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Divergent Changes in the Sensitivity of Maturing T Cells to Structurally Related Ligands Underlies Formation of a Useful T Cell Repertoire

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Summary

CD4$^+$CD8$^+$ thymocyte differentiation requires TCR signaling induced by self-peptide/MHC ligands. Nevertheless, the resulting mature T cells are not activated by these self-complexes, whereas foreign ligands can be potent stimuli. Here, we show that the signaling properties of TCR change during thymocyte maturation, differentially affecting responses to related peptide/MHC molecule complexes and contributing to this discrimination. Weak agonists for CD4$^+$CD8$^+$ thymocytes lose potency during development, accompanied by a change in TCR-associated phosphorylation from an agonist to a partial agonist/antagonist pattern. In contrast, sensitivity to strong agonists is maintained, along with full signaling. This yields a mature T cell pool highly responsive to foreign antigen while possessing a wide margin of safety against activation by self-ligands.

Introduction

Precursor CD4$^+$CD8$^+$ T lymphocytes each express a unique TCR that regulates mature antigen-induced effector function. Because of the quasi-random nature of TCR binding specificity, thymocyte differentiation includes a test of receptor recognition of the internal antigenic environment (von Boehmer et al., 1989; Robey and Fowlkes, 1994; Fink and Bevan, 1995). A key aspect of this process involves the death of thymocytes with TCR whose interactions with self-peptide/MHC molecule ligands might result in full activation of the mature T cell, resulting in autoimmune responses (Kappler et al., 1987; Kisielow et al., 1988). Thymocytes also require signals from receptor recognition of self-ligands for survival, maturation, and the choice between the CD4$^+$ and CD8$^+$ lineages (Fink and Bevan, 1978; MacDonald et al., 1988; Scott et al., 1989; Chan et al., 1993; Lucas and Germain, 1996). Furthermore, TCR recognition of the same or related self-ligands is critical to survival of mature naive T cells (Takeda et al., 1996; Brocker, 1997; Kirberg et al., 1997; Rooke et al., 1997; Tanchot et al., 1997; Markiewicz et al., 1998). This creates an apparent paradox—how self-peptide-associated MHC molecules can deliver both death-inducing signals that purge the repertoire of potentially harmful self-reactive T cells and positive signals ensuring survival but not overt activation of other T cells also recognizing these ligands (Allen, 1994).

Three major alternative hypotheses have been proposed as explanations, the "cosignal/unique peptide," "quantitative threshold or avidity," and "quality of signal" models. The first postulates that thymocytes simultaneously signaled through the TCR and by the CD28 costimulatory pathway undergo apoptosis. Epithelial thymic stromal cells mediating positive selection differ from the hematopoietic cells typically involved in negative selection in lacking expression of the CD80/CD86 costimulatory molecules (Punt et al., 1994; Amsen and Kruisbeek, 1996; Kishimoto et al., 1996; Lesage et al., 1997). Only thymocytes with TCR recognizing peptide/MHC complexes uniquely expressed on the costimulatory-deficient stromal cells (Nakagawa et al., 1998) are presumed to survive the selection process, preventing export of T cells recognizing self-ligands on costimulatory hematopoietic presenting cells in the periphery.

The avidity hypothesis proposes that quantitative thresholds exist for the signals received from the TCR, with signal intensity below one threshold being inadequate for survival (neglect) and that above a second threshold promoting active death (negative selection) (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). Only precursors receiving a signal in between these thresholds survive and mature (positive selection). The actual signal is presumed to be a conjoint product of the TCR affinity for ligand, ligand density, strength of cell adhesion, and possibly cosignaling. An important addition to this model is the proposal that TCR signal generation and/or T cell responsiveness to these messages is actively reduced during maturation due to tonic receptor stimulation, enforcing a lack of reactivity to self (Grossman and Singer, 1996; Sebzda et al., 1996). This "tuning" model derives from theoretic considerations and from evidence that ligands unable to stimulate mature T cell responses can induce thymocyte negative (Pircher et al., 1991; Vasquez et al., 1994) or positive (Hogquist et al., 1994) selection.

The third model is based on the recent recognition that slight structural variations in the peptide/MHC molecule ligand for a TCR control produce agonists, partial agonists, or antagonists, pharmacological differences associated with changes in early TCR intracellular phosphorylation patterns (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Reis e Sousa et al., 1996; La Face et al., 1997; Kersh et al., 1998a). Some thymic ligands could thus deliver a qualitatively distinct signal, with the choice between differentiation and death arising from the differences in intracellular signals induced by agonists versus partial agonists/antagonists. Evidence in favor of a special capacity of antagonist/partial agonist ligands to induce positive selection has been reported (Hogquist et al., 1994) and agrees with data indicating that different

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biochemical pathways may be involved in positive and negative selection (Alberola et al., 1996; Sugawara et al., 1998).

All of these models except that involving tuning do not explicitly consider the impact of developmental changes in TCR, coreceptor, and intracellular signal transduction molecule expression on the outcome of receptor/ligand binding events. Yet, developing T cells increase TCR expression 10- to 50-fold from the $TCR^{lo}CD4^{+}CD8^{+}$ (DP) stage to the mature stage (Guidos et al., 1990), and they alter in complex ways the levels of the key MHC molecule-binding, Lck-associated CD4 and CD8 coreceptors (Lucas and Germain, 1996) as well as intracellular proteins involved in signal transduction (Chan et al., 1994). These events seem likely to contribute to thymocyte fate because the pharmacologic properties of a receptor ligand are determined not only by the kinetics of TCR/ligand interactions (Matsui et al., 1994; Alam et al., 1996; Lyons et al., 1996; Kersh et al., 1998b) but also coreceptor density (Vidal et al., 1996; Hampl et al., 1997; Madrenas et al., 1997) and intracellular regulatory pathways (I. Š. et al., unpublished data).

