrick et al. (14), with 10% of maximal HT-2 response arbitrarily defined as 1 U IL-2/ml. In all experiments, HT-2 assays were capable of detecting as little as 0.25 U IL-2/ml.

**FACS analysis**

The following antibodies and secondary reagents were used in these studies: H129.19-PE (rat anti-mouse CD4; Gibco BRL), 53-6.7-Red 613 (rat anti-mouse CD8a; Gibco BRL), A2B4–FITC [anti-2B4 TCRα; (13)], 14.4.4–biotin [anti-E<sub>a</sub>; (26)], 17.3.3–biotin [anti-E<sub>β</sub>; (26)], Y17 [anti-I-E<sup>+</sup>; (27)], avidin–PE.
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Fig. 1. 2B4 TCR and CD4 expression on different populations of T cells. The long-term 2B4 T cell line (top), freshly isolated lymph node T cells (middle) and CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (bottom) from 2B4 TCR transgenic mice were stained with antibodies against the 2B4α chain (left) and CD4 (right). Histograms of surface staining in the presence (filled histograms) or absence (open histograms) of directly-conjugated antibodies are shown. In each case, forward and side scatter gates were used to eliminate dead cells, additional gates isolated the T cells from the other lymph node cells and the CD4<sup>+</sup>CD8<sup>+</sup> thymocytes from the other thymocytes.

Site-directed mutagenesis of I-<sup>E<sub>k</sub></sup>

A clone containing the I-<sup>E<sub>k</sub></sup> β chain cDNA was used to generate uridine-rich single-stranded DNA template for the dut<sup>+</sup>-ung<sup>+</sup> method of site-directed mutagenesis (29). The sequences of the oligonucleotides used to generate mutations were: TAC AAC GGG ACT GCA GAA GTG CGG CTT CTG (22); AAC CTG CGC TTT AAA AGC GAC GTG GGC (41); CTG GGG CGG CCA GAC GCG CCG AAC TGG AAC AGC CAG (59); GAC GTG GGC GAG TTC GAA GCG GTG AAG CGG CGG CGC (41); and TTC CGG AAT GGC AAG CTT CTG AAA ACA GGA ATT GTG (137). Nucleotides shown in bold face represent changes introduced into the wild type I-<sup>E<sub>k</sub></sup> sequence. Double mutants were created sequentially: uridine-rich single-stranded DNA made from the 137 mutant was annealed to each of the other oligos. Mutant cDNAs were then transferred to the mammalian expression vector pBJneo containing the Srp promoter (30). To generate stable lines of APCs, wild-type or mutant I-<sup>E<sub>k</sub></sup> genes were electroporated along with the wild type I-<sup>E<sub>k</sub></sup> α chain cDNA in the vector pBJ (Srα promoter) into CHO cells. Transfections were carried out with 5 μg of a β chain plasmid and 40 μg of the wild-type α chain plasmid at 230 V and 960 μF. Transfectants were selected in medium containing 0.8 mg/ml G418 (Gibco BRL). Single-cell clones of the selected population were screened for I-<sup>E<sub>k</sub></sup> expression by flow cytometry.

Results

Three T cell populations representing different stages of development or differentiation

2B4 TCR transgenic mice provide a source of T cells at multiple stages of development or differentiation. Freshly isolated T cells from lymph nodes of H-2<sup>k</sup> 2B4 transgenic mice can be activated to proliferate and secrete IL-2 by stimulation with MCC peptide (88–103) plus I-<sup>E<sub>k</sub></sup>-positive APCs. Activation of naive transgenic T cells has been shown to require both a TCR signal and a co-stimulatory signal, and has been found to represent faithfully the primary activation of naive non-transgenic T cells (10,11). These 2B4 lymph node T cells express uniformly high levels of CD4 and the
2B4 TCR (see Fig. 1), and are CD4+CD45RB+ (11 and data not shown).

As a second population, we generated a long-term T cell line from sorted 2B4 CD4+ lymph node T cells from a 2B4 TCR transgenic mouse. Sorted T cells were stimulated with MCC peptide plus H-2k APCs, expanded in exogenous IL-2 and then re-stimulated after a period of rest. After one round of stimulation, these T cells acquire some characteristics of primed cells, becoming co-stimulation independent and secreting higher levels of IL-2 when activated (10,11); in addition, the cells in the established line are CD44+ and CD45RBlo (data not shown). These cells express lower levels of the 2B4 TCR compared with naive lymph node cells, but remain CD4+ even after multiple rounds of stimulation (see Fig. 1).

As a third T cell population, we examined 2B4 CD4+CD8+ thymocytes isolated from H-2b 2B4 transgenic mice. Thymocytes from these mice are ~85% CD4+CD8-, with the remaining cells CD4+CD8-. Fewer than 1% of total thymocytes are CD4+CD8- or CD4-CD8+, as virtually no positive selection occurs in I-Eb mice (31,32) (Fig. 2). The CD4+CD8+ cells express intermediate levels of the 2B4 TCR and a uniformly high level of CD4 (see Fig. 1).

