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Regulation of Thymocyte Development through CD3: Functional Dissociation between p56^{lck} and CD3ζ in Early Thymic Selection

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Summary

We studied the extent of functional linkage between CD3ζ and p56^{lck} in pre-TCR-dependent thymocyte development. Differentiation of DN to DP cells was examined by treatment of *RAG2*/CD3ζ and *RAG1*/p56^{lck} double-deficient mice with anti-CD3ε antibodies. The results suggest that CD3ζ has no specific role in this maturation step, but may be important for amplification of signaling through the pre-TCR. In contrast, p56^{lck} is the main protein tyrosine kinase associated with signaling through the pre-TCR–CD3 complex. In DP thymocytes, the Ca^{2+} response to anti-CD3ε was totally abolished in CD3ζ$^{-/-}$ but only reduced in p56$^{lck-/-}$ mice, and in vivo responses to anti-CD3ε differed from one another. Thus, CD3ζ and p56^{lck} are functionally not tightly associated and their deficiencies cause distinct developmental defects.

Introduction

T cells develop in the thymus, where they undergo multiple steps of differential gene expression and gene rearrangements. Cells having successfully completed one step are selected to mature to the next. At an early stage, defined by the expression of the interleukin 2-Rα (IL-2Rα) chain, thymocytes attempt to produce a functional T cell receptor β (TCRβ) chain gene by random rearrangement of the TCRβ V, D, and J gene segments (Spits, 1994). If rearrangement was successful, the pre-TCR is expressed on the cell surface, consisting of the TCRβ chain, dimerized with the pre-TCRα chain, and members of the CD3 complex (Groettrup et al., 1993; Groettrup and von Boehmer, 1993; Saint-Ruf et al., 1994). The surface expression of the pre-TCR, alone or by interaction with a ligand on thymic stroma cells, selects thymocytes with a functional TCRβ chain to continue maturation. This results in allelic exclusion by arrest of further rearrangement of the TCRβ locus, a burst of cell divisions, down-regulatio of the IL-2Rα chain, and the expression of the coreceptor CD4 and CD8 (Groettrup and von Boehmer, 1993; Leve and Eichmann, 1993). During the CD4$^+$CD8$^+$ double positive (DP) stage, rearrangements in the TCRα locu take place. Thymocytes with a functionally rearrange TCRα chain gene are then selected according to the spec ficity of their mature αβ TCR (Kisielow et al., 1988; Roth enberg, 1994). Thymocytes that are potentially self-reac tive are negatively selected and clonally deleted b apoptosis. Nonselected thymocytes also die. Thymocyte that are self-restricted are positively selected and continu maturation into CD4$^+$ single-positive (SP) or CD8$^+$ SP cells depending on the restriction of their TCR. The differenc between positive and negative selection is most probabl dictated by quantitative parameters of the interactions be tween the TCR and the selecting ligands (Hogquist et al 1994; Ashton-Rickardt et al., 1994), resulting in distinc signal intensities (Eichmann, 1995).

Mice that carry mutations leading to the failure to pro duce a TCRβ chain, such as *scid* mice (Schuler et al 1986), mice deficient for *RAG1* (Mombaerts et al., 1992a or *RAG2* (Shinkai et al., 1992), and mice with a null muta tion in the TCRβ chain genes (Mombaerts et al., 1992b show a nearly complete block in the maturation o CD4$^-$CD8$^-$ double-negative (DN) thymocytes to the D stage. Less severe deficiencies are observed in mice def cient for molecules that are involved in signal transductio through the TCR. For example, mice that are deficient fo CD3ζ (Liu et al., 1993; Ohno et al., 1993; Malissen et al 1993; Love et al., 1993) have a small thymus, with 5% 15% of the normal number of DP thymocytes. Furthe more, positive selection is impaired, with virtually no S cells in the thymus. A phenotypically similar incomplet developmental block is observed in mice deficient fo p56^{lck} (*lck*) (Molina et al., 1992). In contrast, mice that ove express a dominant-negative form of *lck* show a complet block of thymocyte development at the DN stage (Levi et al., 1993), presumably because of competition for adc tional PTK substrates. PTKs are likely also to play a rol in the regulation of allelic exclusion, as a TCRβ transgen does not lead to allelic exclusion in mice overexpressin dominant-negative *lck* (Anderson et al., 1993).