Despite these phenotypic data, little information is available about corresponding changes in either TCR signal transduction or cellular sensitivity to such signals. To address these questions, quantitative functional and qualitative biochemical analyses of antigen-specific signaling at discrete T cell developmental stages are needed. Here, we report such analyses using an in vitro system in which cell death, the expression of marker genes for differentiation, and the biochemical consequences of TCR engagement can be measured at distinct, clearly defined maturation stages as a function of peptide concentration and ligand structure. Our results confirm a decline in TCR-evoked responsiveness during maturation but, importantly, reveal that this loss of sensitivity is ligand selective, not global. Thymocytes bearing a single defined TCR maintain or increase their sensitivity to a foreign agonist ligand not encountered during maturation while showing a decrease in response to related ligands likely to be similar to those involved in positive selection. These diverging changes in functional response during maturation correlate with ligand-specific alterations in proximal TCR-associated tyrosine phosphorylation. Such opposing effects on TCR signaling in response to ligands of related structure provide for sensitive responses to foreign antigens in the face of a reduced likelihood of pathologic autoreactivity among mature T cells exposed to activated antigen-presenting cells.

Results

Failure to See Selective Stimulation of Differentiation versus Death Using a Ligand with Partial Agonist Properties for Mature T Cells

Several reports have described antagonists or weak partial agonists for mature T cells that promote positive selection of thymocytes with the same TCR, whereas full agonist ligands only induce deletion (Hogquist et al., 1994, 1995). Other investigators have reported that low concentrations of an agonist for a mature T cell can positively select thymocytes with the same TCR, although the emerging cells cannot be activated by the selecting ligand (Sebzda et al., 1994, 1996; Girao et al., 1997). These latter findings agree with other evidence that peptide/MHC molecule combinations incapable of eliciting effector functions from mature T cells at any achievable concentration can induce negative selection of developing thymocytes with the same TCR (Pircher et al., 1991). To reconcile these various findings, it has been proposed that signaling upon self-peptide/MHC molecule recognition in the thymus tunes the T cell's response threshold (Grossman and Singer, 1996; Sebzda et al., 1996).

To further investigate these issues, we used an in vitro model in which thymocytes and mature T cells can be exposed to self-peptide/MHC molecule ligands or to graded concentrations of foreign ligands, each in the presence or absence of various cosignaling molecules on MHC class II–expressing transfected L cells. This approach allows direct analysis of the response of pure populations of thymocytes at discrete developmental stages to a homogenous accessory cell population bearing a defined ligand level. In vivo or organ culture models cannot provide such quantitative information on a single cell level, limit the duration of or cell types involved in signal delivery, or restrict the surface molecule display as completely, and they are unsuitable for many biochemical signaling studies. CD69 upregulation, which occurs on all thymocytes undergoing positive as well as negative selection (Swat et al., 1993; Yamashita et al., 1993; Lucas et al., 1994; Lucas and Germain, 1996) and is also upregulated on peripheral T cells by TCR engagement (Ziegler et al., 1994), was analyzed as an indicator of signaling for differentiation, in parallel with induction of cell death (thymocytes) or IL-2 secretion (mature T cells). The T cells were from $RAG2^{-/-}$ TCR transgenic mice in which all T-lineage cells express a single defined TCR.

Using the AND TCR transgenic model, the responses of thymic DP and peripheral $CD4^{+}$ T cells (CD4SP) were examined using PCC 88-104 (the foreign agonist peptide) or a number of variants at residue 99, whose alteration generates peptides that produce partial agonists/antagonists for cytochrome-specific T cells (Page et al., 1994; Spain et al., 1994; Madrenas et al., 1995; Lyons et al., 1996; Combadière et al., 1998). In the case of PCC 88-104, the ED_{50} for IL-2 secretion by CD4SP is between 10- and 20-fold greater than that for induction of CD69 expression, whereas more than a 3 log difference exists for these two parameters for the same peptide with proline substituted at position 99 (P99) (Figure 1). This nonparallel change classifies the P99/I-E^{k} complex as a partial agonist for mature AND TCR T cells. We then asked if this altered ligand shows greater relative induction of thymocyte differentiation responses without accompanying cell death as compared to PCC 88-104, as predicted by the "quality of ligand" model. For DP thymocytes, the absolute amount of peptide required for stimulation of any measured TCR-dependent event is greater for P99 than PCC 88-104. However, the ligand involving this peptide and that containing PCC 88-104 show the same relative difference between the ED_{50} for DP thymocyte differentiation (CD69 expression) and for