We set out to compare the co-receptor dependence of TCR-mediated signaling in these three types of 2B4+ cells. The responsiveness of the T cell line and the lymph node T cells can be assayed by measuring IL-2 production. A different type of assay is necessary to determine the responsiveness of the CD4+CD8+ thymocytes. When stimulated through their TCR with the appropriate MHC-peptide ligand, CD4+CD8+ thymocytes will generally undergo apoptosis (33). However, removed from the environment of the thymus and placed in suspension culture, thymocytes respond somewhat differently to TCR stimulation. In this in vitro assay, TCR stimulation can lead to apoptosis, particularly when the APCs are bona fide splenic presenting cells or phagocytic lines of fibroblasts (34-37). In some cases, though, stimulation of CD4+CD8+ thymocytes with antigenic peptide and APCs results in a response of CD4/CD8 down-regulation, without progression to cell death (38,39). CD4/CD8 down-regulation has also been observed after treatment of CD4+CD8+ thymocytes with anti-TCR-CD3 antibodies (35), calcium ionophores (34) or activators of protein kinase C (40), suggesting that the absence of either co-stimulatory signals or phagocytes accounts for this response. This TCR-mediated CD4/CD8 down-regulation, often referred to as 'CD4/CD8 dulling' has thus been equated with the initial steps of clonal deletion in the thymus during which CD4/CD8 down-regulation can clearly be observed (36,41). Therefore, responses in this sort of system, while not inducing cell death, are likely to be comparable to in vivo deletion; regardless, this assay provides a convenient means for quantitating responses of CD4+CD8+ thymocytes to TCR stimulation. This response will hereafter be referred to as 'thymocyte dulling'.

As can be seen in Fig. 2(A), incubation of thymocytes from H-2b 2B4 transgenic mice with I-Eb-transfected CHO cells...
Fig. 3. Differential sensitivity of a T cell line, naive lymph node T cells and CD4+CD8+ thymocytes to blocking by anti-CD4 Fab fragments. The 2B4 T cell line (A), freshly isolated lymph node cells (B) or thymocytes from a 2B4 TCR transgenic mouse (C) were stimulated with I-E*‐transfected CHO fibroblasts plus a range of concentrations of MCC peptide. Cultures were incubated in the absence of added antibody, or in the presence of intact anti-CD4 antibody or anti-CD4 Fab fragments. For (A) and (B), supernatants were harvested after 24 h and IL-2 concentration was assessed by titration on HT-2 indicator cells. The mean (± SD) concentration of IL-2 produced in duplicate wells is shown. In some cases, error bars are so small that they are imperceptible. In the absence of MCC peptide, <0.25 U/ml IL-2 was secreted by the T cell line or the naive lymph node T cells. Note that 10-fold more responder cells were present in the experiment shown in panel (B). For (C), thymocytes were harvested after 24 h, stained with antibodies against CD4, CD8 and the 2B4α chain, and analyzed by flow cytometry. The graph shows the mean (± SD) of triplicate wells, indicating the percent of CD4+CD8+ thymocytes undergoing dulling, calculated as (1−[%CD4+CD8+ cells in the presence of peptide]/[%CD4+CD8+ cells in the absence of peptide])×100. Again, some error bars are so small that they are imperceptible. For the cultures incubated with anti-CD4 antibodies or Fab fragments, CD4+CD8+ cells were identified by staining with a non-competing second anti-CD4 antibody. Incubation with a control rat IgG2b antibody had no effect (data not shown). In each case, the data shown are representative of four or more experiments with similar results.
are monovalent and have no Fc region which might be used to induce cross-linking. Additionally, GK1.5 Fab fragments 
seem unlikely to interfere with intermolecular interactions 
mediated by the membrane-proximal domains of CD4 (i.e. 
CD4–TCR interactions), since the GK1.5 epitope is primarily 
located in the most membrane-distal domain (D1) of CD4 
(45), a region implicated in class II binding by mutagenesis 
studies (46–49). Overall, GK1.5 Fab fragments provide a well-
characterized means of severely blocking the CD4–MHC 
class II interaction without inducing any undesirable signaling. 

To examine the effects of anti-CD4 blocking on the long-
term T cell line, cells were stimulated with I-E\(^{k}\)-transfected 
CHO cells plus a range of concentrations of the MCC peptide. 
Stimulations were performed in the absence of any antibody, 
in the presence of whole anti-CD4 antibody or with two 
different concentrations of anti-CD4 Fab fragments. After 24 h, 
supernatants were removed and tested for IL-2 content 
by titration on the IL-2 indicator cell line, HT-2. As can be 
seen in Fig. 3(A), whole anti-CD4 Ab effectively inhibited IL-
2 production by the T cell line at all peptide concentrations. 
In contrast, anti-CD4 Fab fragments had no effect on IL-2 
production by the T cell line at high peptide concentrations 
and only a marginal effect at lower peptide concentrations. 

Both the 1 and 3 \(\mu\)g/ml concentrations of anti-CD4 Fab 
fragments were determined to be saturating in these 
assays (data not shown). \[^{3}H\]Thymidine uptake following 
stimulation with peptide and irradiated spleen APCs was similarly 
affected by antibody and Fab fragment addition (data not shown). 
Thus, blocking CD4 interactions with anti-CD4 Fab fragments 
has little effect on the stimulation of a long-term T cell 
expressing the 2B4 TCR. 