In previous work, we have shown that cross-linking CD3ε on DN thymocytes accelerated their maturation DP thymocytes, whereas cross-linking of CD3ε on DP th mocytes induced their deletion (Levelt et al., 1993a). Mor over, cross-linking of CD3ε on DN thymocytes of TCR chain–deficient mice restored their maturation to the D stage (Levelt et al., 1993b). These studies suggested th signaling events through the CD3 complex controlle these early as well as the late selection events. Here, w analyzed mice deficient for CD3ζ and for *lck*, asking th question as to what extent these components of CD mediated signaling are functionally connected with or another during the early pases of thymic developmen

Mice were bred to become double deficient for *RAG1* (or *RAG2*) and either *lck* or CD3ζ, and double-deficient newborn mice were treated with anti-CD3ε, thus addressing the role of CD3ζ and *lck* in the differentiation of DN to DP thymocytes. Furthermore, by studying CD3ζ and *lck* single-deficient mice, we analyzed the responses of immature DP thymocytes upon CD3 cross-linking. The results suggest that CD3ζ and *lck* are involved in partially independent signal transduction pathways with divergent functions in pre-TCR-dependent thymic selection.

Results

Signaling through CD3ζ Is Not Essential for Early Thymocyte Maturation

Newborn F2 generation offspring from intercrosses between CD3ζ-deficient and *RAG2*-deficient parental mice were injected with 10 μg/g bodyweight anti-CD3ε monoclonal antibody (MAb), 1 day after birth. Thymocytes were isolated at day 8 after birth and the mice were typed by intracellular staining for CD3ζ (Levelt et al., 1993c) and by reverse transcription polymerase chain reaction (RT-PCR) for *RAG2*. Remaining thymocytes were stained for CD4, CD8, and IL-2Rα. In Figure 1, the effects of anti-CD3ε MAb treatment on thymic development in *RAG2*-deficient (E–H) and *RAG2*/CD3ζ double-deficient mice (A–D) are shown. As expected from our previous results on *RAG1*-deficient mice (Levelt et al., 1993b), anti-CD3ε MAb caused full induction of CD4/CD8, down-regulation of IL-2Rα, and a substantial increase in cell numbers in *RAG2*-deficient thymi. No significant differences in any of these parameters were detected between *RAG2*-deficient mice and CD3ζ$^{-/-}$/*RAG2*$^{-/-}$ double mutants. Absolute cell numbers of thymocytes in the anti-CD3ε MAb-treated double-mutant mice were similar to those found in untreated wild-type newborn mice, and four to six times greater than those found in untreated CD3ζ single-deficient mice (see Table 1). CD3ζ single-deficient mice also showed full induction of DP cells (see below). These results suggest that the defect in thymocyte development observed in CD3ζ-deficient mice can be overcome by potent signaling through CD3γδε alone. The signaling function of CD3ζ