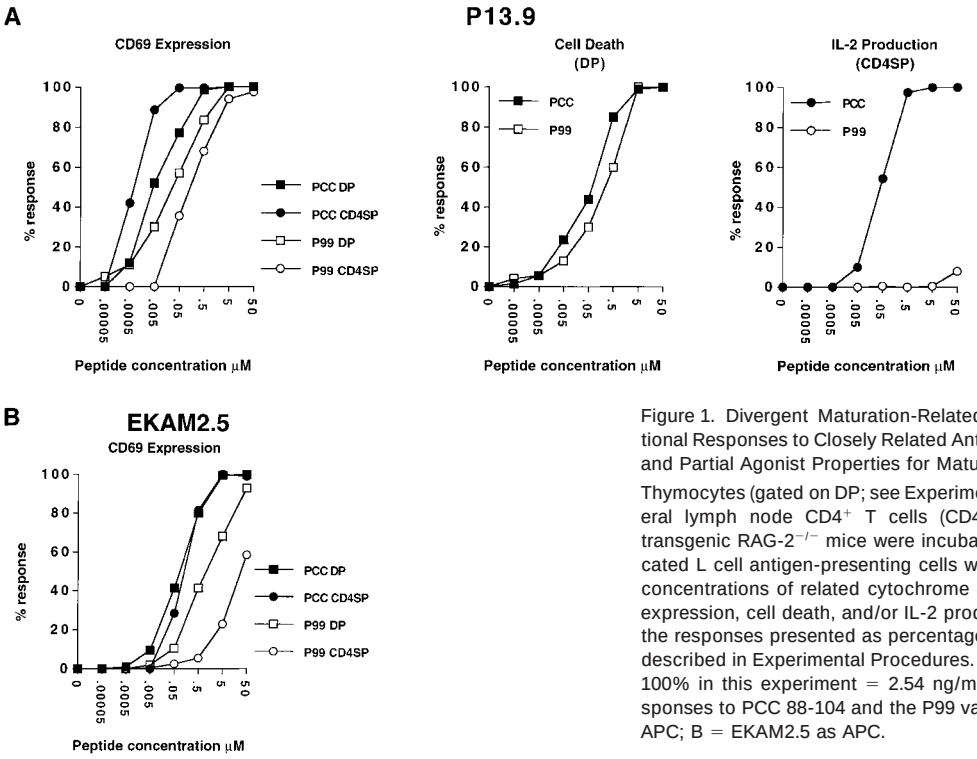

Figure 1. Divergent Maturation-Related Changes in T Cell Functional Responses to Closely Related Antigenic Ligands with Agonist and Partial Agonist Properties for Mature T Cells

Thymocytes (gated on DP; see Experimental Procedures) or peripheral lymph node $CD4^+$ T cells (CD4SP) from $H\text{-}2^b$ AND TCR transgenic $RAG\text{-}2^{-/-}$ mice were incubated overnight with the indicated L cell antigen-presenting cells with or without the indicated concentrations of related cytochrome *c* antigenic peptides. CD69 expression, cell death, and/or IL-2 production were measured and the responses presented as percentage of response calculated as described in Experimental Procedures. For CD4SP IL-2 responses, 100% in this experiment = 2.54 ng/ml. The figure shows the responses to PCC 88-104 and the P99 variant peptide. A = P13.9 as APC; B = EKAM2.5 as APC.

death (Figure 1). Thus, no evidence of selective stimulation of differentiation was seen using a ligand that for mature AND CD4SP clearly has weak partial agonist properties similar to those of some ligands used to induce positive selection of DP thymocytes bearing a class I–restricted TCR (Hogquist et al., 1994; Girao et al., 1997).

Divergent Changes during Development in T Cell Sensitivity to Ligands of Differing Quality

Although P99 and a large series of other position 99 variants with partial agonist or even antagonist properties did not selectively induce differentiation without death of DP thymocytes (data not shown), their use as stimuli for both immature and mature T cells did reveal a remarkable feature of TCR-induced activation during development. If tuning in response to TCR engagement during thymocyte development simply involved an increase in cellular response threshold or a general blunting of signaling, one would expect that ligands similar to the selecting ligand might be unable to generate enough signal to exceed this increased threshold, whereas more potent ligands would still be able to do so, but only at elevated concentrations relative to immature T cells. In contrast to this expectation, we observe diverging changes in the potency of closely related ligands. Although for DP thymocytes only 5- to 10-fold more P99 is required compared to PCC 88-104 to induce an equivalent CD69 differentiation response, with CD4SP 100- to 750-fold more P99 than PCC 88-104 peptide is needed for similar responses, depending on the presence or absence of B7.1 costimulation (Table 1). Thus, sensitivity to stimulation by the variant ligand involving P99 decreases (thymic DP: 4.9×10^{-2} μM versus peripheral $CD4^+$: 4×10^{-1} μM), but reactivity to PCC 88-104 actually increases with maturity when using $B7.1^+$ APC (thymic DP: 1.1×10^{-2} μM versus peripheral $CD4^+$: 4×10^{-3} μM for half-maximal CD69 induction).

Similar data were obtained with another variant peptide, Q99 (Figure 2). Here, thymic $CD4^+$ cells were analyzed in addition to DP and peripheral $CD4^+$ cells. T cell sensitivity to Q99-induced signaling as measured by CD69 expression decreases with T cell maturation from DP to thymic CD4SP. The effect is of such magnitude that no reactivity of peripheral $CD4^+$ T cells to the ligand formed by this peptide could be observed. Moreover, Q99 lacks even the capacity to generate a functional antagonist for mature AND T cells (data not shown).

Table 1. Quantitation of Divergent Changes in Differentiation (CD69) Responses of Immature versus Mature T Cells in Response To Related TCR Ligands

	Thymic DP			Peripheral CD4SP		
50% (μM)	PCC 88-104	P99	Ratio: P99/PCC	PCC 88-104	P99	Ratio: P99/PCC
Medium	1.1×10^{-2}	4.9×10^{-2}	4.5	4×10^{-3}	4×10^{-1}	100
Anti-B7.1	4.4×10^{-2}	4.2×10^{-1}	9.5	4×10^{-2}	30	750
Ratio: B7.1/Medium	4	8.6		10	75	

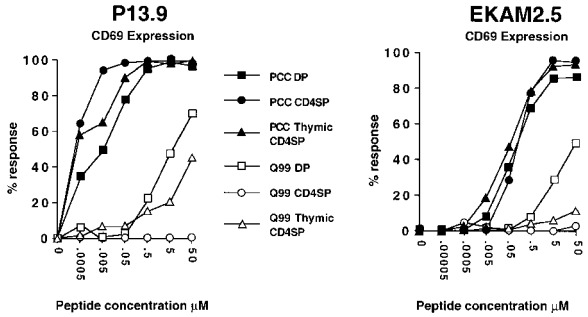

Figure 2. Divergent Maturation-Related Changes in T Cell Functional Responses to Ligands with Agonist and Null Activity for Mature T Cells

The protocol was the same as described in Figure 1. Responses of DP, thymic CD4SP, and peripheral CD4SP to PCC 88-104 and the Q99 variant peptide are shown using P13.9 (left) and EKAM2.5 (right) as APC.