Freshly isolated 2B4 lymph node T cells were then tested 
with anti-CD4 Ab blocking. Again, cells were stimulated with 
I-E\(^{k}\)-transfected CHO fibroblasts plus a range of concentrations 
of MCC peptide and IL-2 production was assessed after 24 h. As can be 
seen in Fig. 3(B), both whole anti-CD4 antibody 
as well as a saturating concentration of anti-CD4 
Fab fragments were capable of inhibiting IL-2 production 
by the naive T cells. However, blocking by the anti-CD4 Fab 
fragments was only partially effective, relative to the whole 
anti-CD4 antibody, at higher peptide concentrations. 
These data indicate that naive T cells expressing the 2B4 TCR 
are more CD4 dependent than the long-term T cell line, 
as anti-
CD4 Fab fragments are more effective at blocking their 
stimulation. 

CD4\(^{+}\)CD8\(^{+}\) thymocytes expressing the 2B4 TCR were the 
most CD4 dependent of the three cell populations tested. 
Thymocytes were cultured with I-E\(^{k}\)-transfected CHO 
fibroblasts plus a range of concentrations of MCC peptide. 
After 20–28 h, thymocytes were stained with antibodies 
against CD4, CD8 and the 2B4 TCR, and analyzed by flow 
cytometry. The percentage of CD4\(^{+}\)CD8\(^{+}\) cells 
undergoing thymocyte dulling at each peptide concentration was then 
calculated as shown in Figs 2 and 3. As with the mature T 
cells, whole anti-CD4 antibody had a dramatic inhibitory effect 
on the response of 2B4 thymocytes in this assay (Fig. 3C). 
However, for the thymocytes, blocking with anti-CD4 Fab 
fragments was as effective as whole anti-CD4 antibody at all 
peptide concentrations. A control isotype-matched antibody 
had no effect on thymocytes in this assay (data not shown). 

Interestingly, even 2B4 thymocytes became CD4 independent 
when stimulated with high enough concentrations of MCC 
peptide (Fig. 3C). On the opposite end of the peptide titration, 
the thymocytes were severely inhibited, with Fab fragments 
completely eliminating thymocyte responses over a large 
portion of their linear range of response in the absence of 
added antibody. These data indicate that compared with the 
two populations of mature T cells, the CD4\(^{+}\)CD8\(^{+}\) thymocytes 
are the most susceptible to inhibition by blocking CD4–MHC 
class II interactions. 

While interpreting these data, we were concerned about 
the propriety of comparing IL-2 assay data with thymocyte 
dulling data. For instance, if the dulling assay tended to 
magnify small differences while the IL-2 assay tended to 
minimize them, this would distort our results. Fortunately, other 
experiments have suggested that these assays are equally 
discriminating. For example, a weak agonist, pigeon 
cytochrome c (PCC) peptide, was substituted for MCC in the 
IL-2 response and thymocyte dulling assays. In both cases, 
three times as much PCC peptide was required for a response 
equivalent to that produced by MCC (50 and data not shown). 
These observations demonstrate that the thymocyte dulling 
assay is not more sensitive to differences in stimulation 
conditions than the IL-2 assay. Therefore, we conclude that 
the observed differences in anti-CD4 Fab fragment blocking 
genuinely indicate that thymocytes are the most dependent 
on CD4. 

To summarize the data from all experiments performed, at 
the concentration of MCC peptide required for 50% response 
in the absence of antibody, anti-CD4 fragments blocked the 
response of the T cell line ~2-fold, the naive T cells 2- to 7-
fold and the thymocytes completely (Fig. 3 and data not shown). 
A similar conclusion can be reached by comparing 
the amount of MCC peptide required to induce half-maximal 
stimulation in the presence versus the absence of Fab 
fragments. For the T cell line, data from all experiments indicate 
that ~3-fold more MCC peptide was required for half-maximal 
IL-2 production in the presence versus the absence of anti-
CD4 Fab. For the naive T cells, the amount of MCC peptide 
required for a half-maximal response increased 6- to 25-fold in 
the presence of anti-CD4 fragments. Finally, to achieve 
50% of the maximal CD4\(^{+}\)CD8\(^{+}\) thymocyte dulling response, 
10- to 60-fold higher concentrations of MCC peptide 
were required in the presence of the Fab fragments. 

MHC class II mutants deficient in CD4 interaction have no 
defect in activating a 2B4 long-term T cell line 

As a second approach to examining the role of CD4 
interactions at multiple stages of T cell development, we chose 
a genetic strategy of disrupting CD4-class II interactions by 
mutating critical residues of I-E\(^{k}\). At the time we began these 
experiments, no CD4-class II binding sites were character-
ized, although direct binding between human CD4 and MHC 
class II had been demonstrated (51). Initially, we designed 
mutations in solvent-exposed loops of all four domains of 
MHC class II, usually altering charged amino acid residues. 
During the course of our studies, a site in the 3–4 (C–D) loop 
of the \(\beta_{2}\) domain of class II was identified through both 
functional and physical binding assays (52,53). One of the 
mutants we had made, a double amino acid substitution in
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ment, but we have been unable to confirm that this mutation 22 seemed slightly deficient in some assays for CD4 engagement and mutant 59 was never significantly different from wild-type. 41 caused a general structural disturbance (see below) with the 137 mutation. Of the mutants tested, only three—

this loop of the β2 domain of I-Ek [Glu137 and Glu138 replaced with Leu-Leu, hereafter referred to as ‘137’; numbering according to Kabat et al. (54)], overlapped with a defective I-Aα mutant reported by König et al. (52).