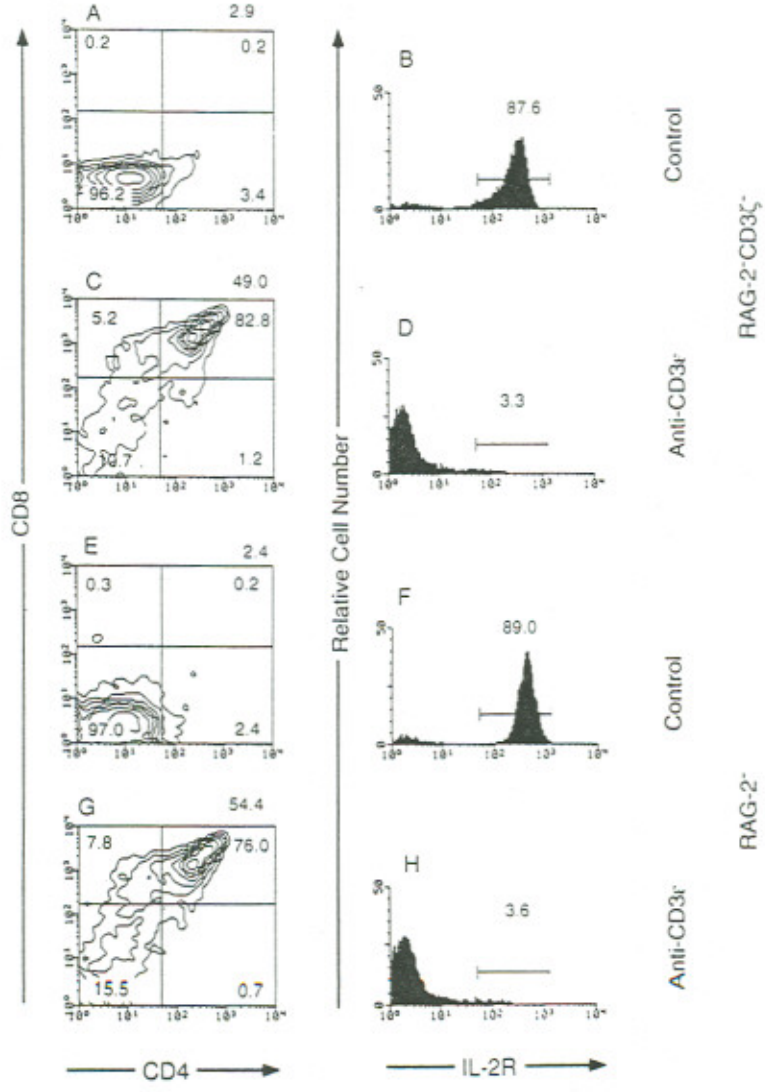

Figure 1. Down-Regulation of IL-2Rα and Induction of CD4 and CD8 on *RAG2*$^{-/-}$/CD3ζ/η$^{-/-}$ Double-Mutant Thymocytes

RAG2$^{-/-}$ newborn mice (E–H) and *RAG2*$^{-/-}$/CD3ζ/η$^{-/-}$ newborn mice (A–D) were injected with anti-CD3ε MAb at day 1 after birth, and thymocytes were analyzed at day 8 after birth for expression of CD4, CD8, and IL-2Rα. Contour plots at the left (A, C, E, and G) represent fluorescence intensities of CD4 and CD8. Histograms at the right (B, D, F, and H) represent the fluorescence intensity of IL-2Rα. Absolute cell numbers (× 10^{-6}) are indicated above the contour plots. Control *RAG2*$^{-/-}$ (E and F) and *RAG2*$^{-/-}$CD3ζ/η$^{-/-}$ (A and B) thymi show that development is blocked at the CD4$^-$CD8$^-$ DN IL-2Rα$^+$ stage. Treatment with anti-CD3ε MAb reconstitutes thymocyte maturation to the CD4$^+$CD8$^+$ DP stage completely in both *RAG2*$^{-/-}$ (G) and *RAG2*$^{-/-}$CD3ζ/η$^{-/-}$ (C) mice. Down-regulation of IL-2Rα is complete in both mouse strains (D and H), and absolute cell numbers increase to ≈50 × 10^6.

Table 1. Total Thymocytes in Wild-Type and Mutant Mice after Injection of Anti-CD3ε MAb* (× 10^6)

Mice	Day after injection 0	1	2	3
WT	58.1 ± 4.6	37.2 ± 5.1	26.4 ± 9.0	5.4 ± 3.3
CD3ζ$^-$	10.7 ± 1.5	22.7 ± 3.8	34 ± 8.5	59 ± 2
Lck$^-$	13 ± 1.6	11.5 ± 1.5	18 ± 2	13.8 ± 0.6
RAG2$^-$	2.9 ± 0.3	3.9 ± 0.9	18 ± 2	31.7 ± 4.9

* Results are means ± SEMs of 2–6 mice for each data point.

during early thymocyte differentiation is therefore either redundant or merely an amplification of the CD3γδε signal. In addition, CD3ζ may increase the surface expression of the immature TCR.