Because tracking CD69 expression allows single cell analysis, these data and the findings with P99 indicate diverging changes in sensitivity to the wild-type versus variant ligands in the entire population of T cells. Comparison of the results obtained using P13.9 and EKAM2.5 presenting cells also confirms the results reported in Figure 1 and Table 1, namely that B7.1 costimulation permits mature $CD4^+$ cells to show heightened reactivity to PCC 88-104 as compared to DP cells, whereas reactivity is simply maintained when this costimulus is absent.

Differential Changes in Proximal TCR Signaling Patterns during Thymocyte Maturation in Response to Distinct Ligands

These data reveal that during maturation, T cell sensitivity to stimuli with the initial characteristics of strong and weak agonists for DP thymocytes can change in opposing directions. Weak agonists acquire the functional characteristics of partial agonists or even nonagonists for CD4SP, whereas initially more potent ligands retain or, in the presence of CD28 costimulation, increase their stimulatory capacity. Such differential, peptide/MHC molecule-specific changes in response cannot be readily ascribed to a generalized decline in TCR signaling capacity during maturation. We therefore examined proximal TCR phosphorylation patterns in DP thymocytes and CD4SP in response to different peptide/MHC molecule combinations, to see if the functional divergence was reflected in early events associated with TCR signal transduction. Transfectants lacking ICAM-1 were used because normal thymocytes and T cells have a high endogenous level of p21 phospho-ζ (van Oers et al., 1994) and in accord with their capacity to induce some CD69 upregulation on their own (B. L. et al., unpublished data), $I\text{-}E^{k+}$, $ICAM\text{-}1^+$ presenting cells add further to this basal signal, obscuring changes specifically induced by the different peptides.

Stimulation of DP thymocytes by PCC 88-104 showed substantial phosphorylation of TCR-associated ζ chains and of ZAP-70 kinase. The appearance of p23 phospho-ζ and phosphorylated ZAP-70 is characteristic of agonist signaling analyzed in mature T cells (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Reis e Sousa et al., 1996; La Face et al., 1997; Kersh et al., 1998a). The less potent P99 or Q99 peptides induce less overall protein phosphorylation when offered at the same concentration as PCC 88-104, but the pattern (generation of p23 phospho-ζ and phosphorylated ZAP-70) remains the same as with PCC 88-104 (Figure 3A). This is consistent with the functional dose-response data classifying the ligands created by the modified peptides as weak agonists for DP thymocytes and with a previous study examining thymocytes stimulated with variant ligands (Smyth et al., 1998).

The wild-type peptide continues to induce significant p23 phospho-ζ and phospho-ZAP-70 formation in CD4SP

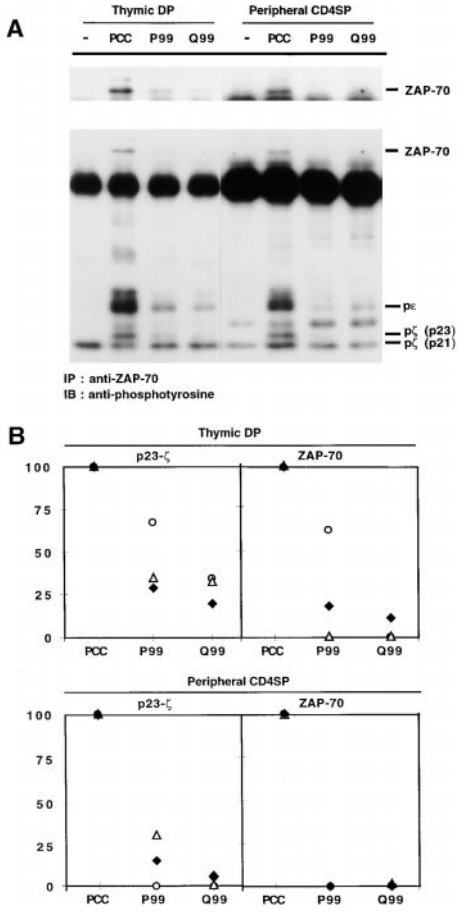

Figure 3. Divergent Changes in Proximal TCR-Associated Tyrosine Phosphorylation Events in Immature and Mature T Cells in Response to Structurally Related Ligands

Thymocytes (from $H\text{-}2^{q/q}$ [nonselecting] AND TCR transgenic $RAG\text{-}2^{-/-}$ mice) or peripheral lymph node $CD4^+$ T cells (CD4SP from selecting $H\text{-}2^{b/b}$ AND TCR transgenic $RAG\text{-}2^{-/-}$ mice) were incubated with EKAM2.5 L cell transfectants ($I\text{-}E^{k+}$, $ICAM\text{-}1^-$, $B7.1^{low}$) previously pulsed with 100 μM of the indicated peptide. After 5 min of cell contact, the cells were lysed and ZAP-70 associated proteins were immunoprecipitated and then analyzed by phosphotyrosine immunoblotting (A). The inset above the full gel shows a longer exposure of the region of the gel containing ZAP-70. In (B), densitometric data from 3 experiments of this type are shown. In each experiment, the p23 phospho-ζ signal or phospho-ZAP-70 signal following stimulation with PCC 88-104 was set as 100%, and the corresponding signals for P99 and Q99 stimulation were expressed relative to these values.