The identification of this CD4 binding site in the β2 domain encouraged us to focus on a subset of our mutations which included 137 and the mutations we had already made in the β1 domain of I-Ek. In light of the extended rod-like structure of domains 1 and 2 of CD4 (55,56), it seemed likely that CD4 could simultaneously contact the membrane-proximal β2 and the membrane-distal β1 domains of MHC class II. We examined four β1 mutations in highly conserved, solvent-exposed residues (see Fig. 4): ‘22’, in which Gin 22 and Arg 23 are replaced with Ala and Glu respectively; ‘41’, in which Asp 41 is replaced with Lys, ‘48’, in which Arg 48 is replaced with Glu; and ‘59’, in which Glu 59 is replaced with Arg. We tested each of these mutations on its own and also in combination with the 137 mutation. Of the mutants tested, only three—137, 48 and a double mutant (48–137) combining the two—showed any reproducible defect in engaging CD4. Substitution at 41 caused a general structural disturbance (see below) and mutant 59 was never significantly different from wild-type in any assays for CD4 interaction (data not shown). Mutant 22 seemed slightly deficient in some assays for CD4 engagement, but we have been unable to confirm that this mutation has no defect in peptide presentation (data not shown). As described below, results with 137, 48 and 48–137 confirmed the acquisition of CD4 independence accompanying T cell development and differentiation.

The mutant I-Ek cDNA clones were co-transfected with a wild type I-Ek cDNA clone into CHO cells. Single cell clones expressing the I-Ek mutants were screened by flow cytometry using antibodies against both the α and β chains of I-E [14.4.4 and 17.3.3 (26)]. All mutant molecules were expressed successfully, except for 41 (data not shown); this mutation disrupts an intramolecular salt bridge which is evidently critical for proper conformation (57). A cell line expressing levels of each mutant comparable to those expressed by a line of wild-type I-Ek-expressing CHO cells was selected for further analysis (cell lines referred to as CHO/wt, CHO/137, CHO/48 and CHO/48–137; see Fig. 5A). A second anti-I-Ek-specific antibody, Y17, was used to assess the levels of I-Ek on the CHO/48 and CHO/48–137 cells, since the anti-I-Ek antibody 17.3.3 no longer recognized the β2 mutant 48 (Fig. 5). These antibody staining data indicate that the overall conformation of each of the I-Ek mutants is intact and confirm previous reports that the epitope recognized by the 17.3.3 antibody is disrupted by a mutation in residue 48 (58).

To confirm that these mutations did not otherwise disrupt the conformation or peptide-binding function of I-Ek, CHO/137, CHO/48 and CHO/48–137 cells were tested for their ability to stimulate 2B4 hybridoma cells in the presence of a range of MCC peptide concentrations. As the 2B4 hybridoma is CD4+, this assay would not distinguish MHC class II variants that differed only in their ability to interact with CD4. As can be seen in Fig. 6(A), the three cell lines expressing mutant I-Ek molecules are as effective as CHO/wt cells at stimulating IL-2 production by the 2B4 hybridoma at all peptide concentrations tested, down to 0.0001 μM (data not shown for lowest peptide concentrations). These data demonstrate that all CD4 independent functions of antigen presentation are intact in the 137, 48 and 48–137 mutants.

CHO/137, CHO/48 and CHO/48–137 cells were then examined for their ability to stimulate the long-term 2B4 T cell line. As can be seen in Fig. 6(B), in comparison to CHO cells expressing wild-type I-Ek, all three cell lines expressing mutant I-Ek molecules were equally effective in stimulating IL-2 production by the T cell line over a range of MCC peptide concentrations, down to 0.0001 μM (data not shown for lowest peptide concentrations). Thus, even a mutation in the previously reported β2 site for CD4 binding has no effect on stimulation of the T cell line. These results are consistent with the outcome of our experiments using anti-CD4 Fab fragments, which indicate that the activation of the 2B4 T cell line is essentially independent of CD4. In addition, these data indicate that anti-CD4 antibody blocking, which does inhibit stimulation of the 2B4 T cell line, is likely to be inhibiting activation by a mechanism other than disruption of the CD4/MHC class II interaction, as has been suggested previously (for review, see 1).