lck Is Important for Efficient Signal Transduction through CD3 during Early Thymocyte Differentiation

The results on CD3ζ-deficient mice suggested that CD3γδε is sufficient for the early signal that induces maturation to the DP stage. If this signal was mediated by PTKs other than *lck*, *lck*-deficient thymi should be fully inducible by anti-CD3ε. This was tested by injecting *RAG1*$^{-/-}$/*lck*$^{-/-}$ double-mutant mice with anti-CD3ε MAb at day 1 after birth. The newborn mice were typed by PCR of tail DNA, and flow cytometry of thymocytes was done on day 8 after birth. As for *RAG2*-deficient mice, expression of CD4 and CD8, down-regulation of IL-2Rα, and proliferation are induced upon anti-CD3ε MAb treatment in thymocytes of *RAG1*-deficient mice (Figures 2E–2H). In contrast *RAG1*$^{-/-}$/*lck*$^{-/-}$ double-mutant mice showed only a poor reconstitution of early thymocyte development upon anti-CD3ε treatment. Down-regulation of IL-2Rα was incomplete (Figures 2B and D), and the absolute number of DP cells was less than 15% of that in thymi of anti-CD3ε MAb-treated *RAG1*-deficient mice (Figures 2A, 2C, and 2G). These results suggest that *lck* is an important element in signal transduction through both the CD3ζ and the CD3γδε modules during this stage of development.

We found that one of the earliest parameters of induction by anti-CD3ε MAb is the expression of CD69, which coincides with the down-regulation of IL-2Rα in *RAG2*-deficien

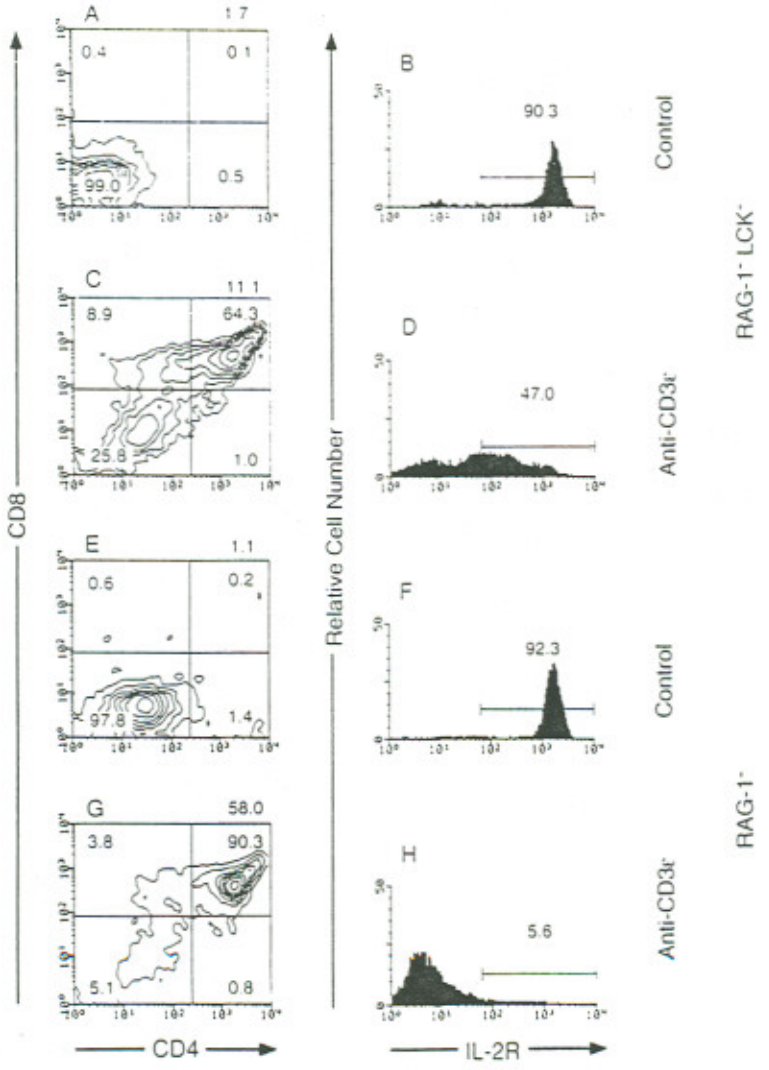

Figure 2. Inefficient Down-Regulation of IL-2R and Induction of CD4 and CD8 in *RAG1*$^{-/-}$/*lck*$^{-}$ Double-Mutant Thymocytes