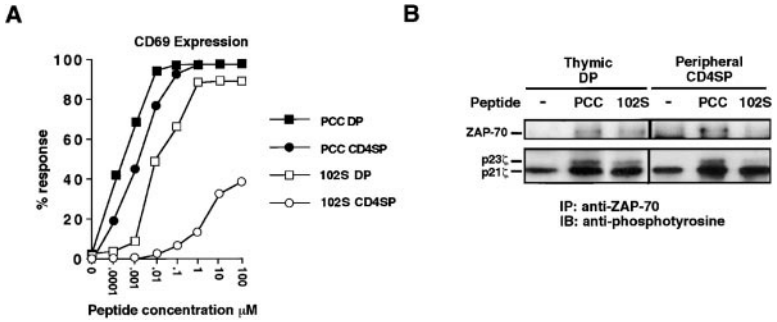

Figure 4. Divergent Maturation-Related Changes in T Cell Functional Responses and Signaling upon 5C.C7 TCR Recognition of Ligands with Agonist and Partial Agonist Activity for Mature T Cells

Thymocytes from nonselecting H-$2^{b/b}$ 5C.C7 TCR transgenic RAG-$2^{-/-}$ mice or mature $CD4^+$ LN cells from selecting H-$2^{a/a}$ 5C.C7 TCR transgenic RAG-$2^{-/-}$ mice were stimulated with DCEKhi7 L cell transfectants (I-E^{k+}, ICAM-1^-, B7.1^{low}) previously pulsed with 100 μM of the indicated peptide. In (A), CD69 responses of DP and CD4SP cells were measured as described in the legend to Figure 1. In (B), proximal signaling was analyzed as described in the legend to Figure 3.

cells. In striking contrast, both P99- and Q99-bearing APCs induce little p21 ζ, marginally detectable p23 ζ, and no measurable phospho-ZAP-70 generation in these cells, a signaling pattern characteristic not of weak agonists, but of very weak partial agonists/antagonists (Figure 3A; summarized quantitatively in Figure 3B). Thus, the divergent change in functional responses seen during thymocyte maturation reflects a change in TCR signal transduction such that strong agonists retain their capacity to signal fully, but weak agonists become ineffective in inducing the biochemical changes necessary for effective downstream signaling, especially ZAP-70 activation.

To extend these observations, we examined a second transgenic model. 5C.C7 is another cytochrome *c*–specific TCR. As seen in Figure 4A, a comparison of CD69 induction by PCC 88-104 and by the variant peptide 102S using unselected 5C.C7 DP thymocytes and selected, mature $CD4^+$ T cells reveals a divergent change in stimulation potency upon maturation. Thus, differential changes in ligand sensitivity during maturation are observed not only with T cells bearing distinct though related TCRs, but whether one examines DP thymocytes from a selecting (AND) or nonselecting (5C.C7) environment. As with the AND TCR, the functional findings are reflected in a change from an agonist-like ζ and ZAP-70 phosphorylation pattern induced by the 102S peptide using DP thymocytes to one typical of partial agonists/antagonists with CD4SP cells, whereas for PCC the pattern is agonist-like with both cell types (Figure 4B).

Alteration in the Molecular Assembly of the TCR Complex as a Possible Basis for Divergent, Ligand-Related Changes in TCR Signaling during Thymocyte Development

To begin to explore how these ligand-selective changes in T cell signaling arise, we examined the state of the TCR in immature versus mature cells. The recruitment of Lck-coupled CD4 or CD8 coreceptors to the TCR is a critical step in the generation of agonist responses (Jameson et al., 1994; Mannie et al., 1995; Vidal et al., 1996; Hampl et al., 1997; Madrenas et al., 1997) and the signaling pattern of full ζ chain phosphorylation and ZAP-70 activation (Madrenas et al., 1997). Besides the Lck that is recruited into the TCR complex via coreceptors, TCR complexes contain a small amount of Fyn (Samelson et al., 1990) and also of Lck that is not CD4- or CD8-associated (Criado et al., 1996; I. Š., unpublished data). Given the key role of Lck in ζ chain and ZAP-70 phosphorylation (Chan et al., 1995; Wange et al., 1995) and these data on the contribution of Lck recruitment to the agonist pattern of modification of these proteins, we examined whether augmented kinase association with the TCR might contribute to better signaling in DP thymocytes by less optimal ligands. Figure 5A shows that despite equivalent total intracellular levels of Lck in DP and CD4SP, isolated TCR complexes from 5C.C7 DP thymocytes contain a substantially greater amount of Lck than an equal number of TCR from CD4SP, in accord with the better signaling seen with 102S in the immature cells. The greater fractional association of Lck with the TCR of DP thymocytes correlates with a higher proportion of associated phosphorylated ζ chains and increased tyrosine kinase activity (Figure 5B). The Lck-TCR association is stable in the presence of an alkylating reagent (data not shown) that efficiently disrupts CD4 and CD8 binding to Lck (Shaw et al., 1990), indicating that the higher level of Lck-TCR association in DP thymocytes is not due to enhanced TCR association with coreceptors. The association is also retained after alkaline phosphatase treatment of the complexes, indicating that the Lck is not bound via its SH2 domain to the phospho-ζ (data not shown).

Discussion

Signals arising from engagement of the TCR determine whether a thymocyte undergoes successful differentiation to a functional, mature T cell or, alternatively, is eliminated by apoptotic cell death. In most experimental systems, ligands able to induce thymocyte maturation are incapable of triggering effector function from mature T cells bearing the same TCR. It has been unclear how self-recognition can deliver signals through the TCR that are adequate for differentiation in the thymus and yet inadequate for activation when recognized in the periphery. Simple avidity models in particular do not account for how a ligand at low concentrations can evoke DP differentiation by TCR signaling and fail at any testable concentration to trigger mature cells. One set of hypotheses proposed to explain this paradox involves invoking distinct biochemical signals as the basis for thymocyte differentiation in comparison to mature T cell activation (Vasquez et al., 1994). Another suggestion is that in re-