CHO cells expressing wild-type and mutant I-Ek molecules were also compared for their ability to activate freshly isolated 2B4 transgenic peripheral T cells. For these experiments, it was necessary to purify the T cells by removing the resident I-Ek-expressing APCs. In light of the moderate inhibition of T cell activation observed with the anti-CD4 Fab fragments (Fig. 3B), we might have expected to see some reduction in stimulation of the naive T cells with the 137 mutant. In three out of four experiments, though, CHO/137 cells were as capable as CHO/wt cells at inducing IL-2 production by the
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A I-E\textsuperscript{a} (14.4.4) I-E\textsubscript{p} (17.3.3) Aji

WT

137

48

48/137

Fig. 5. Surface I-E expression on CHO cells transfected with wild-type or mutant I-E\textsuperscript{k} genes. CHO fibroblasts were transfected with wild-type I-E\textsuperscript{k} \(\alpha\) and \(\beta\) chains, or wild-type I-E\textsuperscript{k} plus the indicated E\textsubscript{p} mutatns. (A) Lines expressing comparable levels of surface I-E\textsuperscript{k} were chosen, and are shown after surface staining with antibodies against I-E\textsubscript{a} or against I-E\textsubscript{p} (filled histograms) or in the absence of primary antibody (open histograms). Note the loss of the 17.3.3 epitope in mutant 48. (B) CHO cells expressing wild-type I-E\textsuperscript{k} or mutant 48 were stained with an additional I-E-specific Ab, Y17.

naive 2B4 T cells over a range of peptide concentrations (data not shown). In one experiment, the CHO/137 cells could only induce ~75% as much IL-2 production as CHO/wt cells. CHO/48 cells were more defective in this assay; in three out of four experiments, they were ~70% as effective as CHO/wt cells (data not shown). In the remaining experiment, no deficiency was apparent. Overall, the naive T cells seem minimally sensitive to these mutations. However, since we cannot conclusively rule out the possibility that these experiments were contaminated with small numbers of \textit{bona fide} I-E\textsuperscript{k}-positive APCs, it is difficult to quantitate the small inhibitory effect of these mutations on the naive T cells. Since our Fab fragment blocking data indicated that the naive cells are less CD4 dependent than the thymocytes, we then examined the effects of the I-E\textsuperscript{k} mutations in the thymocyte dulling assay, anticipating a greater impact.

MHC class II mutants reduced in their ability to interact with CD4 are less efficient at inducing dulling of CD4\textsuperscript{+}CD8\textsuperscript{+} thymocytes

We compared the ability of CHO/wt, CHO/137, CHO/48 and CHO/48-137 cells to induce dulling of 2B4 thymocytes, the most CD4-dependent assay. As can be seen in Fig. 7(A), CHO/137 cells were slightly less efficient than CHO/wt cells at inducing dulling of CD4\textsuperscript{+}CD8\textsuperscript{+} thymocytes over a range of MCC peptide concentrations. The magnitude of the difference between the CHO/wt and CHO/137 cells is small, but statistically significant (see Fig. 8A). CHO/48 cells were even further reduced in their ability to induce dulling of CD4\textsuperscript{+}CD8\textsuperscript{+} thymocytes (Fig. 7A and Fig. 8A and B). Interestingly, CHO cells expressing the double 48-137 mutant, while also defective in inducing dulling of 2B4 thymocytes compared with CHO/wt and CHO/137 cells, were not significantly worse than cells expressing the single 48 mutation (Fig. 7B). These results indicate that the 48 mutation causes a more severe defect in CD4-dependent stimulation than the 137 mutation. For both the 137 and 48 mutants, this deficiency is overcome at high peptide concentrations. These data are consistent with our anti-CD4 Fab blocking studies, which indicate, first, that the thymocyte dulling assay is the most dependent on CD4-MHC class II interactions, and, second, that the requirement for this interaction is overcome at high peptide concentrations.

Since the effects observed with these mutant I-E\textsuperscript{k} molecules were small in magnitude, statistical analyses were performed to determine the significance of the data. Each of the three mutants (137, 48 and 48-137) was compared to wild-type I-E\textsuperscript{k}, and to each other, at peptide concentrations which generate ~50% of maximal thymocyte dulling with the CHO/wt cells. Sets of data points, each of which represents percent dulling in an individual assay well, were compared in pairs, using the one-tailed t-test (28); for instance, 137 was compared to wild-type, 48 was compared to wild-type, 48 was compared to 137, etc. A summary of the data showing all experiments used for the analyses is shown in Fig. 8. As Fig. 8 demonstrates, the precise magnitude of the deficiencies...
Fig. 6. I-E^k mutants deficient in CD4 interaction are fully competent at stimulating the 2B4 hybridoma and the 2B4 T cell line. The 2B4 hybridoma (A) or the 2B4 T cell line (B) were stimulated with wild-type or mutant I-E^k-transfected CHO fibroblasts plus a range of concentrations of MCC peptide. Supernatants were harvested after 24 h and IL-2 concentration was assessed by titration on HT-2 indicator cells. In the absence of peptide, 2B4 hybridoma cells and the T cell line secreted <0.25 U/ml IL-2. In the absence of APCs and the presence of peptide, the T cell line also secreted <0.25 U/ml of IL-2, demonstrating that no stimulatory H-2^k APCs remained in this population. The graphs show the mean (±SD) concentration of IL-2 produced in duplicate wells. In some cases, error bars are so small that they are imperceptible. The data shown are representative of three experiments with similar results.