RAG1$^{-/-}$ newborn mice (E–H) and *RAG1*$^{-/-}$/*lck*$^{-}$ newborn mice (A–D) were injected with anti-CD3ε MAb at day 1 after birth, and thymocytes were analyzed at day 8 after birth for expression of CD4, CD8, and IL-2Rα. Contour plots at the left (A, C, E, and G) represent fluorescence intensities of CD4 and CD8. Histograms at the right (B, D, F, and H) represent the fluorescence intensity of IL-2Rα. Absolute cell numbers (× 10^{-6}) are indicated above the contour plots. Control *RAG1*$^{-/-}$ (E and F) and *RAG1*$^{-/-}$/*lck*$^{-/-}$ (A and B) thymi show that thymocyte development is blocked at the DN IL-2Rα$^+$ stage. Treatment with anti-CD3ε MAb reconstitutes thymocyte maturation to the DP stage completely in *RAG1*$^{-/-}$ (G) mice, accompanied by down-regulation of IL-2Rα (H) and an increase of the absolute cell number to 58 × 10^6. In *RAG1*$^{-/-}$/*lck*$^{-/-}$ thymi, the reconstitution of thymocyte development by anti-CD3ε MAb treatment is incomplete. A smaller proportion of thymocytes reaches the DP stage (C), down-regulation of IL-2Rα is inefficient (D), and the absolute cell number reaches only 11 × 10

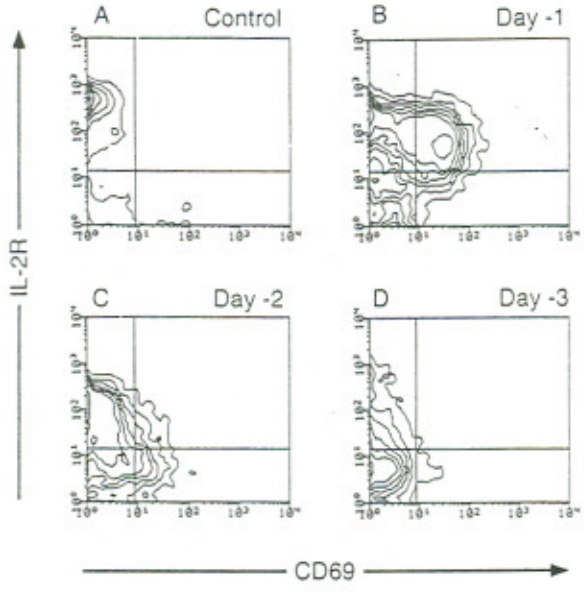

Figure 3. Expression of CD69 during Down-Regulation of IL-2Rα

RAG2$^{-/-}$ newborn mice were injected 1–3 days before analysis with anti-CD3ε MAb. At day 8 after birth, thymocytes were isolated and stained. Contour plots represent fluorescence intensities of IL-2Rα and CD69. Control thymocytes (A) are predominantly IL-2Rα^{+}CD69^{+}. After 1 day of treatment (B), thymocytes down-regulate IL-2Rα expression, and show high expression of CD69. After 2 and 3 days (C and D), expression CD69 is lost again, together with IL-2Rα.