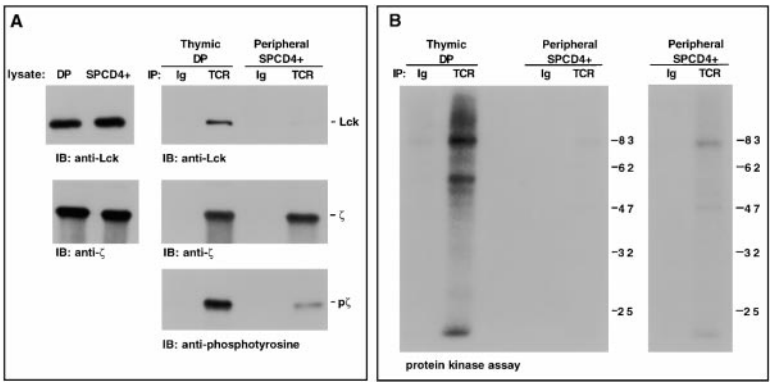

Figure 5. Changes in TCR Association with Total and Kinase-Active Lck during Maturation

The total levels of Lck and ζ chain were analyzed using an equal number of DP thymocytes and SP CD4$^+$ T cells (A). TCR were isolated from both DP thymocytes and SP CD4$^+$ T cells and analyzed for the presence of Lck and ζ chain, as well as for tyrosine phosphorylation of the ζ chain (A) or the kinase activity associated with the TCR complex (B). A longer exposure of phosphoproteins labeled during an in vitro kinase assay using the TCR complexes immunoprecipitated from CD4SP T cells is shown in a replicated set of lanes on the right.

sponse to positively selecting ligands, a thymocyte reduces its sensitivity to TCR signaling to prevent self-recognition of the same ligand(s) from reaching the threshold necessary for activation of the mature cell. This process has been called tuning or adaptation (Jameson et al., 1994; Grossman and Singer, 1996; Sebzda et al., 1996) and it is consistent with evidence that signaling through the TCR both persists and is critical throughout thymocyte development (Kisielow and Miazek, 1995; Wilkinson et al., 1995; Schmitt et al., 1997; Yasutomo et al., submitted), providing a tonic TCR signal that can actively induce and maintain a lowered state of responsiveness. Furthermore, a lower capacity of variant ligands to induce peripheral T cell activation as compared to thymocyte positive (Hogquist et al., 1994) or negative (Pircher et al., 1991) selection has been documented. Despite these functional data, only limited information is available concerning possible changes in the biochemical signals produced by TCR engagement at various stages of T cell development (Vasquez et al., 1994; Davey et al., 1998).

Here, we have used an in vitro model that permits a quantitative analysis of the differentiation and/or death responses of individual cells at distinct stages of T cell maturation to TCR ligands of varying structure to gain insight into how self-ligands can select a useful repertoire. An important feature of T cell development was revealed by these studies. Maturing thymocytes expressing a single TCR showed divergent rather than unidirectional changes in sensitivity to signaling induced by peptide/MHC molecule complexes with the properties of a strong agonist versus those DP thymocytes initially perceive as weak agonists. As thymocytes differentiate from the DP to the SP state, they maintain or increase sensitivity to strong agonists and maintain an agonist proximal phosphorylation pattern. At the same time, these cells lose sensitivity to weak agonists, in concert with a change in the biochemistry of TCR signaling induced by these ligands to that typical of partial agonists/antagonists. The functional dose-response changes seen here are in full agreement with a report that appeared while this paper was under review (Davey et al., 1998). Comparing DP thymocytes to mature CD8$^+$ T cells, these investigators noted a loss in sensitivity to altered peptide ligands but not wild-type agonist ligand, as measured by CD69 upregulation. These independent results suggest that conclusions we reach here concerning CD4$^+$ T cells are likely to be general properties of αβ TCR-bearing cells.

Our findings of changing patterns of proximal signaling additionally provide direct evidence that thymocyte sensitivity to ligands incapable of activating mature T cells arises at least in large measure from greater TCR signal generation rather than from just a lower response threshold for selection events as compared to effector gene activation. Both wild-type and variant peptides induced fully phosphorylated p23 ζ and phosphorylation of associated ZAP-70 in DP thymocytes, although the P99 and Q99 ligands did so more weakly for the AND TCR, as did 102S for the 5C.C7 TCR. For CD4SP, however, the wild-type ligand retained the capacity to induce both of these markers of effective TCR signaling, whereas the variant ligands lost their capacity to phosphorylate ZAP-70 to detectable levels. In agreement with our findings using DP thymocytes, altered peptide antigens also have been shown to elicit weak but detectable ZAP-70 phosphorylation and p23 phospho-ζ generation in another TCR transgenic model. In this case, however, the relationship between the signaling patterns of immature thymocytes and mature cells was not analyzed (Smyth et al., 1998). The divergent changes reported here in downstream TCR signaling ("signal 1") in response to ligands of slightly distinct initial potency are precisely those needed to ensure that maturing T cells (1) delete in response to any strong agonist self-ligands present in the medullary thymic environment, (2) remain highly sensitive to activation by agonist foreign antigens they do not encounter as they mature, and (3) acquire a substantial barrier against signaling beyond the effector activation threshold in response to distinct self-ligands. Not all capacity to signal through the TCR is lost with peptide/MHC molecule combinations one might consider to be analogs of the self-ligands contributing to positive selection, however, perhaps explaining how self-MHC molecules help foster naive T cell survival (Takeda et al., 1996; Brocker, 1997; Kirberg et al., 1997; Rooke et al., 1997; Tanchot et al., 1997; Markiewicz et al., 1998).

Differential activation of ZAP-70 at distinct stages of T cell maturity using the same TCR/ligand pair helps explain past paradoxical observations that stimuli with pure antagonist quality for mature T cells, which are incapable of eliciting detectable activation of this kinase, could promote positive selection, a process shown to depend on functional ZAP-70 (Negishi et al., 1995; Wiest et al., 1997). This appears to result from the capacity of such antagonists to show weak agonist signaling with

DP thymocytes, resulting in the necessary induction of ZAP-70 kinase function, followed by the loss of this capacity as the cell matures. These findings are also likely to explain previous observations that such nonagonist ligands for mature cells can induce negative selection in defined transgenic TCR models (Pircher et al., 1991; Vasquez et al., 1994).

