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Lck Activity Controls CD4/CD8 T Cell Lineage Commitment

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Summary

Thymocytes carrying MHC class I–restricted TCRs differentiate into CD8 T cells, while those recognizing MHC class II become CD4 T cells. The mechanisms underlying how MHC class recognition, coreceptor expression, and effector function are coordinated are not well understood. Since the tyrosine kinase Lck binds with more affinity to CD4 than CD8, it has been proposed as a candidate to mediate this process. By using transgenic mice with altered Lck activity, we show that thymocytes carrying a class II–restricted TCR develop into functional CD8 T cells when Lck activity is reduced. Conversely, thymocytes carrying a class I–restricted TCR develop into functional CD4 T cells when Lck activity is increased. These results directly show that quantitative differences in the Lck signal control the CD4/CD8 lineage decision.

Introduction

During T cell development, a common precursor population of $CD4^+CD8^+$ double-positive (DP) thymocytes gives rise to two main cell lineages of $\alpha\beta$ T cells: CD4 and CD8 T cells. CD4 T cells recognize peptides bound to MHC class II and have helper activity. CD8 T cells recognize peptides bound to class I MHC and have cytotoxic activity. Defining the mechanisms used by DP thymocytes to choose the CD4 or CD8 lineage and how this process operates to coordinate MHC class recognition, CD4/CD8 coreceptor expression, and effector function is central for our understanding of T cell development.

Two main models, stochastic and instructional (von Boehmer, 1996), have been proposed to explain how MHC specificity and coreceptor expression are linked during development. The stochastic model proposes that commitment to the CD4 or CD8 lineage occurs independently of TCR specificity (Robey et al., 1991). Expression of either CD4 or CD8 is terminated stochastically. Since MHC recognition by a specific TCR usually requires coengagement of class I by CD8 and of class II by CD4, only cells with an appropriately matched TCR and coreceptor mature. Studies supporting this model have demonstrated that the in vivo maturation of T cells with mismatched MHC recognition and coreceptor expression (class I–specific TCR with CD4 and class II–specific TCR with CD8) can be rescued by constitutive expression of CD8 or CD4 transgenes, respectively (Davis et al., 1993; Chan et al., 1994; Corbella et al., 1994; Robey et al., 1994). However, the rescue was very inefficient in all cases when the transgene-encoded coreceptor was expressed at physiological levels (Robey and Fowlkes, 1994; Fowlkes and Schweighoffer, 1995). In addition, the existence of "transitional" subsets of thymocytes that are $CD4^+CD8^{lo}$ in class II–deficient mice (S. H. Chan et al., 1993; Suzuki et al., 1995) and $CD4^{lo}CD8^+$ in class I–deficient mice (van Meerwijk and Germain, 1993) has been offered as evidence for a stochastic mechanism of lineage commitment, but it is unclear whether CD4/CD8 lineage commitment has occurred in these cells. For example, both intrathymic transfer experiments (Lundberg et al., 1995) and coreceptor reexpression assays (Suzuki et al., 1995) have shown that $CD4^+CD8^{lo}$ thymocytes can give rise to both CD4 and CD8 T cells.

The instructional model proposes that the recognition and coengagement of class I MHC by a specific TCR and CD8 activates a differentiation program that includes the turn-off of CD4, thus generating a CD8 T cell. Recognition and coengagement of class II by a specific TCR and CD4, conversely, promotes a program that includes the downregulation of CD8, producing a CD4 T cell. Given that it seems unlikely that the TCR could distinguish between a class I or class II MHC ligand, it has been proposed that the distinct instructional signal would be delivered through the coreceptor. Strong support for this model comes from experiments in which a CD8 extracellular/CD4 intracellular chimeric receptor was shown to direct differentiation of a significant number of T cells with a class I–restricted TCR into the CD4 lineage (Seong et al., 1992; Itano et al., 1996). Recently, the instructional model has been modified to include the notion that the difference between the signals that induce generation of CD4 T cells or CD8 T cells is a quantitative one. This is based on experiments where cross-linking of the TCR with conventional antibodies generated CD4, but not CD8 T cells (Bommhardt et al., 1997; Suzuki et al., 1998), presumably because these antibodies aggregate and give a strong signal. Conversely, stimulation with antibodies genetically modified so that they do not aggregate can induce generation of CD8 T cells (Basson et al., 1998). Furthermore, it has been shown that in the absence of CD4, class II–restricted T cells can differentiate into CD8 T cells, presumably because they receive a weaker signal (Matechak et al., 1996). Together, these results have led to the notion that the CD4 coreceptor would contribute a strong signal and induce the generation of CD4 T cells, while the weaker signal that the CD8 coreceptor would bring to the TCR complex would result in the generation of CD8 T cells.

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An attractive candidate for a mediator of the difference in instructive signaling is the *src*-family protein tyrosine kinase Lck, which is expressed mainly in T cells throughout development (reviewed by Perlmutter et al., 1993). Lck associates noncovalently with the cytoplasmic tails of both CD4 and CD8 and becomes catalytically activated when the coreceptors are cross-linked (Veillete et al., 1988; Shaw et al., 1989; Veillette and Zúñiga-Pflücker, 1989; Luo and Sefton, 1990; Turner et al., 1990). Lck associates more efficiently with CD4 than CD8; on DP cells, 25%–50% of surface CD4 but only 2% of surface CD8 molecules are found to be associated with Lck (Weist et al., 1993). Therefore, it has been proposed that the amount of Lck signal brought by the coreceptor into the TCR complex could play a central role in CD4/CD8 lineage commitment (Matechak et al., 1996; Bommhardt et al., 1997; Basson et al., 1998). However, CD4 and CD8 could deliver qualitatively different intracellular signals through other associated signaling molecules or could exert their effects by modifying the avidity of the TCR–MHC interactions, leading to general alterations in TCR signaling. Thus, the role of Lck in CD4/CD8 lineage determination needs to be addressed directly.

