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CD4 function in thymocyte differentiation and T cell activation

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SUMMARY

The ectodomains of the T cell surface glycoproteins CD4 and CD8 bind to membrane-proximal domains of MHC class II and class I molecules, respectively, while both cytoplasmic domains interact with the protein tyrosine kinase (PTK) $p56^{lck}$ (lck) through a shared cysteine-containing motif. Function of CD4 and CD8 requires their binding to the same MHC molecule as that recognized by the T cell antigen receptor (TCR). *In vitro* studies indicate that CD4-associated lck functions even in the absence of kinase activity. *In vivo* experiments show that, whereas helper T cell development is impaired in CD4-deficient mice, high level expression of a transgenic CD4 that cannot bind lck rescues development of this T cell subset. These studies suggest that CD4 is an adhesion molecule whose localization is regulated through protein–protein interactions of the associated PTK and whose function is to increase the stability of the TCR signalling complex by binding to the relevant MHC.

The function of CD4 in development has been further studied in the context of how double positive ($CD4^+CD8^+$) thymocytes mature into either $CD4^+$ T cells with helper function and TCR specificity for class II or into $CD8^+$ T cells with cytotoxic function and specificity for class I. Studies using CD4-transgenic mice indicate that development of single positive T cells involves stochastic downregulation of either CD4 or CD8, coupled to activation of a cytotoxic or helper program, respectively, and subsequent selection based on the ability of the TCR and remaining coreceptor to engage the same MHC molecule.

1. INTRODUCTION

The CD4 molecule is a critical component in the activation of multiple signalling pathways during T cell development and activation (Janeway 1992; Julius *et al.* 1993). This cell surface glycoprotein binds to a membrane-proximal domain of MHC class II molecules on antigen presenting cells (König *et al.* 1992), thus facilitating signal transduction via the T cell antigen receptor (TCR). The cytoplasmic domain of CD4 interacts with the cytoplasmic tyrosine kinase lck, a member of the src family of protein tyrosine kinases (reviewed in Xu & Littman 1993). These two different interactions, occurring at the cell surface and on the cytoplasmic face of the plasma membrane, are key elements in the establishment of a repertoire of helper T lymphocytes as well as in the activation of mature T helper cells. Modulation of these interactions is likely to play a major role in determining whether a helper T cell initiates an autoimmune response or remains non-reactive against self-antigen. In addition, the human CD4 molecule serves as the receptor for human immunodeficiency viruses (HIVs) and is likely to be involved in cell death-inducing signals resulting from virus–host interactions (Sattentau & Weiss 1988; Gougeon & Montagnier 1993). CD4 thus plays a major role in host immune responses and in disease, and a full understanding of its role in signalling is likely to contribute significantly to combatting the loss of T helper function in AIDS as well as the hyper-reactivity of these same cells in transplantation rejection and autoimmunity.

The ectodomain of the CD4 glycoprotein, which binds to a segment of the non-polymorphic β2 domain of MHC class II molecules, consists of four distinct domains that are members of the immunoglobulin superfamily. Only the N-terminal domain can be easily assigned to this family, and it was through the insight of the Oxford group led by Alan Williams that the Ig-like structure of the other domains was correctly predicted (Clark *et al.* 1987; Brady *et al.* 1993). The sequence of the cytoplasmic segment of CD4 is highly conserved through evolution, and contains a short motif involved in interaction with the N-terminal domain of lck (Shaw *et al.* 1990; Turner *et al.* 1990). This motif, defined by two closely-spaced

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cysteine residues, is present in the cytoplasmic tails of both CD4 and CD8, and allows both of these T cell surface molecules to interact with the cytoplasmic PTK.

Based on studies from numerous laboratories during the past decade, a number of models have been postulated for the functions of CD4 and CD8. Early experiments suggested that these molecules serve primarily to increase the strength of adhesion between T cells and antigen presenting cells (APCs) or target cells, especially in those cases in which the T cell receptor has a relatively low affinity for the antigen/MHC complex (Marrack *et al.* 1983; Doyle & Strominger 1987). The discovery that CD4 and the TCR complex could be co-modulated at the cell surface gave rise to the 'coreceptor' model, which suggested that CD4 and CD8 associate with the TCR complex and recognize the same MHC molecule as that bound by the TCR (Janeway 1989). There is now extensive evidence that CD8 and the TCR must bind to the same MHC class I molecule for effective signal transduction in development of class I-reactive T cells and in activation of cytotoxic T cells (Wallace *et al.* 1993). Since CD4 binds to a region of class II that is analogous to the region of class I recognized by CD8, it is likely that CD4 can also bind to an MHC molecule that is simultaneously recognized by a TCR (Parham 1992). The coreceptor model was strengthened by the finding of lck association with both CD4 and CD8, suggesting that bridging of the TCR complex with CD4/8 recruits this PTK into the complex and contributes to the transduction of activating signals (Julius *et al.* 1993). A somewhat perplexing twist was introduced by the finding that treatment of cytotoxic T cell clones with anti-TCR antibodies increased the avidity of CD8 on these cells for immobilized MHC class I molecules; this result, which was independent of the MHC specificity of the TCR, suggested that a signal from the TCR can influence the activity of CD8 (O'Rourke & Mescher 1993).

In this review, we describe recent results from our laboratory which suggest that CD4 functions primarily as an enhancer of the avidity of the TCR–MHC interaction. Our results suggest that the activity of CD4 is, at least in part, regulated through the associated lck, and thus may relate to the findings of O'Rourke & Mescher with CD8. These and other results that will be discussed below provide strong evidence that CD4 controls the decision of immature thymocytes to become $CD4^+$ helper cells by facilitating the positive selection of precommitted cells rather than by transducing signals that influence commitment to the helper cell pathway.