The biochemical basis of maturation-related changes in ligand pharmacology is likely to be multifactorial. One developmental molecular alteration that is concordant with existing knowledge about proximal TCR signal transduction involves src-family kinase Lck. Direct examination of the TCR from DP versus CD4SP revealed a much higher fractional association of Lck with the receptors of immature thymocytes. Previous studies have demonstrated that decreasing the availability of (Lck-coupled) coreceptors on a mature T cell alters TCR proximal signaling to agonist ligand so that it resembles that seen with partial agonists/antagonists (Madrenas et al., 1997). Together, these data suggest that the contribution of coreceptor-associated Lck to effective signal generation may be more critical for mature cells and play a smaller role in determining the signaling quality of a ligand for DP thymocytes due to higher direct TCR coupling to Lck. Because coreceptor recruitment to an occupied TCR requires a certain period of time, ligands whose dissociation rate from the TCR is slow enough to permit this coreceptor coupling would be better able to generate a biochemically complete signal in mature T cells that are more dependent on the coreceptor-supplied pool of Lck. This could explain how for AND, PCC 88-104, P99, and Q99-containing ligands, and for 5C.C7, PCC 88-104 and 102S-containing ligands, could all show agonist-like activity with DP cells but only PCC 88-104-containing complexes would retain this property for mature $CD4^+$ cells, because their binding would be sufficiently long-lived to permit a high rate of effective CD4-Lck association (Hampl et al., 1997; Madrenas et al., 1997; Kersh et al., 1998b). Variations in the expression of other molecules regulating TCR function may also contribute to changes in the stimulatory properties of related ligands. SHP-1 negatively regulates TCR signaling (Plas et al., 1996), and preliminary studies in nontransgenic mice suggest that SHP-1 levels rise during the DP to SP transition (our unpublished data). Thus, immature thymocytes would be less subject to this negative feedback pathway, allowing better signaling in response to nonoptimal ligands.

A remaining issue is the extent to which these or other changes affecting TCR signaling are induced in proportion to the strength of the selecting signal, as postulated in tuning models (Sebzda et al., 1996; Grossman and Singer, 1996), or arise from a developmental program that operates in concert with negative selection of those cells not achieving an adequate suppression of TCR signaling. The failure to induce overwhelming negative selection at high concentrations of certain altered ligands (Sebzda et al., 1996) may be taken as evidence against the latter model. However, it is possible that only some cells in the precursor pool can respond to this weak ligand and the deletion of these few cells might be missed. It seems to us more appealing to imagine that a combination of programmed and actively regulated events is at work. The decline in fractional Lck-TCR association documented here, along with a rise in SHP-1 and other changes such as a decrease in Syk (Chan et al., 1994), seem to be developmentally controlled events that would change the signaling capacity of all TCR. These effects may be fine tuned in response to active signaling by specific ligands at particular concentrations, as the tuning models postulate.

An emerging theme in T cell biology is the ability of the TCR to vary signal strength and quality in the face of small differences in ligand structure. This flexibility is clearly related to the inability of any individual TCR to anticipate its affinity for either the large pool of self-ligands or the foreign ligands to which effector responses are required. By evolving the capacity to signal in a discriminate fashion upon engagement of ligands of distinct affinity, it is possible to establish a repertoire of mature T cells that can use self-recognition to monitor interaction with critical antigen-presenting cells, without undergoing undesirable activation to effector function and while maintaining a high sensitivity to ligands involving foreign peptides. The data reported here begin to detail how developing T cells achieve this remarkable property.

Experimental Procedures

Mice

$H\text{-}2^b$ as well as $H\text{-}2^q$ AND TCR transgenic (Kaye et al., 1989) $RAG\text{-}2^{-/-}$ (Shinkai et al., 1992) mice were generated by breeding and maintained in NIAID Research Animal Facilities. They were generously provided by Dr. B.J. Fowlkes. 5C.C7 (Seder et al., 1992) $RAG\text{-}2^{-/-}$ mice of the nonselecting $H\text{-}2^b$ as well as the selecting $H\text{-}2^a$ ($I\text{-}E^k$) haplotypes were obtained from Taconic or bred in NIAID Research Animal Facilities.

Cells

DAP.3 is a MHC class II–negative, ICAM-1-negative, B7-negative/low fibroblast cell line derived from $H\text{-}2^k$ mice. DCEKhi7 and P13.9 L cells are daughter cell lines of DAP.3 stably transfected with cDNA expression constructs encoding $I\text{-}E^k$ or $I\text{-}E^k$, B7.1, and ICAM-1, respectively (Ding et al., 1993). The former expresses high levels of $I\text{-}E^k$ and very low levels of B7.1, without ICAM-1, whereas the latter expresses high levels of all three molecules. EKAM2.5 was derived from DCEKhi7 by transfection with pβA-ICAM (Siu et al., 1989) encoding the costimulatory molecule ICAM.1, but which does not express this molecule, and therefore is phenotypically identical to the parental cell line DCEKhi7.

Monoclonal Antibodies for Cell Surface Staining

Antibodies were purchased from Pharmingen. For flow cytometry, the following antibody combination was used: PE anti-CD4 (RM4-5), FITC anti-CD8 (53-6-7), and biotinylated anti-CD69 (H1-2F3) revealed by TC-streptavidin (Caltag). Surface staining was performed as previously described (Lucas and Germain, 1996). For inhibition of cell surface molecule function during cell culture, purified anti-B7.1 (16-10A1) was used.

Peptides

The following peptides were used for these experiments: PCC 88-104 is KAERADLIAYLKQATAK; P99, Q99, and 102S peptides are synthetic peptides in which the lysine in position 99 is changed to proline or glutamine or the threonine at position 102 is changed to serine, respectively. All peptides were synthesized and purified by the NIAID Peptide Synthesis Facility, NIH, Bethesda, MD.