Lack of functional Lck in a mutant Jurkat subclone (JCaM1) blocks activation through the TCR, providing genetic evidence for the essential role of Lck in TCR signaling (Straus and Weiss, 1992). In mice, targeted disruption of the *lck* gene or transgenic (Tg) expression of catalytically inactive Lck under the control of the *lck* proximal promoter, which drives transgene expression in double-negative (DN) and DP thymocytes, interferes with both proliferation and TCRβ allelic exclusion during transition from the DN to DP stage (Molina et al., 1992; Levin et al., 1993). In addition, expression of the activated form of Lck (LckF505) under the same promoter can drive development of RAG$^{-/-}$ and pre-T$\alpha^{-/-}$ thymocytes into the DP compartment (Mombaerts et al., 1994; Fehling et al., 1997). These studies indicated that Lck controls signals induced through the pre-TCR complex. The *lck* distal promoter, which becomes active in DP thymocytes and drives expression throughout positive selection and in mature T cells (Wildin et al., 1995), has been used to study the effects of Lck activity at later stages of development. Transgenic mice expressing a catalytically inactive form of Lck under the control of this promoter (dLGKR; Hashimoto et al., 1996) show a reduction in the number of positively selected CD4 and CD8 cells in the thymus. Conversely, expression of a constitutively active form of Lck under the control of the same promoter (dLGF) increases the number of positively selected CD4 and CD8 thymocytes (S. J. S. et al., unpublished data). However, these mice do not exclusively generate CD8 or CD4 mature T cells, respectively, demonstrating that changes in Lck activity per se do not determine the fate of developing DP thymocytes.

To test directly the contribution of Lck to CD4/CD8 lineage determination, we bred dLGKR mice with mice expressing a class II–restricted Tg TCR (AND) and dLGF mice with mice expressing a class I–restricted Tg TCR (OT-I) and analyzed the development of T cells in these animals. If the difference between the signals contributed by the intracellular domains of CD4 and CD8 is merely the amount of Lck that they can bring into the TCR signaling complex, then a decrease in the Lck activity associated with CD4, holding TCR specificity constant, should change lineage commitment toward a CD8 fate. Similarly, an increase in Lck activity associated with CD8 should shift lineage commitment toward the CD4 fate.

Our results show that in the presence of dLGKR, class II–restricted T cells develop into CD8 T cells with cytotoxic potential, while in the presence of dLGF, class I–restricted T cells develop into CD4 T cells with helper characteristics. Therefore, alterations in the intensity of the Lck signal brought to the TCR complex by the coreceptor can result in a complete change in the developmental program of maturing T cells.

Results

AND$^{+/-}$dLGKR$^{+/-}$ Mice Develop Class II–Restricted CD8 T Cells

To test if a reduced Lck signal during positive selection determines development of cells into the CD8 lineage, we crossed dLGKR mice with mice Tg for the class II–restricted TCR AND. AND mice carry a Tg TCRαβ (Vβ3, Vα11.1) that in animals of the H-2^b background recognizes a peptide derived from pigeon cytochrome C (PCC) in the context of I-E^k and induces development of thymocytes into the CD4 T lineage (Kaye et al., 1989). We crossed AND$^{+/+}$ with dLGKR$^{+/-}$ mice to generate animals with the genotypes AND$^{+/-}$dLGKR$^{+/-}$ (AND/dLGKR) and AND$^{+/-}$dLGKR$^{-/-}$ (AND) and analyzed their thymocyte populations between 6 to 12 weeks of age; all mice were homozygous for H-2^b. Expression of the catalytically inactive dLGKR transgene in AND mice dramatically blocked development of CD4 single-positive (SP) thymocytes and led to development of a significant number of CD8 SP cells (Figure 1). The CD8 SP cells, mostly positive for TCRVα11.1, underwent complete maturation (Figure 1) followed by migration into the spleen, where the CD4/CD8 ratio of the Vα11.1$^+$ cells was reversed from 40 to $<$0.1 (Figure 1). Although the AND/dLGKR mice showed a 3- to 4-fold reduction in total thymocyte numbers when compared to AND mice (Figure 1), the absolute number of HSA$^{lo/-}$Vα11.1$^+$CD8 SP cells increased 2- to 10-fold, while that of the corresponding CD4 SP population was reduced 300-fold (Figure 3). Thus, the increased percentage of CD8 SP thymocytes found in AND/dLGKR mice could not be accounted for by the exclusive block of CD4 T cell development. These results indicate that the intensity of the Lck signal is crucial for development of AND thymocytes into the CD4 lineage. Most striking is the fact that a significant number of these cells carrying a class II–restricted TCR developed into the CD8 lineage.

OT-I$^{+/-}$dLGF$^{+/-}$ Mice Develop Class I–Restricted CD4 T Cells

To test the complementary part of the hypothesis, that is, whether increased levels of Lck activity lead to development of class I–restricted thymocytes into the CD4 lineage, we bred dLGF with OT-I mice. OT-I mice bear a Tg TCRαβ (Vβ5, Vα2), specific for an OVA peptide in the context of H-2K^b class I, that directs thymocyte development into the CD8 lineage in the H-2^b back-

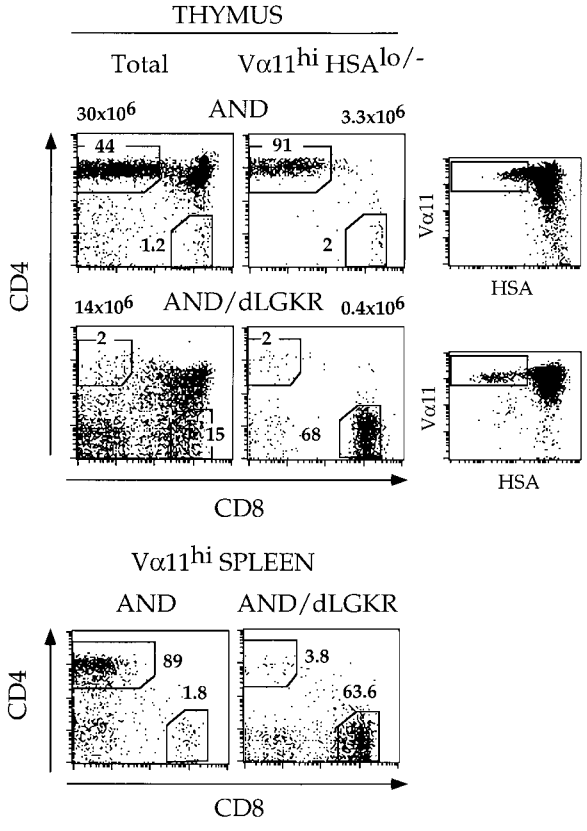


Figure 1. Reduced Lck Activity Induces Class II–Restricted Thymocytes to Develop into CD8 SP Cells