2. FUNCTION OF CD4 IN T CELL DEVELOPMENT

Eighty to ninety percent of thymocytes coordinately express both CD4 and CD8 on their surface. These cells are developmental intermediates between precursor cells that are double negative ($CD4^-CD8^-$) and mature single positive cells which express either CD4 or CD8, but not both. The pathway that leads to maturity is narrow and selects only for those cells which correctly pair expression of class I-restricted TCRs with retention of CD8 (i.e. loss of CD4) or expression of class II-restricted TCRs with retention of CD4 (loss of CD8). To provide genetic proof for the involvement of CD4 in T cell development, we have used gene targeting technology to generate a mutant mouse strain which lacks expression of the CD4 molecule (Killeen *et al.* 1993). The mutation was achieved by insertion of a neomycin resistance gene into the fifth exon of CD4, thus disrupting the protein coding sequence just after the amino-terminal IgV-like domain. T lymphocytes from mice homozygous for this mutation had no cell surface staining with anti-CD4 antibodies and no detectable CD4 protein on immunoprecipitation analysis.

Absence of CD4 expression had dramatic consequences on the constitution of the mature T cell repertoire (see below; compare $+/+$ and $-/-$ control panels in figure 4*b*). Most strikingly, there was a large drop in the representation of those T cells which would normally express CD4, i.e. the $CD8^-$ helper cell lineage. In a normal mouse, typically 60–70% of T cells in lymph nodes express CD4 and 30–40% express CD8. Without CD4, the total number of T cells was within the normal range, but 90% of the cells were $CD8^+$. The reduction in CD4 lineage cells in CD4-null mice was accompanied by a decrease in helper T cell activity, such as the ability to generate antibodies following immunization with a T cell-dependent antigen or to mediate class II-restricted mixed lymphocyte responses (Killeen *et al.* 1993). Thus, a simple analysis of mature T cells in the mutant mice indicates that the development of the CD4 helper lineage is significantly compromised in the absence of the CD4 glycoprotein.

These results suggest that CD4 function is required for thymic selection events and, in particular, for the positive selection and development of helper cells. None the less, $\sim$10% of mature $\alpha\beta$ T cells in the mutant mice were $CD8^-$ and some of these had normal helper cell functions (Locksley *et al.* 1993). Therefore, while the function of CD4 is to facilitate positive selection of CD4 lineage cells, its requirement is apparently not absolute. The nature of the helper cells that can differentiate in the absence of CD4 is not yet understood; it is not clear if these cells constitute a separate thymocyte lineage, if they simply have higher affinity T cell receptors, or if they have molecules other than CD4 that can compensate to take over the function of this glycoprotein.

We have also studied the potential role of the CD4 molecule in signal transduction resulting in negative selection by examining superantigen-mediated deletion of T cells in the mutant mice. Superantigens preferentially stimulate $CD4^+$ T cells, are presented by MHC class II molecules, and cause the deletion of immature thymocytes bearing specific Vβ chains within their TCR. Interestingly, both $CD4^+$ and $CD8^+$ mature cells are deleted in animals that express particular superantigens encoded by endogenous

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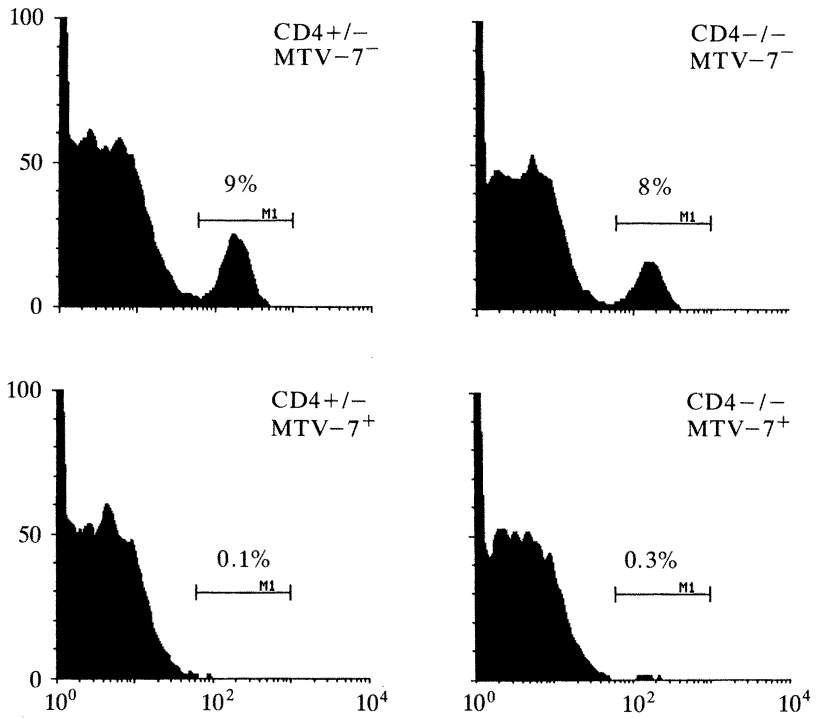

Figure 1. MTV-7-mediated deletion of $V_\beta6^+$ T cells in the absence of CD4. Histograms show $V_\beta6$ expression on T cells from the lymph nodes of mice of the indicated CD4 and MTV-7 genotypes. The MTV-7 retroviral integrant was introduced from the DBA/2 background and was detected by Southern blot using an MMTV LTR probe and *Pvu II*-digested tail DNA. Mice were typed for H-2^d expression by FACS. Cells were analysed using a Becton Dickinson FACScan and Lysys II software.

retroviruses (MTVs); since loss of $CD8^+$ cells is inhibited with anti-CD4 antibody (Fowlkes *et al.* 1988, MacDonald *et al.* 1988), the CD4 molecule has been thought to be required for the apoptotic signal occurring in double positive cells. Paradoxically, we find that $CD8^+$ cells which develop in CD4-deficient mice remain sensitive to superantigen-mediated deletion. Thus, CD4−/− mice delete $CD8^+$ T cells bearing Vβ-3,5,6, and 8 in the appropriate situations where MTV-6,7,9 or 13 are expressed (see for example figure 1) or following neonatal injection of staphylococcal enterotoxin B. In a similar study, MTV-7-mediated deletion of $CD8^+$ T cells expressing Vβ7 did not occur in the absence of CD4 (Wallace *et al.* 1992). Thus, the function of CD4 in superantigen-mediated deletion is largely dispensable, but may be significant for some TCR variable regions. In general, interactions between superantigens and TCR variable regions may be of sufficiently high affinity to obviate the contribution of CD4, and in this sense they may be poor models for class II-restricted peptide antigens. Other antigenic systems may therefore be more appropriate for studying CD4 function in negative selection.