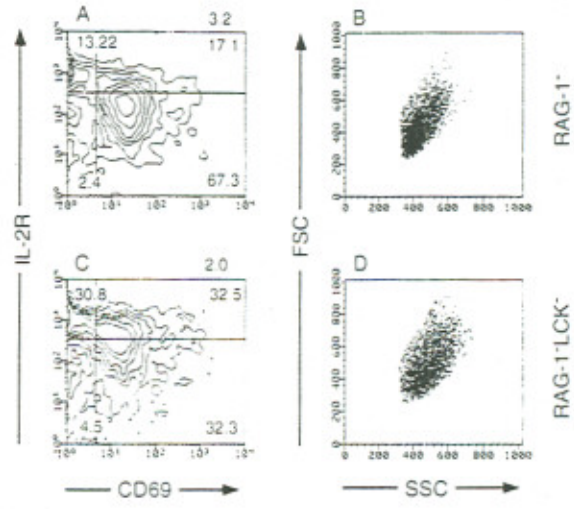

Figure 4. Early Events after Cross-Linking of CD3ε in *RAG1*$^{-/-}$*lck*$^{-/-}$ Newborn Mice

RAG$^{-/-}$ and *RAG1*$^{-/-}$*lck*$^{-/-}$ newborn mice were injected at day 7 after birth with anti-CD3ε MAb. At day 8, thymocytes were isolated and stained. Contour plots (A and C) represent fluorescence intensities of IL-2Rα and CD69. Absolute thymocyte numbers ($\times 10^{-6}$) are indicated above the contour plots. In the *RAG*$^{-/-}$*lck*$^{-/-}$ thymi, down-regulation of IL-2Rα and expression of CD69 occurs on less cells, and less efficiently than in *RAG1*$^{-/-}$ thymi (A and C). Thymocytes proliferate less in *RAG1*$^{-/-}$*lck*$^{-/-}$ mice (A and C). The dot plots (B and D) show that in *RAG1*$^{-/-}$*lck*$^{-/-}$ thymi, cells initially remain larger than in *RAG1*$^{-/-}$ thymi.

thymi. (Figures 3A and 3B). CD69 is lost 1 day later, together with IL-2Rα (Figures 3C and 3D). It is likely that this occurs also in physiological thymocyte differentiation, regulated by the pre-TCR rather than by anti-CD3ε treatment: CD69 expression was detected on IL-2Rα^{lo} and IL-2Rα^{-} thymocytes from day 16 wild-type embryos before expression of CD4 or CD8 (data not shown). These and additional early consequences of CD3ε cross-linking were compared in *RAG1*-deficient and *RAG1*$^{-/-}$*lck*$^{-/-}$ double-mutant mice. In the *lck*-deficient thymi, fewer cells expressed high levels of CD69 and more cells were found with intermediate levels (Figures 4A and 4C); down-regulation of IL-2Rα was less effective in all thymocytes, including those that expressed CD69; and expansion of DN thymocytes and reduction of cell size were less pronounced (Figures 4B and 4D). This demonstrates that a number of early events in the maturation of DN thymocytes to the DP stage are impaired in the absence of *lck*.

Responses of DP Thymocytes to Anti-CD3ε Treatment in Mice with Defects in Signal Transduction through TCR-CD3

The responses to CD3ε cross-linking of thymocytes that spontaneously matured to the DP stage in CD3ζ-deficient or *lck*-deficient mice were studied. Newborn mice deficient for either *lck* or CD3ζ were treated with anti-CD3ε MAb 1–3 days before analysis. As controls, wild-type newborn mice, and *RAG2*-deficient newborn mice were used. Table 1 shows the absolute numbers of thymocytes in these mice over time. In wild-type mice, the antibody treatment resulted in a rapid decrease of the absolute number of thymocytes, reflecting negative selection by deletion. In contrast, CD3ζ-deficient mice and *RAG2*-deficient mice responded with an increase in the absolute numbers of thymocytes. *lck*-deficient mice showed no significant changes in the absolute thymocyte number.

Apoptosis upon anti-CD3ε treatment, as analyzed by gel electrophoresis or intracellular DNA staining with propidium iodide, was marginal or undetectable in CD3ζ- or *lck*-deficient thymocytes (data not shown). Because a relative resistance to dexamethazone-induced apoptosis was also observed, it is likely that DP thymocytes in CD3ζ- or *lck*-deficient mice do not efficiently mature to an apoptosis-sensitive stage.