Thymocytes and splenocytes from AND ($AND^{+/-}$) and AND/dLGKR ($AND^{+/-}dLGKR^{+/-}$) littermate mice were stained for CD4, CD8α, TCRVα11.1, and HSA (thymus only) and analyzed by flow cytometry as described in Experimental Procedures. Top panels display total thymocytes and $HSA^{lo/-}V\alpha11.1^{+}$ thymocytes, electronically gated as shown in the smaller panels; bottom panels display $V\alpha11.1^{+}$ spleen cells. The numbers above the thymocyte panels represent the number of total or $HSA^{lo/-}$TCRVα11.1^{+}-gated live thymocytes; the percentages of CD4 and CD8 SP cells within each panel are shown. Percentages of double-positive thymocytes were 52.6% in AND and 40.7% in AND/dLGKR. Similar results were obtained when cells were stained with anti-CD8α or β (data not shown). Animals were genotyped by PCR from tail DNA; a minimum of 10 animals of each AND and AND/dLGKR 6–12 weeks of age were analyzed, and representative results are shown.

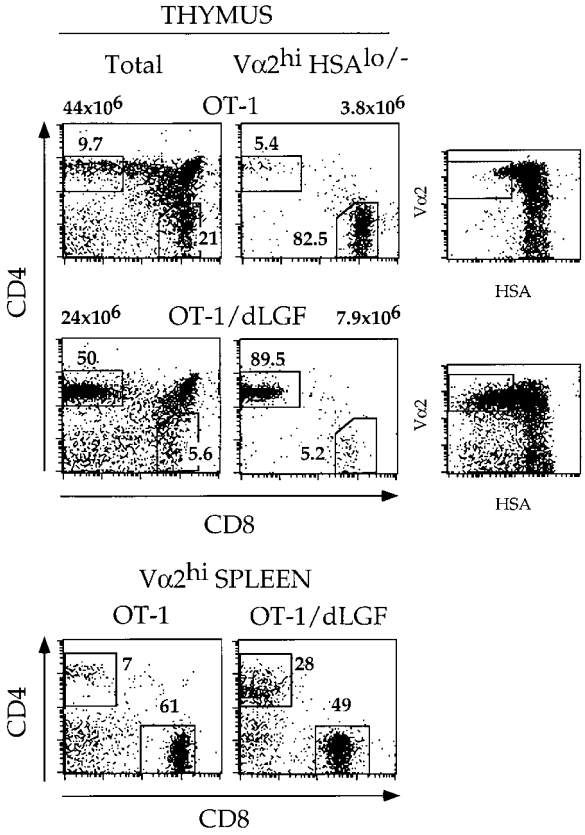


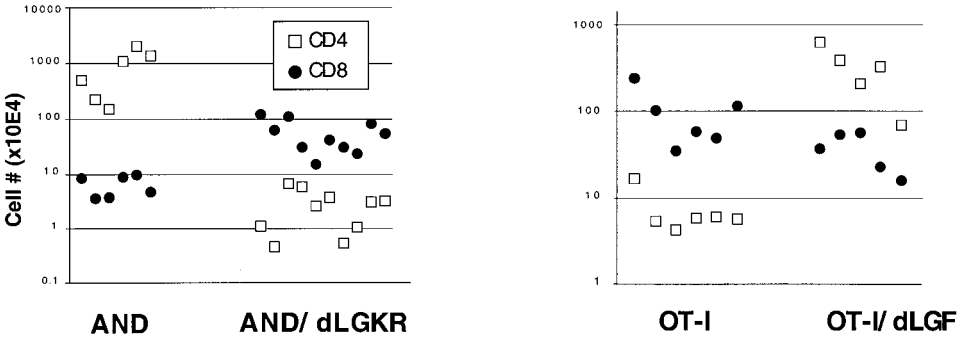
Figure 2. Increased Lck Activity Induces Class I–Restricted Thymocytes to Develop into CD4 SP Cells

Thymocytes and splenocytes from OT-I ($OT\text{-}I^{+/-}$) and OT-I/dLGF ($OT\text{-}I^{+/-}dLGF^{+/-}$) littermate mice were labeled for CD4, CD8α, TCRVα2, and HSA (thymus only) and analyzed by flow cytometry as described in Experimental Procedures. Top panels display total thymocytes and $HSA^{lo/-}V\alpha2^{+}$ thymocytes, electronically gated as shown in the smaller panels; bottom panels display $V\alpha2^{+}$ spleen cells. The numbers above the thymocyte panels represent the number of total or $HSA^{lo/-}$TCRVα2^{+}-gated live thymocytes; the percentages of CD4 and CD8 SP cells within each panel are shown. Percentages of double-positive thymocytes were 52.6% in OT-I and 22.5% in OT-I/dLGF. Animals were genotyped by PCR from tail DNA; a minimum of 10 animals of each OT-1 and OT-1/dLGF 6–12 weeks of age were analyzed, and representative results are shown.

ground (Hogquist et al., 1994). We bred $OT\text{-}I^{+/-}$ with $dLGF^{+/-}$ mice and analyzed the development of T cells in their progeny: $OT\text{-}I^{+/-}dLGF^{+/-}$ (OT-I/dLGF) and $OT\text{-}I^{+/-}dLGF^{-/-}$ (OT-I); all animals were homozygous for $H\text{-}2^{b}$. Expression of the constitutively active dLGF transgene in OT-I animals induced a dramatic decrease in the number of CD8 SP thymocytes and a substantial increase in the number of CD4 SP thymocytes (Figure 2). Most of the CD4 SP thymocytes expressed the Tg Vα2 chain. The $V\alpha2^{+}$CD4 SP population underwent complete maturation, as determined by the reduction in surface expression of HSA and their migration into the spleen, where a vast increase of the CD4 T cell population was also observed (Figure 2). Although expression of the dLGF transgene in OT-I mice led to a 2- to 3-fold reduction in the number of total thymocytes, the absolute number of mature $HSA^{lo/-}V\alpha2^{+}$CD4 SP thymocytes was increased 30- to 70-fold, whereas that of the corresponding CD8 SP population was reduced 2- to 8-fold (Figure 3). The increased generation of mature CD4 T cells modified the splenic CD8/CD4 ratio of the $V\alpha2^{+}$ cells from 8 to 1.5. The increase in peripheral $CD4^{+}$ T cells is not as striking as would be expected from the vast increase of mature CD4 T cells observed in the thymus. This may be the result of secondary effects in the periphery such as reduced survival of the CD4 T cells, as has been observed in the dLGF mice (S. J. S., unpublished data).