3. FUNCTION OF CD4 IN TCR-MEDIATED SIGNAL TRANSDUCTION

We have studied CD4 function in a reconstituted T cell hybridoma, 171.3, that is dependent on CD4 for antigen-specific activation. This cell, which lacks expression of endogenous TCR-α and TCR-β chains, as well as of CD4 and CD8, has been reconstituted with genes encoding a TCR that recognizes a peptide of hen egg lysozyme (HEL) in the context of the class II molecule $I\text{-}A^b$. Secretion of IL-2 upon addition of peptide in the presence of antigen presenting cells requires that the hybridoma expresses CD4. Moreover, only CD4 molecules that can bind lck are effective in this system. Thus, mutations of the cysteines involved in the binding of the cytoplasmic PTK abrogate the response (Glaichenhaus *et al.* 1991).

To study the role of the CD4-associated lck molecule, we expressed chimeric proteins containing the CD4 extracellular and transmembrane domains linked directly to cytoplasmic PTKs of the src family (Xu & Littman 1993). Such chimeric molecules, whether they contained $p56^{lck}$, $p60^{c\text{-}src}$, or $p59^{fyn\text{-}T}$, functioned well in this assay. A chimeric molecule containing $p62^{hck}$, though having high tyrosine kinase activity, was relatively inefficient in this system, providing the first hint that kinase activity may not be the primary function of CD4-associated lck.

A structure–function analysis of the chimeric proteins was then initiated, and the results are depicted schematically in figure 2. Most surprisingly, point mutations of lysine-227 within the ATP binding site to either alanine or arginine, which resulted in complete abolition of phosphotransferase activity, had only a minor effect on the antigen-response curve. Whereas a mutation in the Lck-binding region of CD4 reduced the response by more than 1000-fold, mutations that destroyed kinase activity in the chimera decreased responses only about three- to fivefold. Even more

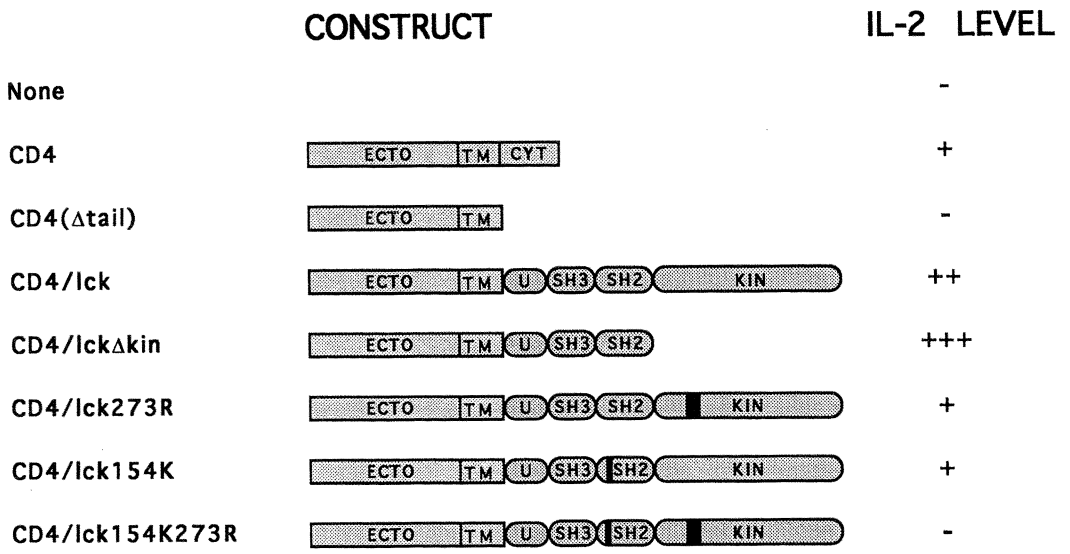

Figure 2. Activity of CD4/lck chimeric proteins in the antigen specific T cell hybridoma. The various constructs were introduced into 171.3 cells by retroviral mediated gene transfer, and cells expressing equivalent levels of cell surface CD4 were sorted and tested for their response to HEL peptide in the presence of the appropriate antigen presenting cells. Segments of CD4 are indicated by rectangles, and consist (from left to right) of the extracellular, transmembrane, and cytoplasmic domains; oval segments represent regions of the linked lck molecule. Mutations of lck residues 154 and 273 (marked by black inserts) inactivate the SH2 and kinase domains, respectively. Relative levels of IL-2 production are indicated.

strikingly, deletion of the entire kinase domain in the chimeric protein resulted in hyper-responsiveness to antigen. These results suggested that the kinase domain of CD4-associated lck regulates the ability of other regions of the molecule to participate in protein–protein interactions. As in all src family PTKs, the kinase domain of lck is preceded by a unique myristylated N-terminal segment, an SH3 domain, and an SH2 domain. It has been proposed that tyrosine-505 of lck, when phosphorylated, participates in an intramolecular interaction with the SH2 domain, thus essentially keeping the molecule in an 'off' configuration; upon dephosphorylation mediated by the protein tyrosine phosphatase (PTPase) CD45, the molecule may open up, thus freeing up both the kinase domain and the SH2 site within lck (reviewed in Xu & Littman 1993). Our results are consistent with this model. Thus, when the kinase domain is present but is defective in enzymic activity, the accessibility of other regions of the molecule continues to be regulated, but there is a slightly diminished response due to the loss of the kinase activity. The increased response upon complete removal of the kinase domain is consistent with constitutive access to domains within the rest of the protein. Further support for this interpretation comes from the finding that a point mutation within the chimeric protein's SH2 domain (arginine 154 changed to lysine, destroying P-tyr binding activity) decreased response to antigen about fivefold relative to the wild-type chimera. Moreover, when this mutation was coupled to the point mutation in the kinase domain, there was virtual elimination of the activity of the CD4 chimeric protein.