Fig. 7. Dulling of 2B4 CD4^+CD8^+ thymocytes is diminished by β2 or β2 mutations in I-E^k. Thymocytes from an H-2^k 2B4 TCR transgenic mouse were incubated in triplicate wells with wild-type or mutant I-E^k-transfected CHO fibroblasts plus a range of concentrations of MCC peptide. Panel (A) compares wild-type I-E^k, mutant 137 and mutant 48. Panel (B) shows a separate experiment comparing wild-type, mutant 48 and double mutant 48-137. Thymocytes were harvested after 24 h, stained with antibodies against CD4, CD8 and the 2B4α chain, and analyzed by flow cytometry. The mean (±SD) percent dulling, calculated as described in the legend to Fig. 3, is shown in the graphs. In some cases, error bars are so small that they are imperceptible. All results were confirmed with multiple independent clones of CHO transfectants expressing each I-E^k mutant (data not shown). The data shown are representative of at least five experiments with similar results. Some experiment-to-experiment variation was seen in the absolute concentration of MCC peptide inducing maximal dulling.

Discussion

We have shown that cells expressing the 2B4 TCR lose CD4 dependence as they differentiate; two methods of interference with CD4 function—antibody/Fab blocking and class II mutants—lead us to the same conclusion. The long-term 2B4 T cell line is relatively CD4 independent compared to naive 2B4 T cells, which are less dependent on CD4 than the 2B4 CD4^+CD8^+ thymocytes. Simultaneous analysis of co-receptor dependence of thymocytes, naive cells and effector cells has not been done for any other TCR nor has the co-receptor dependence of a peptide-specific thymocyte response for any TCR been studied in this manner. Additionally, it is
important to recognize that the course of this study has also led to the identification of residue β48 as a potential CD4-binding site.

Previous studies of co-receptor contributions during thymic deletion suggested that the degree of contribution of co-receptor in mature and immature cells expressing a particular TCR would be similar. For example, naive cells expressing the alloreactive 2C TCR can be CD8 independent and, correspondingly, 2C cells can be efficiently deleted on the appropriate MHC background even in the absence of CD8 contributions (2, 8, 59, 60). In contrast to the general CD8 independence of 2C, the H-Y TCR, which recognizes a male-specific peptide presented by Dp, is CD8 dependent in the periphery and in the thymus (8, 61, 62). Consistent with the CD8 dependence of the H-Y TCR is the observation that tolerance in male H-Y transgenic mice can be mediated by down-regulation of CD8 (63).

Given these precedents, and the CD4 independence of the 2B4 T cell line and hybridoma, it is surprising that 2B4 thymocytes are demonstrably CD4 dependent. However, in each of the other studies described above, the density of ligand [e.g. allospecific ligand or male (H-Y) ligand] in the thymus compared to that present on peripheral APCs is unknown. Also, the thymic APCs may express a different set of co-stimulatory molecules than the peripheral APCs. Due to these uncontrolled variables, it is difficult to assess co-receptor dependence in a quantitative way. An advantage of our experimental design is that all three cell types are presented with a range of peptide concentrations on identical APCs. Our experiments demonstrate that for each T cell population, ligand density does in fact contribute to the degree of CD4 dependence: for all three 2B4 cell populations analyzed, we observed a decreasing effect of anti-CD4 Fab fragment-mediated inhibition as the concentration of stimulatory peptide increased. Therefore, experiments conducted with controlled ligand densities and APCs might reveal that 2C and H-Y thymocytes are in fact more co-receptor dependent than their peripheral counterparts.

Our studies indicate that variables regulated during T cell development and differentiation have a critical influence on co-receptor dependence. One obvious characteristic which changes as a thymocyte becomes a peripheral T cell is the level of TCR expression CD4+CD8+ thymocytes have substantially lower surface TCR levels than mature T cells (see Fig. 1). Therefore, the increased requirement for CD4 on thymocytes may compensate for low avidity TCR interactions and the higher TCR levels on peripheral T cells may relieve some amount of this co-receptor dependence. However, while potentially playing an important role, TCR levels cannot explain all of our observations. The long-term 2B4 T cell line, which is the least CD4-dependent population analyzed, has lower levels of TCR than the naive 2B4 lymph node T cells (see Fig. 1).

Other changes in the overall avidity between the T cells and APCs may compensate for the low levels of TCR on the T cell line and play a role in its CD4 independence. For example, it is well known that pruned or activated T cells express higher levels of adhesion molecules than naive, resting T cells (64). However, it is unknown whether up-regulation of adhesion molecules would actually enhance avidity in our system, considering that their ligands may not be expressed by the CHO fibroblast cell line. It is important to note that the CD4 independence of the T cell line may be a consequence of repeated in vitro stimulation, rather than a reflection of its maturity. Ideally, it would be more appropriate to compare the naïve peripheral T cells to in vivo primed T cells. Unfortunately, in vivo priming of TCR transgenic T cells does not result in the normal consequences of priming, such