Analysis of Tg TCRα Expression on "Mismatched" T Cells

While forced expression of rearranged TCRα transgenes is known to induce strict allelic exclusion of endogenous TCRα genes, differentiation of DP into the "wrong"



EXP	Mouse	CD4	CD8	CD4:CD8
1	A	217.7	3.4	64.0
	A	147.0	3.6	40.8
	A/KR	0.5	28.6	0.02
	A/KR	1.0	22.1	0.05
2	A	487.0	7.9	61.6
	A/KR	5.4	28.4	0.2
	A/KR	2.5	14.2	0.2
	A/KR	3.6	38.9	0.1
3	A	1065.5	8.4	126.8
	A	1977.1	8.9	222.1
	A	1337.8	4.5	297.3
	A/KR	2.9	78.8	0.04
	A/KR	3.1	50.9	0.06

EXP	Mouse	CD4	CD8	CD8:CD4
1	O	16.5	236.1	14.30
	O/F	613.8	36.9	0.06
2	O	5.3	101.6	19.30
	O/F	379.8	52.2	0.13
3	O	5.7	112.8	19.80
	O/F	318.0	22.6	0.07

Figure 3. Total Numbers of CD4 and CD8 SP Thymocytes Obtained from Individual Animals of Each Cross

Thymocyte populations from a total of six AND, ten AND/dLGKR, five OT-I, and six OT-I/dLGF were analyzed by flow cytometry and the absolute number of $HSA^{lo/-}$ Tg $TCRV\alpha^{+}$CD4 or CD8 SP cells was calculated using the gates shown in Figures 1 and 2. In the top panels, each vertical pair of symbols represents the CD4 and CD8 SP cells found per mouse analyzed. The table shows the absolute number of CD4 and CD8 SP $HSA^{lo/-}$ Tg $TCRV\alpha^{+}$ thymocytes and the CD4:CD8 or CD8:CD4 ratio per mouse, obtained from three individual experiments of each AND (A) and AND/dLGKR (A/KR), and OT-I (O) and OT-I/dLGF (O/F). Animals within each experiment are littermates. Notice that within each individual experiment, there is a clear inversion of the numbers of mature SP thymocytes generated.

lineage could be due to rearrangement of endogenous TCRα genes, leading to surface expression of a second TCR with different specificity (Borgulya et al., 1992). The surface expression of a second TCRα chain leads to reduced surface expression of the first without altering the total levels of surface TCR expression (Heath and Miller, 1993; Hardardottir et al., 1995). Comparison of surface Vα2 or Vα11.1 and total surface TCRβ (determined by a pan-TCRβ mAb) revealed that the expression level of the Tg Vα perfectly correlated with the expression of TCRβ in all T cells (Figure 4). This remained true despite the changes imposed by alterations in Lck activity (increased TCR expression in dLGKR animals and decreased expression in dLGF animals) or lineage of the cells (CD4 T cells express higher levels of TCR than CD8 T cells in wild-type mice; data not shown). Furthermore, if the expression of a TCR with an alternative specificity had allowed these cells to develop into the wrong lineage, the expression of this alternative TCRα chain would be expected to be preferred during the selection process. These results indicate that the "mismatched" Tg $V\alpha^{+}$ cells bear mainly the Tg TCR receptor.

CD8 T Cells in AND/dLGKR Mice Behave as Competent Cytotoxic T Cells

The defining feature of lineage commitment is the programming of CD4 and CD8 T cells with helper and cytotoxic functions, respectively. Therefore, to determine whether the phenotypic differentiation of cells into the "wrong" lineage was associated with the corresponding changes in functional differentiation, we tested some of these functional characteristics. Perforin expression, which is characteristic of cytotoxic CD8 T cells, can be detected at much higher levels in mature, unstimulated CD8 SP thymocytes than in mature CD4 SP thymocytes (Vandekerckhove et al., 1994; Wang et al., 1998). Therefore, to determine if AND/dLGKR CD8 SP thymocytes were committed to the cytotoxic lineage, we sorted $V\alpha11.1^{+}$CD8 SP thymocytes from AND/dLGKR mice, $V\alpha11.1^{+}$CD4 SP thymocytes from AND mice, wild-type TCR^{hi}CD4, and CD8 SP thymocytes and compared their perforin expression by RT–PCR. CD8 SP cells from AND/dLGKR mice expressed perforin levels comparable to those found in wild-type CD8 SPs (Figure 5A, left panel). Under the same conditions, perforin expression was not detected in wild-type or AND CD4 SPs, and upon further PCR amplification was detected at much lower levels in these populations (data not shown; Wang et al., 1998). Similarly, sorted OT-I $V\alpha2^{+}$ CD8 SP expressed perforin at similar levels to that of wild-type CD8 SPs, while OT-I/dLGF $V\alpha2^{+}$ CD4 SP thymocytes did not express detectable perforin under these conditons (Figure 5A, right panel). Since cytotoxicity is the hallmark of CD8 T cells, we also tested the ability of the CD8 splenocytes generated in AND/dLGKR mice to mediate PCC peptide class II $I\text{-}E^{k}$–restricted killing after in vitro stimulation

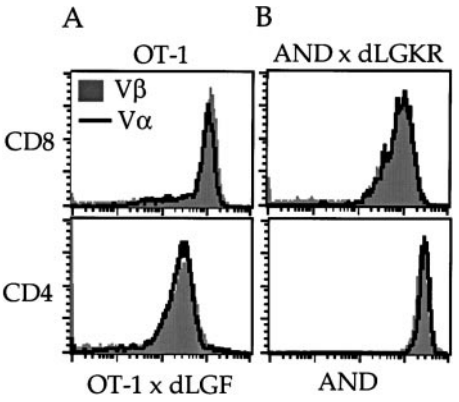


Figure 4. Comparison of the Surface Expression of Tg TCRVα and Total TCRβ Chains in Double Transgenic Mice

(A) Thymocytes from OT-I and OT-I/dLGF mice were labeled for CD4 and CD8 and either Vα2 or TCRβ. The histograms show the overlay of the expression of Vα2 and TCRβ on gated CD8 SP OT-I or CD4 SP OT-I/dLGF thymocytes.