In Vitro Culture Assay

Thymi or pooled mesenteric lymph nodes and spleens were homogenized in complete RPMI-1640 medium with 10% heat-inactivated

FCS. 1×10^6 thymocytes or 0.5×10^6 peripheral T cells and 1×10^6 or 0.5×10^6 fibroblasts of the indicated cell line, respectively, were centrifuged and then incubated in 6 ml polypropylene, round-bottom tubes with cap (Falcon) at 37°C in 5% CO_2. Where indicated, peptides were added at 5×10^{-5} μM to 50 μM. After 18 to 21 hr of culture, thymocyte cell death, IL-2 production by peripheral T cells, and CD69 expression on thymic DP cells, or thymic and peripheral $CD4^+$ cells, were determined.

IL-2 Production

Pooled peripheral mesenteric lymph node and spleen cells were cultured with peptide and APCs overnight in 2.5 ml of complete medium. Supernatants were assayed in triplicate for the presence of IL-2 by ELISA, using the jES6-1A12 mAb (Pharmingen) as a capture reagent. Bound cytokine was detected with biotinylated jES6-5H4 mAb, alkaline phosphatase-conjugated avidin, and p-nitrophenyl phosphate (Sigma). The reaction product was measured at 405 nm.

In Vitro Cell Death Assay

After overnight culture, thymocytes were harvested and cell death measured as described (Combadière et al., 1998). In brief, all recovered cells were stained for CD4, CD8, and CD69, washed, and then resuspended in identical volumes. Data were acquired for 60 s at a constant flow rate using a FACScan equipped with CellQuest software (Becton Dickinson). Total viable cells were enumerated using a restricted gate defined by FSC/SSC parameters and verified by the absence of propidium iodide incorporation. Viable DP thymocytes were measured by setting a gate on the basis of CD4/CD8 fluorescence intensity that identified $CD4^{low}CD8^{low}$ thymocytes (Lucas and Germain, 1996). The results were converted to percentages of maximal cell death as explained below. No increase in cell death was observed among peripheral $CD4^+$ cells during culture at any peptide concentration.

Calculations

Because reactivity against some fibroblast cell lines was observed without adding any source of peptide, CD69 expression by all cells, IL-2 production by peripheral CD4SP, and death of DP thymocytes were expressed as "% response," calculated after correction for this background by subtraction of the response obtained with the same APCs not exposed to peptide. "100% response" for all peptide titrations was considered to be the induction of CD69 upregulation by all cells, the maximal cell death induced by PCC 88-104 in the experiment, or the amount of IL-2 produced using the highest tested concentration of PCC 88-104. Because a significant percentage of DP thymocytes died during culture both without, and to a greater extent with, peptide exposure, and because CD69 expression on DP thymocytes was routinely measured by flow cytometry only on viable cells, we have corrected for this by assuming that all DP cells induced to die by peptide exposure also expressed CD69, based on the following calculation: percentage of CD69 induced on DP cells = (percentage of viable DP cells × percentage of CD69 measured by cytometry) + (percentage of dead DP cells).

Stimulation and Processing of T Cells for Analysis of ζ and ZAP-70 Tyrosine Phosphorylation

DP thymocytes were from AND TCR transgenic mice on a RAG-$2^{-/-}$, H-$2^{q/q}$ background or 5C.C7 mice on an H-$2^{b/b}$ background. Because these MHC haplotypes do not induce positive or negative selection of these TCR, all the cells are immature and can be directly analyzed without exposure to antibodies that might affect the signaling response. Peripheral T cells were from AND TCR transgenics on a RAG-$2^{-/-}$, H-2^b background, or 5C.C7 RAG-$2^{-/-}$ on an H-2^a background, which positively select only $CD4^+$ mature T cells. Cells (10×10^6) were added to 2.5×10^6 EKAM2.5 cells (for AND) or DCEKhi7 cells (for 5C.C7) previously pulsed with 100 μM of peptide and after centrifugation at 1000 rpm for 10 s, incubated at 37°C for 2 or 5 min. After incubation, cells were washed once with PBS and placed in lysis buffer containing 1% NP-40, 10 mM Tris-HCl (pH 7.2), 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, 1 mM Na_3VO_4 (Sigma), and complete protease inhibitor cocktail (Boehringer Mannheim, Germany) for 25 min on ice. Following removal of nuclear debris, the resultant supernatants were subjected to immunoprecipitation using optimized amounts of polyclonal antiserum to ZAP-70 (Burkhardt et al., 1994). After incubation of cell lysates and antibody on ice for 4 hr, immune complexes were collected with *S. aureus* protein A (Pansorbin, Calbiochem) and washed in lysis buffer. The proteins were eluted in SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting (Stefanova et al., 1993). Immunoblots were developed using 4G10, a mouse mAb to phosphotyrosine (Upstate Biotechnology), peroxidase-linked goat antibodies to mouse IgG (BioRad Laboratories), and SuperSignal enhanced chemiluminescence (Pierce). Quantitative data were obtained from film exposures with a Molecular Dynamics Laser Densitometer.

Immunoprecipitation and Immunoblotting Analysis of Immature and Mature TCR Complexes

The following antibodies were used: anti-mouse TCR β chain mAb H57-597 (Pharmingen), rabbit antiserum to ζ chains (Burkhardt et al., 1994), mouse mAb 3A5 to Lck (Santa Cruz Biotechnology), and peroxidase-linked goat antibodies to mouse and rabbit IgG (BioRad Laboratories). TCR were immunoprecipitated by incubation of lysates with optimized amounts of mAb on ice for 4 hr. Immunocomplex protein kinase assays, SDS-PAGE, and immunoblotting were performed as previously described (Stefanova et al., 1993).

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