![Fig. 8. Summary of thymocyte dulling assays comparing wild-type and mutant I-Ek molecules. (A) Sets of data points, each of which represents percent dulling in an individual assay well, are compared for wild-type I-Ek and each I-Ek mutant, or wild-type I-Ek in the presence and absence of anti-CD4 Fab fragments. In (B), mutants are compared to each other. For each pair compared, data points at the peptide concentration inducing ~50% maximal dulling with wild-type I-Ek were chosen. In each experiment, thymocytes from one animal were used in duplicate or triplicate wells; therefore, each panel summarizes three or more experiments, each done on a different day with a different animal. Not all mutants were tested in every experiment, so some data points differ between comparisons. Horizontal bar represents mean value of all observations within a set. Each pair of data sets was analyzed with the one-tailed t-test (see Methods). In all sets of pairwise comparisons, except the comparison of 48 with 48-137 (data not shown), a statistically significant difference was found. The P values were as follows: wt versus 137, P < 0.01; wt versus 48, P < 0.01; wt versus 48-137, P < 0.01; wt versus Fab, P < 0.01; 137 versus 48, P = 0.01; 137 versus 48-137, P = 0.035; 48 versus 48-137, P = 0.28 (data not shown for the final pair).]
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Where it participates in T cell activation (69-71). In addition, pathways simultaneously (72). Finally, clonal deletion of mature T cells which require activation of both signaling protein kinase C activation separately (34,40), in contrast to \( + \)CD4 \( + \)CD8 thymocytes respond to calcium signaling and TCR may differ in these three populations. For example, the T cell maturation. There is a significant amount of evidence of human MHC class II DR1 complexed with hemagglutinin peptide (HA 306-318, shown as ball and stick in yellow) (88) Residue 48 is shown in blue, and residues 137 and 138 are shown in red. The figure was generated using GRASP (89).

Fig. 9. Molecular surface representation of MHC class II indicating the proposed sites of CD4 interaction in the \( \beta_1 \) and \( \beta_2 \) domains. The surface was calculated from the three-dimensional crystal structure of human MHC class II DR1 complexed with hemagglutinin peptide (HA 306-318, shown as ball and stick in yellow) (88) Residue 48 is shown in blue, and residues 137 and 138 are shown in red. The figure was generated using GRASP (89).

as dramatic expansion of activated antigen-specific cells, and therefore is not an advantageous approach (65).

Although alterations in avidity may explain some of our results, developmentally regulated changes in signal transduction pathways within T cells may also account for the decrease in CD4 dependence observed over the course of T cell maturation. There is a significant amount of evidence indicating that the biochemical pathways downstream of the TCR may differ in these three populations. For example, the co-receptor associated signaling protein, \( \epsilon \)k, is known to have a different function early in thymic development, mediating the CD4\(^+\)CD8\(^+\) to CD4\(^+\)CD8\(^-\) transition and ensuring TCR \( \beta \) chain allelic exclusion (66-68), than in mature T cells, where it participates in T cell activation (69-71). In addition, CD4\(^+\)CD8\(^-\) thymocytes respond to calcium signaling and protein kinase C activation separately (34,40), in contrast to mature T cells which require activation of both signaling pathways simultaneously (72). Finally, clonal deletion of CD4\(^+\)CD8\(^+\) thymocytes is independent of calcineurin (73,74), unlike mature T cell activation (75). Furthermore, a number of studies have documented alterations in the activation requirements of primed, as compared with naive, T cells. For instance, unlike naive T cells, primed cells are no longer co-stimulation dependent (10,11). Also, more specific differences in CD4 signaling have been observed between naive and memory CD4\(^+\) T cells, correlating with a change in surface expression of the isoforms of the tyrosine phosphatase, CD45 (76). Further studies will be required to determine which developmentally regulated extracellular or intracellular factors contribute to the changing role of CD4 documented here.

It is interesting to note that the differences in CD4 dependence between these three populations of T cells are reinforced by experiments not involving antibodies or antibody fragments which may induce undesired conformational changes or signals. We have shown that a mutation in the \( \beta_2 \) domain of I-E\(^\text{k}\), corresponding to the previously reported CD4-binding site on I-A\(^\text{d}\) (52) and human DR4 (53), has an effect on stimulation of CD4\(^+\)CD8\(^-\) thymocytes, but not on mature T cells. However, the magnitude of the deficiency of our \( \beta_2 \) mutation in our thymocyte dunning assay appears to be quite small compared to the effects of an E137A I-A\(^\text{d}\) mutation in different functional assays (52). Several studies have suggested that I-E may have a weaker overall interaction with CD4 than I-A (52,77,78); this may explain the discrepancy between our results. For example, residues Glu 137, Val 140 and Val 142 of I-A\(^\text{d}\) were found to be involved in interactions with CD4 (52). In these experiments, a 137-142 double mutation was found to be no more defective than a 137 mutant in interactions with mouse CD4; however, a mutation in 140 was only tested independently and was not as striking as the 137 mutant. Of these three residues, only Glu 137 is conserved between I-A\(^\text{d}\) and I-E\(^\text{k}\). I-E\(^\text{k}\) contains residues Thr 140 and Ile 142. Therefore, a wild-type I-E molecule is already somewhat 'mutated' in the essential CD4-binding residues of this loop; in this context, the impact of mutating residue 137 may be less striking. In an effort to find a more defective mutation in this \( \beta_2 \) loop, we have compared a double mutant in residues 137 and 140 with the 137 mutant described here. We find that the 137-140 mutant is no more defective than the 137 mutant (data not shown).