(B) Thymocytes from AND and AND/dLGKR mice were labeled as in (A) except that anti-Vα11.1 was used instead. The histograms show the overlay of the expression of Vα11.1 and TCRβ on gated CD4 SP AND or CD8 SP AND/dLGKR thymocytes.

Fine-line histograms show Tg Vα chain; filled histograms show TCRβ chain. Analyses were performed as described in Experimental Procedures.

with peptide and APCs. In spite of the lack of appropriate coreceptor expression and reduced Lck activity, splenocytes from AND/dLGKR mice killed PCC-loaded I-E^k tumor cells (A2252, H-$2^{b/k}$), while spleen cells from AND mice did not (Figure 5B). The rate of killing was comparable to that of OT-I splenocytes against the same tumor cells loaded with OVA peptide (Figure 5B). Thus, the CD8 SP thymocytes generated in AND/dLGKR mice express perforin at levels similar to those expressed by wild-type CD8 SP thymocytes and behave as fully competent cytotoxic T cells.

CD4 T Cells in OT-I/dLGF Mice Respond to Cognate Antigen by Upregulating CD40-L

Activation of helper CD4 T cells triggers the transient surface expression of CD40-L, which is required for appropriate costimulation between B and T cells (Spriggs et al., 1992; Roy et al., 1993) and has been used previously as a test of helper activity in CD4 T cells (Robey et al., 1994). We stimulated OT-I, OT-I/dLGF, and wild-type splenocytes with plate-bound anti-CD3 and anti-CD28 or with their cognate peptide OVA and used flow cytometry to analyze the surface expression of CD40-L on CD4 and CD8 T cells. After stimulation with CD3 + CD28, we detected CD40-L expression almost exclusively on CD4 cells, of which 30%–40% were positive for CD40-L. OT-I/dLGF CD4 TCRV$\alpha 2^+$ cells and wild-type CD4 T cells showed similar patterns of CD40-L expression (Figure 6). Conversely, CD8 T cells from wild-type and Tg mice showed very little (<5%) or no expression of CD40-L. Stimulation with OVA peptide also induced CD40-L expression on CD4 cells of OT-I/dLGF but not wild-type mice, demonstrating specific recognition by the OT-I TCR. Thus, the CD4 lymphocytes generated in OT-I/dLGF mice display functional characteristics of CD4 T cells and respond to their cognate peptide even in the absence of the CD8 coreceptor, indicating that alteration in Lck activity induces a complete shift in the maturation program.

The Reciprocal Mouse Crosses OT-$I^{+/-}$dLGKR$^{+/-}$ and AND$^{+/-}$dLGF$^{+/-}$ Do Not Display Changes in Thymocyte Lineage Decision

To test that the effects we observed were dependent on the MHC restriction of the developing thymocytes, we analyzed the thymocyte populations of the reciprocal mouse crosses OT-$I^{+/-}$dLGKR$^{+/-}$ and AND$^{+/-}$dLGF$^{+/-}$ (Figure 7). The expression of the dLGF transgene in AND mice led to changes in levels of CD4 and CD8 expression, characteristic of the dLGF transgene, but did not lead to changes in lineage commitment. Expression of the dLGKR transgene in OT-I mice, similarly, did not change the expected fate of the cells.

Discussion

The objective of the present study was to test directly the role of Lck signaling in CD4/CD8 lineage determination. For this purpose, we crossed mouse lines that express catalytically inactive (dLGKR) or constitutively active (dLGF) Lck under the control of the *lck* distal promoter with mouse lines expressing class II–restricted

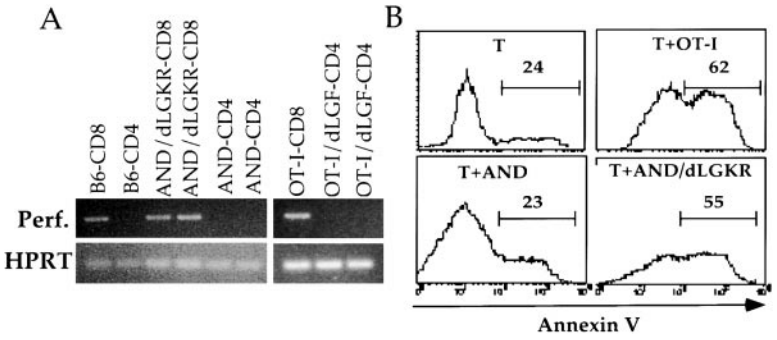


Figure 5. Commitment of AND/dLGKR CD8 T Cells to the Cytotoxic Lineage

(A) Perforin expression of CD8 thymocytes. Vα11.1^{hi}CD4 SP thymocytes from AND mice, Vα11.1^{hi}CD8 SPs from AND/dLGKR mice, Vα2^{hi}CD8 SPs from OT-I mice, and Vα2^{hi} CD4 SPs from OT-I/dLGF mice were sorted and analyzed by RT–PCR for their expression of perforin in samples normalized for HPRT expression. Sorted CD4 and CD8 SP thymocytes from wild-type C57BL/6 (B6) mice were included for comparison.