Based on these results, we propose that a major function of CD4-associated lck is to regulate the location of CD4 within the plasma membrane, as shown in the model in figure 3. CD4 is likely to be present at a concentration that is too low to permit substantial contacts with the few MHC class II molecules that are simultaneously recognized by the TCR. To ensure that CD4 'finds' the relevant class II molecules, the Lck molecule helps guide it to a partially activated TCR complex, where it can stabilize the TCR–MHC interaction and thus permit signalling to proceed. The model can be divided into the following sequential steps: (i) contact of the TCR with the peptide/class II complex results in a weak activation of a receptor-associated PTK(s) (candidates for these include ZAP-70 and fyn-T as well as lck); (ii) the activated PTK(s) phosphorylate tyrosines within the TCR complex (such as those on the CD3 chains or on ζ chain); (iii) the SH2 domain of CD4-associated lck binds to the P-tyr within the partially activated TCR complex; (iv) the CD4 molecule now contacts the 'correct' MHC class II molecule, thus stabilizing the complex by lowering the effective off-rate of the TCR–MHC interaction; (v) a strong signal is transmitted through tyrosine phosphorylation of appropriate substrates by the TCR-associated PTKs (which may include, if necessary, the CD4-bound lck). This model requires that the SH2 domain of CD4-associated lck be accessible; this may involve the function of CD45, as discussed above.

There are a number of previous observations that support this model. For example, following cross-linking of the TCR complex with antibodies, the CD4 molecules redistribute adjacent to the TCR. This migration is dependent on interaction of CD4 with lck (Collins *et al.* 1992). As previously mentioned, in cytotoxic T cells, the avidity of CD8 for MHC class I is increased upon antibody crosslinking of the TCR, suggesting that there is a change in CD8 conformation

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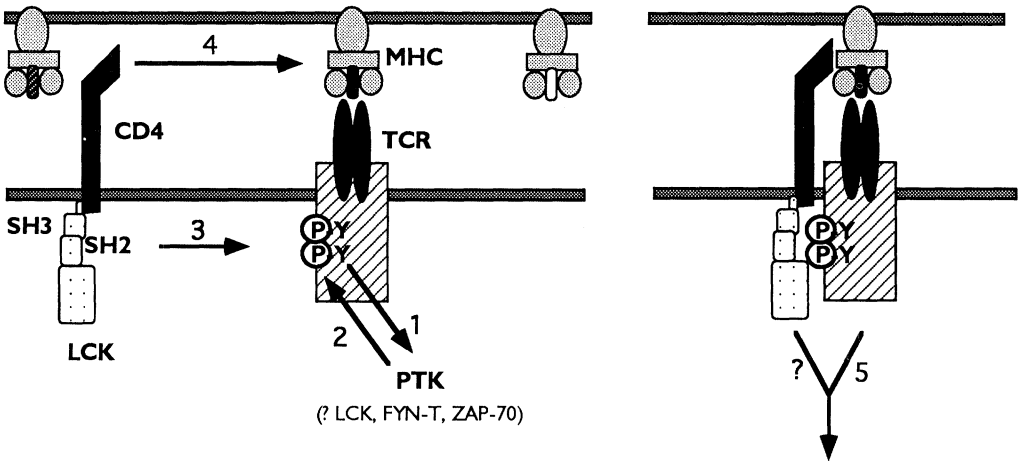

Figure 3. Proposed function of CD4 in the stabilization of the TCR signalling complex.

or its state of oligomerization secondary to a primary signal from the TCR. This model is also supported by several recent findings of molecules consisting only of SH2 or SH3 domains, such as Sem5/GRB2, nck, and crk, that are likely to function as adaptors for protein-protein interactions (McCormick 1993). It is tempting to speculate that lck has several functions; one would be to participate in kinase-dependent signalling when bound to the TCR complex, as suggested by the finding that a cell line lacking lck cannot be activated with anti-TCR antibodies (Straus & Weiss 1992); the second role would be similar to that postulated for adaptor molecules, regulating the migration of the CD4 or CD8 co-receptors into the TCR complex.

The nature of the weak activation signal (step 1) early in signal transduction remains a matter of conjecture. The inability to activate lck-deficient T cells is due to an early block in the PTK pathway (Straus & Weiss 1992). In addition, we have found that crosslinking of the CD4/lck chimera to a CD8/ζ chimeric molecule in the 171.3 hybridoma results in phosphorylation of tyrosines in CD8/ζ and in the association of a 70 kDa phosphoprotein, most likely ZAP-70, with CD8/ζ. Moreover, the CD4/lck chimera can phosphorylate CD8/ζ in transiently transfected COS cells and in *in vitro* kinase assays (K. Chu & D. R. Littman, unpublished results). Taken together, these results suggest that an early event in T cell activation is the triggering of TCR-associated lck (or another src family member), which phosphorylates TCR-associated CD3 chains (including ζ chain). The availability of phosphorylated tyrosines on these chains is then likely to result in recruitment of ZAP-70 via its SH2 domains and in the subsequent phosphorylation of tyrosines that interact with the SH2 domain of lck. Thus, a kinase-competent form of lck is likely to be required in step 1 of the activation pathway, but a kinase-defective lck can still participate in CD4 function.