Our studies have also identified a novel site in the \( \beta_1 \) domain of I-E\(^\text{k}\). Our mutation in residue 48 does not inhibit the activation of a CD4\(^+\) T cell hybridoma, nor is it deficient in stimulating the relatively CD4 independent 2B4 T cell line. In contrast, this mutant I-E\(^\text{k}\) molecule is substantially reduced in its ability to stimulate the CD4 dependent CD4\(^+\)CD8\(^-\) thymocytes. In addition, dose-response curves with CHO/48 cells show similar behavior to the anti-CD4 Fab fragment blocking data, indicating a stronger deficiency at low peptide concentrations and wild-type behavior at high peptide concentrations. Based on these functional data, it is likely that this region of the \( \beta_1 \) domain directly binds CD4. This possibility is supported by a number of experiments demonstrating inhibition of CD4 dependent T cell activation with MHC class II-derived peptides spanning this region (79-82). Ideally, physical binding assays would directly demonstrate that residue 48 binds to CD4. Unfortunately, these assays are not technically feasible due to the low affinity of murine CD4 for
murine MHC class II molecules (83). Without direct CD4 binding evidence, we cannot rule out the possibility that the 48 mutation in the β1 domain of I-Ek has a more direct effect on CD4 dependent T cell stimulation, such as interfering with MHC class II or class II–CD4 oligomerization. In addition, it is possible that residues other than 48 in this loop are responsible for the direct interactions with residues on CD4. Evidence from the X-ray crystal structure of human DR1 indicates that residues 46 and 48 in the β1 domain can form a salt bridge (57). Consequently, mutation of residue 48 would be predicted to disrupt this interaction, potentially altering the conformation of the entire loop.

Interestingly, our data indicate that the mutation we tested in the β1 domain site is more detrimental than the mutation we tested in the β2 domain. It is difficult to conclude that the β1 site is actually more critical for CD4 interaction without comparing the worst possible mutation in each region. For instance, our 137 mutation may not represent the maximum possible damage to this loop. However, our observation that the double β1/β2 mutant is not deceptively more defective than the single β1 mutant is consistent with the idea that disruption of the β1 site induces the maximal loss of CD4 interaction with this face of MHC class II. As can be seen in Fig 9, both the β1 and β2 sites are solvent-accessible and could simultaneously bind the same CD4 molecule. CD4 mutagenesis studies also support the conclusion of multiple binding sites on different regions of MHC class II (46–49). For instance, mutations in both domains 1 and 2 of CD4 have been shown to diminish binding to MHC class II, consistent with complementary binding sites in both the membrane-proximal and membrane-distal domains of MHC class II. A similar model of binding has recently been proposed for the CD8–MHC class I interaction (84,85).

None of our β chain mutations have an effect equivalent to blocking with anti-CD4 Fab fragments. This observation suggests that MHC class II molecules may contain additional sites of CD4 interaction. Again, this possibility is supported by CD4 mutagenesis data, which indicate binding sites for MHC class II on opposite faces of the CD4 molecule (46–49). An interesting possibility is that CD4–class II oligomers may form, involving multiple faces of both partners. The formation of oligomers which group multiple CD4 and class II molecules together has also been suggested by data indicating CD4 interaction sites in additional regions of the α2 and β2 domains of MHC class II (86,87), as well as the class II dimer of dimers observed in the class II X-ray crystal structure (57).

These data provide some resolution to the conundrum posed by the discrepancy between the CD4+ 2B4 hybridoma and the CD4 developmental bias observed in the 2B4 TCR transgenic mice. We find that the role of CD4–MHC class II interactions is substantially more important for immature thymocytes expressing the 2B4 TCR than for mature T cells. Further investigations will reveal whether this is also true for other TCRs; it will be particularly interesting to compare other TCRs of known affinity as this information becomes available. These data further suggest a mechanism for the enhanced sensitivity of clonal deletion in the thymus relative to peripheral T cell activation. It is well documented that a lower quantity or quality of antigen which is insufficient to activate peripheral T cells can be sufficient for thymic deletion (19,20). Paradoxically, in spite of their low levels of TCR, thymocytes are exquisitely sensitive to low concentrations of antigen. Perhaps the tendency of thymocytes to involve increased co-receptor participation in TCR signaling relative to peripheral cells, as observed here, accounts for this increased level of antigen detection. A remaining puzzle is the role of CD4 in CD4 lineage commitment during the maturation of 2B4 CD4+CD8- thymocytes. An analysis of positive selection in transgenic mice expressing the CD4 binding-deficient I-Ek mutants is underway to address this complex issue.

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Abbreviations

APC antigen-presenting cell
MCC moth cytochrome c
PCC pigeon cytochrome c
PE phycoerythrin
PHA phytohemagglutinin

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