(B) AND/dLGKR CD8 T cells display cytotoxic acitivity. Stimulated AND, AND/dLGKR, and OT-I splenocytes were incubated for 12 hr with peptide-loaded H-$2^{b/k}$ target cells (T) at an effector:target ratio of 10:1 and then analyzed by staining cells with annexin V and anti-H-$2K^k$. The panels show the percentage of annexin V^+ cells in electronically gated target cells. Annexin V staining of target cells in the absence of peptide was similar to that of background. The AND effector population was composed of >90% CD4 Vα11.1^+ cells, and the AND/dLGKR effectors were composed of 45% Vα11.1^+CD8^+, 10% Vα11.1^+CD4^-CD8^-, 10% Vα11.1^+CD4^+, and 35% Vα11.1^- cells.

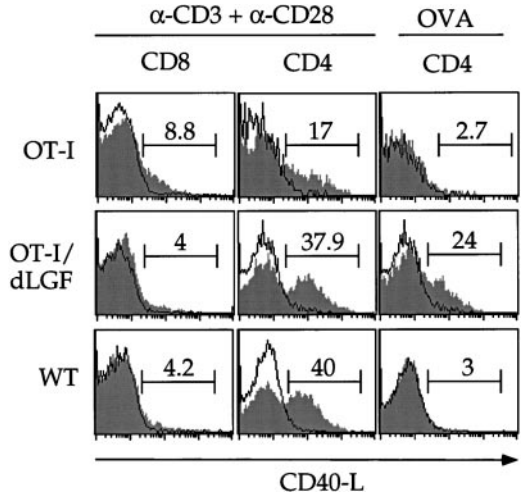


Figure 6. Upregulation of CD40-L upon Stimulation of OT-I/dLGF CD4 T Cells

OT-I, OT-I/dLGF, and wild-type splenocytes stimulated with plate-bound anti-CD3 and anti-CD28 (two first columns) or 10 μM OVA peptide (last column) were labeled for CD4, CD8, TCRVα2, or TCRβ (wild-type cells only), and CD40-L for flow cytometric analysis. Filled histograms show the expression of CD40-L on CD8 Vα2$^+$OT-I and OT-I/dLGF cells; CD4 Vα2$^+$OT-I and OT-I/dLGF cells; and on CD8 or CD4 wild-type cells. The percentage of CD40-L$^+$ cells is shown in each case. The fine-line histograms show the corresponding CD40-L expression in cultured, unstimulated cells. The percentage of unstimulated cells expressing CD40-L was <3.5%.

(AND) or class I–restricted (OT-I) Tg TCRs. This allowed us to increase or decrease the levels of Lck activity during positive selection. There are three critical features of the transgenic models employed in these experiments: (1) the *lck* transgene-encoded proteins are

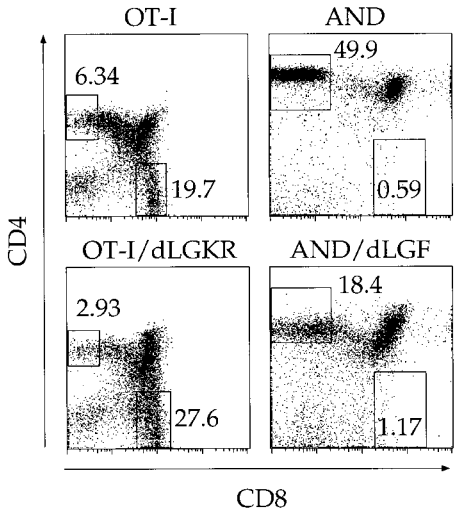


Figure 7. The Reciprocal Mouse Crosses OT-I/dLGKR and AND/dLGF Do Not Display Changes in Thymocyte Lineage Decision

Thymocytes from OT-I$^{+/-}$ and OT-I$^{+/-}$dLGKR$^{+/-}$ or AND$^{+/-}$ and AND$^{+/-}$dLGF$^{+/-}$ littermate mice were labeled for CD4, CD8α, TCRVα2, or Vα11.1 and analyzed by flow cytometry as described in Experimental Procedures. The absolute numbers of thymocytes were similar in all animals. When cells were gated for TCRα^{hi} small cells, OT-I/dLGKR mice had a small decrease in the numbers of mature CD8 cells (10%) and AND/dLGF mice had a 40% decrease in the numbers of mature CD4 cells, with respect to controls.

expressed at 1.6- (dLGKR; Hashimoto et al., 1996) and 0.7- (dLGF; S. J. S. et al., unpublished data) fold levels over wild-type Lck, that is, within a narrow range that does not completely block positive selection; (2) the *lck* distal promoter maintains expression of the transgenes throughout positive selection, which is essential because full effector function develops only at the latest stages of positive selection (Ramsdell et al., 1991); (3) the TCR Tgs used can function in a coreceptor-independent manner (Matechak et al., 1996; Goldrath et al., 1997), which allows the functional characterization of the mature T cells even in the absence of the appropriate coreceptor.

Our results show that class II–restricted AND T cells develop preferentially into the CD8 lineage in the presence of a catalytically inactive Lck, whereas increased Lck activity results in the preferential development of class I–restricted OT-I T cells into the CD4 lineage. Furthermore, the class II–restricted CD8 cells generated in the AND/dLGKR mice behave functionally as killer cells, indicating that the change in surface phenotype is associated with the corresponding change in function. Similarly, the class I–restricted CD4 cells generated in the OT-I/dLGF mice respond to cognate stimulation by upregulating CD40-L, as is characteristic of T helper cells (Robey et al., 1994).

We examined the possibility that the mismatched cells (CD8 in AND mice and CD4 in OT-I mice) we observed appeared simply as a consequence of the inhibition in the development of the normal populations (CD4$^+$ in AND mice and CD8$^+$ in OT-I mice) and the survival of preexisting mismatched cells. However, the numbers of mismatched mature HSA$^{lo/-}$ cells with high expression of the Tg TCR were dramatically increased, indicating that our observations were not due to a selective survival of a small population of mismatched cells present in AND and OT-I mice. It is also formally possible that the increase in absolute numbers of mismatched cells was due to the abnormal expansion of a preexisting population. Several lines of evidence argue against this. First, positive selection in adult mice occurs in the almost total absence of proliferation (Hare et al., 1998). Expression of the dLGF Tg does not lead to aberrant proliferation of DP or SP thymocytes, as no increase in cells at the G2/M stage of the cell cycle was observed in either of these two populations (S. J. S., unpublished data). Second, although it could be argued that expression of the constitutively active Lck Tg could lead to undetectable but significant levels of proliferation, expression of a catalytically inactive Lck Tg would certainly not promote proliferation. Third, the fact that the experiment works in both directions makes it highly unlikely that they result from proliferation artifacts. It should be noted that both the OT-I and AND mice have lower numbers of thymocytes than wild-type mice and that the addition of the Lck transgenes decreased the numbers further. This likely reflects an increase in cell death caused by the extreme conditions under which thymocytes were forced to develop.