Our model predicts that, since the CD4 interaction with the 'correct' MHC molecule is limiting, it should be possible to circumvent the need for a CD4–lck interaction by increasing the surface concentration of CD4. This prediction has been satisfied in studies on T cell development, in which the importance of the CD4–lck interaction was examined *in vivo* by using CD4-deficient mice as recipients for CD4 transgenes (figure 4*a*). In one series of experiments, CD4 deficient mice were bred to a previously described mouse harbouring minigenes encoding wild-type or cytoplasmic domain-deleted CD4 under transcriptional regulation of the *lck* proximal promoter. The ability of the transgene-encoded CD4 to substitute for endogenous CD4 in lineage development was then evaluated by flow cytometry. As shown in figure 4*b*, expression of transgene-derived wild-type CD4 restored the proportions of T cell subsets to normal levels, compared to the markedly impaired CD4 lineage representation in non-transgenic littermates. Interestingly, tailless CD4 was also capable of mediating a partial restoration of lineage development back to approximately one half of the wild-type complement. Accompanying lineage repopulation, there was also a correction of the helper cell defects (T-dependent antibody responses and class II-specific alloreactivity) which characterize non-transgenic CD4-deficient mice (Killeen & Littman 1993).

In a more extensive analysis of multiple transgenic lines expressing tailless CD4 under transcriptional control of the CD3δ promoter, we have found a direct correlation between level of transgene expression and extent of lineage rescue (Killeen & Littman 1993). The demonstration of adequate replacement of wild-type CD4 by a tailless variant argues against an absolute requirement for CD4–lck association in T cell development. Rather, this observation is consistent with the above-proposed function for CD4 which does not necessarily involve direct signal transduction through the associated PTK. The high concentration of CD4 on the surface of developing thymocytes thus appears to increase the probability that CD4 will interact with the relevant MHC class II molecule, permitting signal transduction through the TCR complex and positive selection (figure 5).

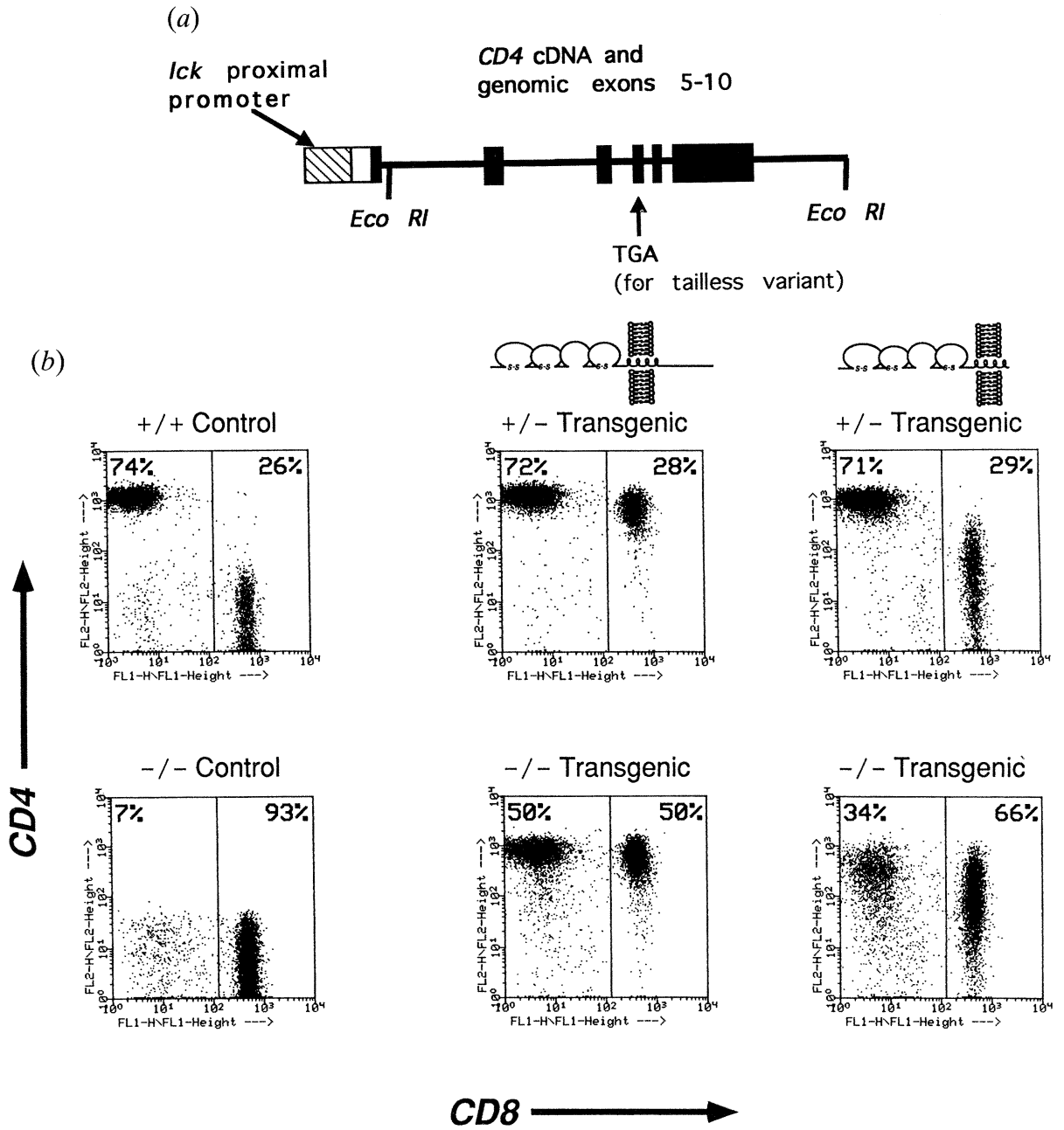

Figure 4. (*a*) Structure of constructs for expressing CD4 in transgenic mice. Minigenes encoding wild-type or cytoplasmic domain-deleted CD4 molecules were fused to the enhancer and promoter elements of the mouse *lck* gene. (*b*) Rescue of the CD4 lineage by wild-type and tailless transgene-encoded CD4 molecules. Lymph node cells from mice of the indicated genotypes were stained with anti-CD4, anti-CD8 and anti-CD3ε. Dot plots were generated from files of 10 000 events by gating for expression of CD3.