It should be emphasized that the effect of Lck activity on lineage determination may depend on the intrinsic affinity of the TCR. dLGKR mice develop both CD4 and CD8 T cells (Hashimoto et al., 1996). Thus, reduced Lck activity per se does not limit development of DP thymocytes to the CD8 lineage. Conversely, increased Lck

activity per se does not limit development to the CD4 lineage, as dLGF mice develop CD8 T cells as well (data not shown). Therefore, choice of lineage is most likely determined by the combination of (1) a particular TCR bearing a certain intrinsic affinity for its selecting MHC + peptide and (2) the strength of the Lck signal during positive selection, as has been previously proposed (Matechak et al., 1996; Bommhardt et al., 1997; Basson et al., 1998). This hypothesis would predict that changing the CD4/CD8 lineage determination of cells expressing different TCRs is likely to require different amounts of Lck activity as long as the overall Lck activity is within the window permissive for positive selection. This would explain results of a previous study that used a different class II–restricted TCR (DO11.10; Hashimoto et al., 1996). The presence of the dLGKR Tg did not result in the generation of CD8 cells; instead, only a reduction in the frequency of mature CD4 T cells was observed. Thus, differences in the intrinsic affinity of different TCRs for their selecting MHC + peptide ligand in the thymus (Goldrath et al., 1997) or in the precise developmental regulation of expression of different TCR transgenes could affect the outcome if tested in our system.

The biochemical basis of the cross-talk between TCR and coreceptor is unknown. Some Lck activity is probably necessary to initiate signaling through the TCR (Straus and Weiss, 1992; Iwashima et al., 1994; van Oers et al., 1996), but the coreceptor-associated Lck is not absolutely necessary for this processs, as shown in experiments where tailless coreceptors rescue development of T cells (I. T. Chan et al., 1993; Killeen and Littman, 1993). Besides phosphorylating the ITAM motifs in the CD3 complex, Lck signaling may have other specific roles. For example, signaling through the TCR in a Jurkat Lck null mutant can be reconstituted by transfection of Lck. If an Lck mutant with a nonfunctional SH3 domain is used instead of wild-type Lck, Zap-70 activation and recruitment of some effectors (PLC-γ) is rescued, but other signal transduction pathways like MAPK are not activated (Denny et al., 1999), suggesting that the SH3 domain of Lck contributes something unique to signaling through the TCR complex. One can postulate that, following the recruitment of ZAP-70 to the TCR, the intensity of the signal emanating from the TCR/coreceptor complex through ZAP-70 is proportional to the affinity of this receptor for its ligand, MHC + peptide. If so, the amount of Lck signal present in the complex, different depending on which coreceptor is associated and possibly recruiting different downstream effectors, could be measured against the ZAP-70 signal to regulate specific downstream events or patterns of gene expression that would contribute to specify the lineage fate of the developing thymocyte.

To understand in molecular terms how developing T cells interpret quantitative differences in Lck signals to determine their lineage choice, the downstream efffectors of Lck involved in this process need to be identified. In some recent experiments, alterations in Erk activation by expression of a hypersensitive Erk allele (SM; Sharp et al., 1997) or by addition of Mek inhibitors to fetal thymic organ cultures (Sharp et al., 1997; Bommhardt et al., 1999) resulted in an increased development of CD4 cells when Erk activity was increased and of CD8 cells when it was decreased. These results suggest that activation of the Ras/MAPK cascade may be downstream of Lck for the control of this cell fate decision. This is also indirectly supported by biochemical evidence that shows that an intact Lck is necessary to activate MAPK in Jurkat cells (Denny et al., 1999). However, overexpression of DN Ras and Mek blocked development of both CD4 and CD8 T cells, and no clear preferential effect was observed (Alberola-Ila et al., 1996). Expression of the hypersensitive Erk allele SM did not result in generation of CD4 T cells in the context of the OT-I TCR (Sharp et al., 1997). Furthermore, the Mek inhibitors used in the experiments described above have proved to be less specific than previously thought (Kamakura et al., 1999). It is possible that there are other downstream mediators of Lck besides Erk and that for the full effect, changes in Erk activation have to be balanced with changes in activation of these other mediators.

To explain how MHC specificity and coreceptor expression are coupled during T cell development, the instructional model proposes that the recognition and coengagement of class I MHC by a specific TCR and CD8 directs the turn-off of CD4, thus generating a CD8 T cell. Recognition and coengagement of class II by a specific TCR and CD4 promotes the downregulation of CD8, producing a CD4 T cell. This model has been further refined in recent years by ascribing the discrimination between CD4 and CD8 coreceptor contributions to a quantitative difference in the signals provided by these coreceptors. Therefore, it has been proposed that the amount of Lck signal brought by the coreceptor into the TCR complex could play a central role in CD4/CD8 lineage committment (Itano et al., 1996; Matechak et al., 1996; Bommhardt et al., 1997; Goldrath et al., 1997; Basson et al., 1998). Our experiments directly identify Lck activity as one of the determining factors in this decision and strongly support this quantitative instructional model to explain CD4/CD8 lineage commitment of T cells.