4. MECHANISM OF THYMOCYTE LINEAGE COMMITMENT AND THE ROLE OF CD4 IN REPERTOIRE SELECTION

The mechanism involved in the differentiation of double positive ($CD4^+CD8^+$) thymocytes into single positive mature cells has recently been a subject of considerable interest. It has been proposed that instructional signals, potentially involving CD4 and CD8 as well as T cell receptors, result in commitment to either the CD4 or CD8 lineage (discussed in Janeway 1993). The appearance of some CD4-lineage helper cells in the CD4-null mice (Locksley *et al.* 1993) as well as the ability of tailless CD4 to rescue development of this T cell lineage argue strongly that the choice of developmental pathway does not require instructional signals dependent on co-recognition of MHC class II by CD4 and TCR. These results favor a second model, in which there is stochastic down-regulation of CD4 or CD8 and subsequent selection of cells bearing the appropriate TCR-coreceptor combination. Therefore, a signal for selection of $CD4^+$ cells would only be possible if these cells express a class II-restricted TCR, permitting co-engagement of CD4 and TCR on the MHC molecule.

To further distinguish between the instructional

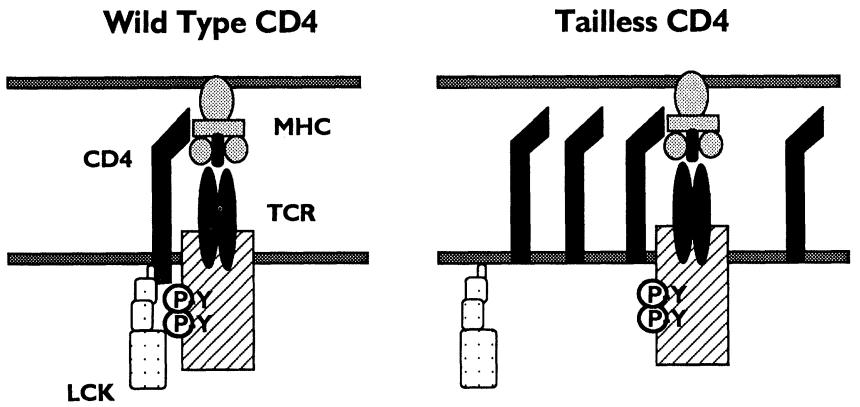

Figure 5. Proposed molecular mechanism in rescue of helper T cell development by tailless CD4 molecules.

and stochastic models, we analyzed development of class II-restricted thymocytes in mice that express a CD4 transgene in all T cell lineages. If CD4 is involved in an instructional signal, all cells bearing a class II-specific TCR should shut off CD8 and commit to the CD4 lineage. However, if single positive cells arise stochastically independent of TCR specificity, $CD8^+$ cells with class II-specific TCRs should be detected due to their rescue in the presence of transgenic CD4 (figure 6).

To specifically examine the development of class II-restricted thymocytes, experiments were carried out in mice whose T cells were heavily skewed for class II specificity (Davis *et al.* 1993). These included mice lacking class I-restricted cells due to a targeted disruption of the β_2-microglobulin (β_2m) locus and

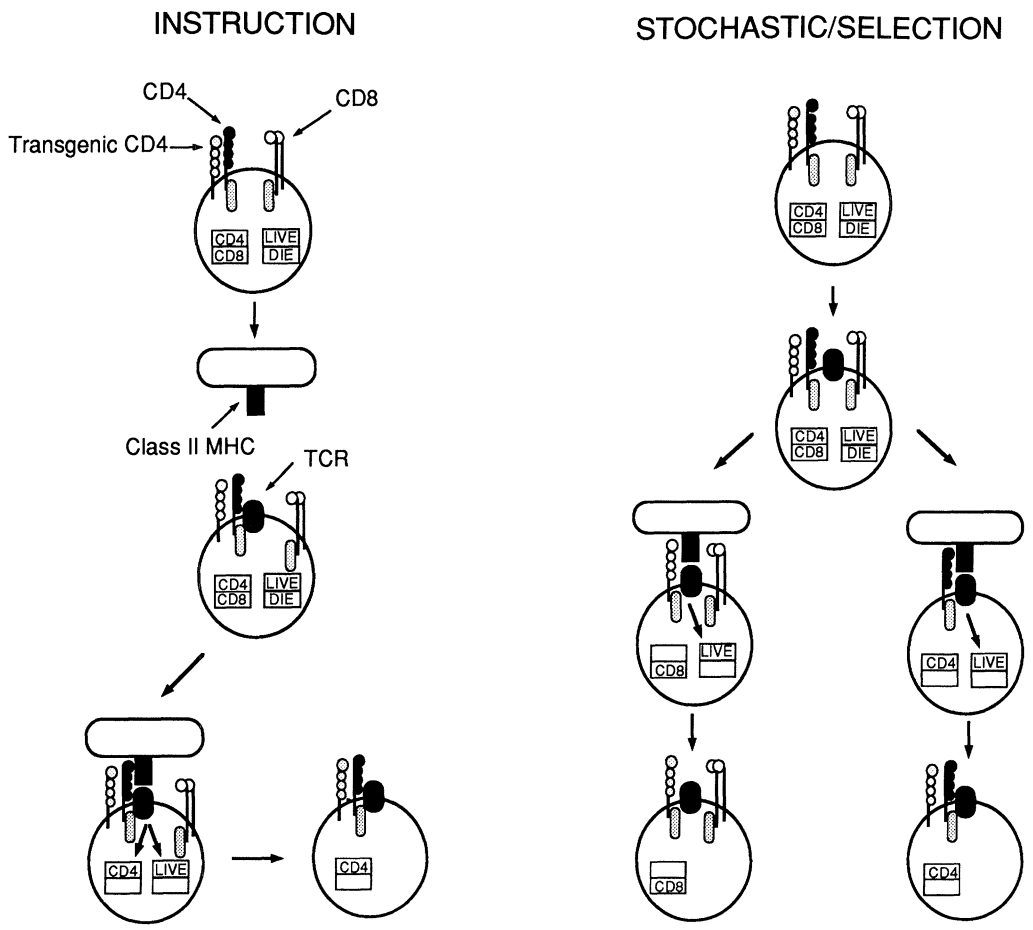

Figure 6. The instruction and stochastic/selection models of development predict different outcomes for development of MHC class II-reactive thymocytes in CD4 transgenic mice. The instruction model predicts that class II-reactive thymocytes will always commit to the CD4 lineage and shut off CD8. The stochastic/selection model predicts that some class II-reactive thymocytes will commit to the CD8 lineage. Ordinarily these $CD8^+$ cells die in the thymus because coengagement of MHC by coreceptor and TCR does not occur, but they can be rescued by expression of transgenic CD4.