Still, it is formally possible that in the case of the AND/dLGKR mice, reduced Lck activity could allow or promote the development of stochastically CD8-committed AND T cells, while blocking that of stochastically CD4-committed T cells, rather than instructing AND DP cells that would normally become CD4 T cells, to become CD8 T cells. The same applies to the complementary case. We would have to postulate that the Lck signal provides a survival signal for the stochastically committed cells and that a strong signal is permissive for CD4-committed cells and a weak signal for CD8-committed cells. As the majority of all DP thymocytes never develop into SP cells, it is not possible to discard the latter possibility completely, although we think that the instructional model provides a more parsimonious explanation for our results. To clarify this issue, the stage at which Lck activity normally operates during positive selection to determine lineage committment needs to be elucidated, as does the "point of no return," where changes in Lck activity can no longer change the fate of the cells. Expression of a constitutively active Lck transgene under the control of the proximal instead of the distal Lck promoter in OT-I mice does not lead to the substantial generation of class I–restricted CD4 T cells that we observed (Sharp and Hedrick, 1999). As

transcription from the proximal Lck promoter is downregulated at the onset of positive selection, it appears that prolonged exposure to the Lck signal during positive selection is required for effective CD4 lineage commitment to occur.

In conclusion, our results indicate that changes in Lck activity during positive selection result in changes of CD4/CD8 lineage determination. Therefore, developing thymocytes can interpret quantitative differences in the Lck signal they receive and translate them into lineage determination decisions. These results provide direct evidence to support the notion that the coreceptors inform the CD4/CD8 lineage decision by virtue of the different amounts of Lck they bring to the TCR/CD3 complex during positive selection.

Experimental Procedures

Mice

AND (B6, SJL-TgN(TcrAND)53Hed, the Jackson Laboratory); OT-I; C57BL/6; dLGF (see below); dLGKR, all homozygous for H-2^b; and C3H (H-2^k) strains were maintained at Caltech under SPF conditions. Animals were genotyped by PCR from tail DNA. dLGF Tg mice (S. J. S. et al., unpublished data): the *lck* gene containing the F505 mutation and a 0.6 kb fragment of the 3′ human growth hormone gene was isolated from a previously reported construct, pLGF (Abraham et al., 1991), and cloned into pW120, the vector containing the *lck* distal promoter (Wildin et al., 1995). The A-to-T point mutation converting the tyrosine to a phenylalanine and the joining region of the distal promoter and the lck gene were verified by nucleotide sequencing. The transgene DNA was microinjected into fertilized (C57BL/6 × DBA2)F2 embryos to generate transgenic founders. Transgene integration was determined by Southern blot analysis of tail DNA, and four founders were backcrossed with C57BL/6 animals to establish transgenic lines. The ratio of F505Lck to wild-type Lck in the A16924 line used in these experiments is 0.7. dLGF transgenic animals appear healthy and do not develop tumors of the thymii or peripheral lymphoid organs (up to 5 months of age). The thymus cellularity is normal, but thymii from dLGF transgenic animals contain 3-fold as many single-positive thymocytes as wild-type thymii.

Flow Cytometric Analysis

Thymus and erythrocyte-depleted spleen cell suspensions of 5- to 12-week-old mice were prepared. Cells were stained with monoclonal antibodies for CD4 (GK1.5), CD8α (53-6.7), Vα2 (B20.1), Vα11.1 (RR8-1), pan-TCRβ (H57-597), HSA (M1/69), CD40-L (MR1), and H-2K^k (AF3-121) and annexin V, as indicated, and analyzed by flow cytometry in a Becton Dickinson FACScalibur. A total of 60,000 (Figures 1–4), 30,000 (H-2K^{k+}) (Figure 5B), and 250,000 (Figure 6) events per sample were collected for analysis. In all cases, except Figure 5B, live cells were electronically gated using forward and side scatter parameters. In Figure 4, we selected a combination of Vα2, Vα11.1, and TCRβ antibody batches that showed similar mean fluorescence intensity at saturating concentrations to simplify comparison.

Perforin Detection

CD8 SP (CD8$^+$CD4$^-$Vα11.1^{hi}) cells from four individual AND/dLGKR mice, CD4 SPs (CD4$^+$CD8$^-$Vα11.1^{hi}) from four AND mice, CD8 SPs (CD8$^+$CD4$^-$Vα2^{hi}) from two OT-I mice, and CD4 SPs (CD4$^+$CD8$^-$TCRVα2^{hi}) from four OT-I/dLGF mice were sorted from freshly stained thymocytes in a Becton Dickinson FACSvantage SE at the Caltech cell-sorting facility. The purity of the various sorted populations was always ≥95%, as determined by after-sort analyses. RT–PCR conditions and perforin and HPRT primers were as previously described (Wang et al., 1998). HPRT primers were used to amplify products from serially diluted cDNA samples, and cycle numbers were minimized to prevent saturation of the PCR reaction. Perforin and HPRT products were amplified for 38 and 34 cycles, respectively. Approximately, 1/200 of the cDNA obtained from 50,000 sorted cells was used per PCR reaction. To ensure reproducibility of the results, each reaction was performed at least three times.

Cytotoxic Assays

Erythrocyte-depleted splenocytes were stimulated for 5 days in vitro, in DMEM 10% FCS, as follows: AND and AND/dLGKR cells were cultured with irradiated (2,000 rads) C3H (H-2^k) spleen cells (at a 1:1 ratio) with 10 μM PCC (KAERADLIAYLKQATAK) peptide; OT-I spleens were cultured with 10 μM OVA (SIINFEKL) peptide. Viable cells were then isolated using Ficoll-isopaque gradients and cultured with peptide-loaded or -unloaded H-$2^{b/k}$ tumor cells A2252 (Klinken et al., 1988) at a 10:1 ratio for 12 hr, after which cells were analyzed by flow cytometry as indicated to determine the percentage of apoptotic cells in the target population (Goldberg et al., 1999).

CD40-L Expression Assays

Fresh erythrocyte-depleted splenocytes were cultured at 10^7 cells/well in 200 μl of medium and in flat-bottom 96-well plates in the presence of plate-bound anti-CD3 and -CD28 monoclonal antibodies or 10 μM OVA peptide for 4 hr at 37°C and analyzed by flow cytometry as indicated. The time for in vitro stimulation was chosen based on preliminary experiments showing maximum expression of CD40-L on CD4 T cells at 4 hr. CD8 T cells showed very little (<5%) or no expression of CD40-L at all time points tested. Plates were coated with 75 μl of anti-CD3ε (145-2C11) and anti-CD28 (37.51) at 10 μg/ml each in PBS for 1 hr at 37°C and then washed three times with medium before plating the cells.

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