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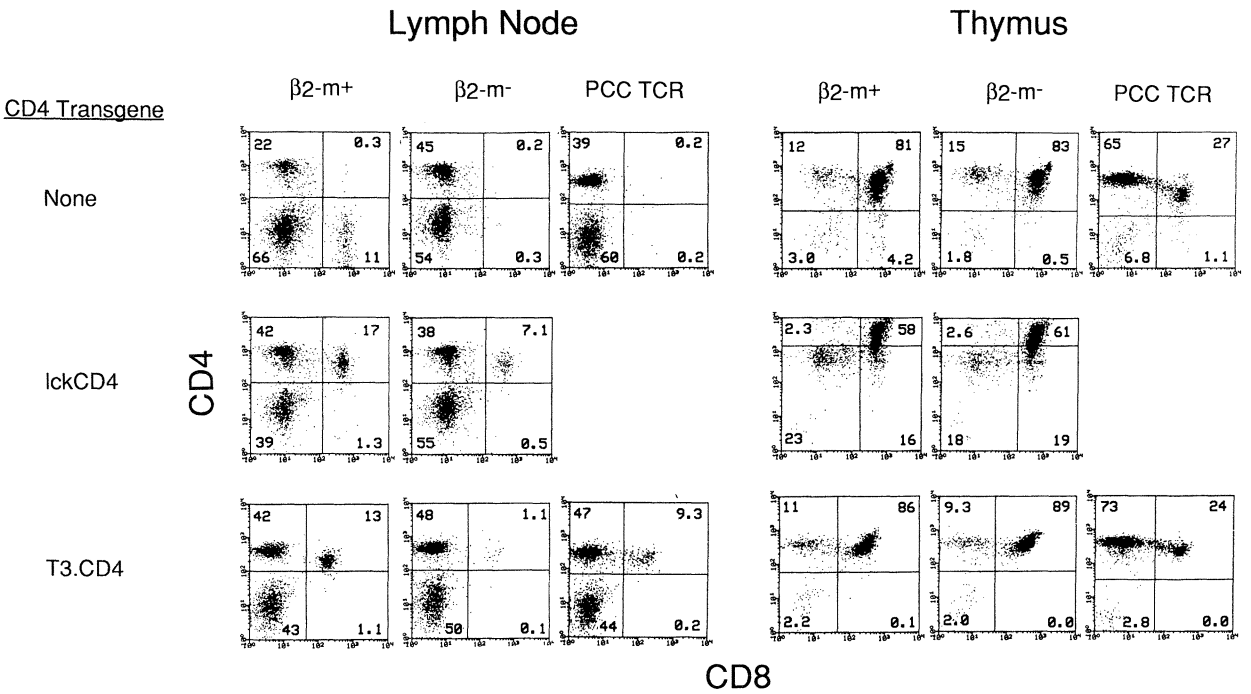

Figure 7. CD4 and CD8 expression on thymocytes and lymph node cells from CD4 transgenic mice. The percentage of cells in each compartment is indicated. β_2m, β_2-microglobulin; PCC, pigeon cytochrome c.

mice expressing a transgenic class II-restricted pigeon cytochrome c (PCC)-specific TCR. Both lines of mice have mature $CD4^+$ T cells, but essentially no $CD8^+$ cells. These mice were mated to CD4 transgenic mice in which expression of a CD4 minigene was directed either by the proximal promoter of the murine lck gene (lckCD4 mice) or by the human CD3δ promoter (T3.CD4 mice; Davis *et al.* 1993). The lckCD4 transgene was expressed at 10- to 15-fold normal levels in immature thymocytes, but dropped to normal level expression in mature thymocytes and T cells. In contrast, the T3.CD4 transgene was expressed at levels similar to endogenous CD4 throughout development.

Expression of the CD4 transgenes resulted in the appearance of $CD8^+$ T cells in both the β_2m-null and PCC-TCR mice (figure 7). There was rescue of mature $CD8^+$ thymocytes, having low HSA expression, high TCR-αβ expression, and resistance to hydrocortisone treatment. Commitment of the T cells to the CD8 lineage was indicated by the down-regulation of endogenous CD4, high level expression of CD45RB, and acquisition of cytolytic activity. Transgenic CD4 is therefore capable of rescuing the CD8 lineage when only class II molecules can serve as substrates for positive selection, thus confirming a prediction of the stochastic/selection model of thymocyte development (figure 6).

The outcome of class II-restricted thymocyte development is critically dependent on the level of transgenic CD4 expression. In the β_2m-null mice, the highly-expressing lckCD4 transgene was more effective at rescuing the CD8 lineage than the lower-expressing T3.CD4 transgene (figure 7). However, the T3.CD4 transgene rescued the CD8 lineage very efficiently in PCC TCR transgenic mice. One possible interpretation of this result is that the PCC-specific TCR has higher affinity for class II than the majority of TCRs in mice with a broad receptor repertoire, so that thymocytes in these mice are less dependent on CD4 for positive selection. Mating of the PCC TCR mice to the lckCD4 animals resulted in a phenotype strongly suggestive of intrathymic deletion: approximately a tenfold drop in the number of thymocytes, a pronounced reduction in the immature DP subpopulation, and the appearance in the periphery of mature T cells that expressed the PCC-specific TCR and the CD4 transgene, but not endogenous CD4 or CD8 (C. B. Davis, unpublished results). Therefore, increasing the avidity of thymocyte–class II interactions up to a certain point increases the efficiency of CD8 lineage rescue, but increasing avidity beyond that point adversely affects generation of both CD4 and CD8 lineages. These results are consistent with models of thymocyte development in which a threshold TCR avidity for self MHC is required for positive selection and a higher avidity threshold, when surpassed, results in negative selection (Travers 1993).

Rescue of the CD8 lineage is dependent on an intact cytoplasmic domain of CD4. β_2m-null mice expressing an lckCD4 transgene encoding a protein with a deleted cytoplasmic domain failed to rescue the CD8 lineage. The association of lck with CD4 is therefore likely to be important for sufficient stabilization of the association between CD4, TCR, and class II molecules to obtain rescue. It is interesting to note that tailless CD4 rescued the CD4 lineage in CD4-null mice (see above) but failed to rescue the CD8 lineage in the β_2m-null mice, even though positive selection is mediated by interactions with class II molecules in

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both cases. This observation, along with the inefficient rescue of the CD8 lineage by normal levels of transgenic CD4, suggests that commitment to the CD8 lineage is accompanied by some alteration in these thymocytes that makes positive selection by CD4 less favorable.

5. REGULATION OF CD4 GENE EXPRESSION

The finding that shutoff of CD4 gene expression in CD8 lineage cells is correlated with commitment to a program of CTL differentiation suggests that a common signal may regulate shutoff of CD4 and activation of CTL-specific genes. To study this problem, we have begun to dissect the mechanism of transcriptional regulation of the CD4 gene. A sequence 13 kilobases (kb) upstream of the transcription initiation site was found to have T cell-specific enhancer activity (Sawada & Littman 1991). One of the three nuclear protein binding sites in this region has an E-box consensus sequence to which a T cell-specific bHLH heterodimer, containing the proteins HEB and E2A (E12/E47), was found to bind. Mutations that abrogated binding of these proteins also abolished enhancer activity (Sawada & Littman 1993). In addition, co-transfection with Id, an HLH protein lacking the DNA-binding basic region that forms inactive heterodimers with E12/E47, inhibited enhancer function. These results suggest that T cell specificity is achieved due to pairing of HEB, which is preferentially expressed in T cells, with the ubiquitous E2A protein(s).

Using the combination of the CD4 enhancer and promoter in transfection studies, expression was observed in all T cell lines regardless of their subset phenotype. Moreover, inclusion of sequences from several DNAse hypersensitive sites within the CD4 gene did not result in repression of expression in CD4-negative cell lines. We therefore chose to study subset specificity using transgenic mice. Mice with a human CD4 transgene containing the CD4 promoter alone or the promoter plus the 0.3 kb enhancer were initially analyzed (Killeen *et al.* 1993). Whereas there was no expression of the transgene in mice bearing the promoter-only DNA, inclusion of the enhancer resulted in copy number-dependent expression of human CD4 only in cells of the murine CD4 lineage. The human gene was appropriately expressed in the double positive thymocytes, and was shut off in CD8 lineage cells. To further map sequences involved in shutting off CD4 in the CD8 lineage, we have made transgenic mice with a construct containing only the minimal CD4 enhancer and promoter, the first (non-coding) exon, the first intron, and part of exon 2, into which the human CD2 cDNA was introduced as a reporter gene. These mice expressed human CD2 only in the CD4 T cell subset, thus localizing the subset specificity in the enhancer, promoter, and/or the first intron (S. Sawada, J. D. Scarborough, N. Killeen and D. R. Littman, unpublished results). Studies are now under way to identify sequences in these regions that may silence CD4 expression in the CD8 subset of mature T cells.

6. CONCLUSIONS

A central unsolved issue in immunology is the nature of the difference between positive and negative selection signals in the thymus. A qualitative difference in the means of engagement of MHC by the TCR may result in the activation of distinct pathways. Alternatively, quantitative differences in the avidity of TCR–MHC interactions may trigger distinct signals that have different thresholds. This second possibility is favored by results suggesting that the level of CD4 or CD8 coreceptor can determine whether self-MHC recognition by a specific transgenic TCR results in positive versus negative selection of thymocytes. Thus, overexpression of CD8 with a class I-specific receptor (Robey *et al.* 1992; Lee *et al.* 1992) or of CD4 with a class II specific receptor (C. B. Davis, unpublished results) resulted in intrathymic deletion of cells bearing these receptors, which are normally positively selected. In addition, cells bearing an autoreactive class I-specific receptor have been shown to develop to maturity in peripheral lymphoid organs, but only after down-regulating CD8 (Schonrich *et al.* 1991). This apparent modulation of coreceptor may be a mechanism for preventing appearance of T cells having high avidity self-reactive TCRs.

If avidity differences govern fates of T lymphocytes, it is likely that CD4 also plays important roles in shaping the TCR repertoire and in regulating the reactivities of mature T cells. Modulation of CD4 activity may therefore influence the 'trigger point' of helper T cells, and can be used as a means of regulating the autoreactive potential of these cells. Ongoing studies are aimed at determining whether there are differences in the properties of TCR complexes on class II-restricted T cells selected in the presence or absence of CD4. In addition, a potential role for CD4 as a direct signalling molecule has not yet been elucidated; numerous studies suggest that 'negative' signalling via CD4, independent of the TCR complex, inhibits activation of the TCR and may even result in an apoptotic signal upon TCR stimulation (Julius *et al.* 1993). This phenomenon may involve sequestration of lck away from the TCR complex, but the mechanism has yet to be fully worked out.

Another central issue is the relationship between CD4 expression or extinction and the commitment of thymocytes to a functional program of differentiation. It is not yet clear whether there is coupling, at the gene expression level, between shutoff of CD4 and commitment to a cytotoxic program. Such coupling would ensure that CTLs would preferentially interact with endogenously processed antigen (presented on class I molecules) and that, conversely, helper cells (that shut off CD8) would interact with antigen on specialized antigen presenting cells (presented on class II). Studies aimed at identifying factors that shut off CD4 expression in CD8 single positive thymocytes may provide the necessary tools for future studies on the molecular basis of the stochastic process that gives rise to these mature T cell subsets.

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