The kinetics of expansion of DN and DP thymocytes in CD3ζ-deficient mice after cross-linking of CD3ε were studied in more detail. Figure 5 shows the absoute numbers of DN and DP thymocytes with or without functionally rearranged TCRβ genes of newborn mice on days 1–3 after anti-CD3ε MAb treatment. Beginning from day 1, the increase in cell numbers was largely restricted to DP thymocytes expressing intracellular TCRβ chain. Production of new DP thymocytes from DN thymocytes upon anti-CD3ε treatment takes 3 days, and results in a block in rearrangement of the TCRβ locus (Levelt et al., 1995), also in CD3ζ-deficient animals (Wang et al., 1995). Therefore, these data indicate that CD3 cross-linking induces proliferation of preexisting DP TCRβ^{+} thymocytes in CD3ζ-deficient mice.

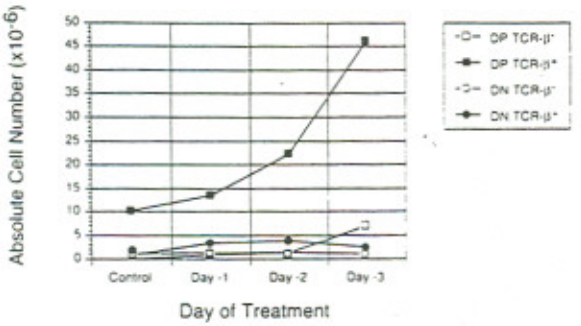

Figure 5. Expansion of the DP Population in CD3ζ-Deficient Thymi upon anti-CD3ε MAb Treatment

CD3ζ$^{-/-}$ newborn mice were injected with anti-CD3ε MAb, 1–3 days before analysis, and absolute cell numbers of different thymocyte populations were determined, as indicated. The DP cells that proliferate in the CD3ζ-deficient mice during the first 2 days after treatment consist completely of thymocytes expressing intracellular TCRβ chain (DP TCRβ$^+$). A few DP thymocytes not expressing TCRβ (DP TCRβ$^-$) are detected 3 days after treatment. The results represent mean cell numbers of 2–5 mice for each data point; variations between individual mice were less than ± 25% on day 2, less than ± 15% on all other days.

Differences in Calcium Mobilization between *lck*-Deficient and CD3ζ-Deficient DP Thymocytes

Calcium mobilization upon stimulation with anti-CD3ε was measured in thymocytes derived from mice deficient for CD3ζ or *lck* or from heterozygous littermates. This was done by flow cytometry with and without gating for DP cells. In CD3ζ-deficient thymocytes, no calcium mobilization could be induced upon stimulation with up to 6 μg/ml anti-CD3ε MAb followed by 60 μg/ml anti-hamster immunoglobulin G (IgG) (data not shown). *lck*-deficient DP thymocytes showed a reduced calcium response compared with that of littermate thymocytes, when stimulated with 3 μg/ml anti-CD3ε MAb followed by 30 μg/ml anti-hamster IgG (Figure 6A). Using a 10-fold dilution of the anti-CD3ε MAb, Ca^{2+} mobilization was virtually absent in *lck*-deficient DP thymocytes, but still clearly demonstrable in DP thymocytes of littermates (Figure 6B). Similar results were obtained when the analysis was performed with ungated thymocytes, and when 10 μg/ml anti-CD3ε MAb without cross-linking by anti-hamster IgG were used (data not shown).

Discussion

Mutant mice that are unable to produce a TCRβ chain, such as Scid mice, *RAG1*- or *RAG2*-deficient mice, and mice with a mutation in the TCRβ locus itself, show a nearly complete block in thymocyte differentiation at the DN stage, which can be overcome by cross-linking of CD3ε (Levelt et al., 1993b). In mice with null mutations in either CD3ζ or *lck*, thymocyte maturation is blocked incompletely, with the generation of about 5%–15% of the normal number of DP thymocytes (Liu et al., 1993; Ohno et al., 1993; Malissen et al., 1993; Love et al., 1993; Molina et al.,1992). In addition, positive selection is impaired in such mice, resulting in the nearly complete absence of

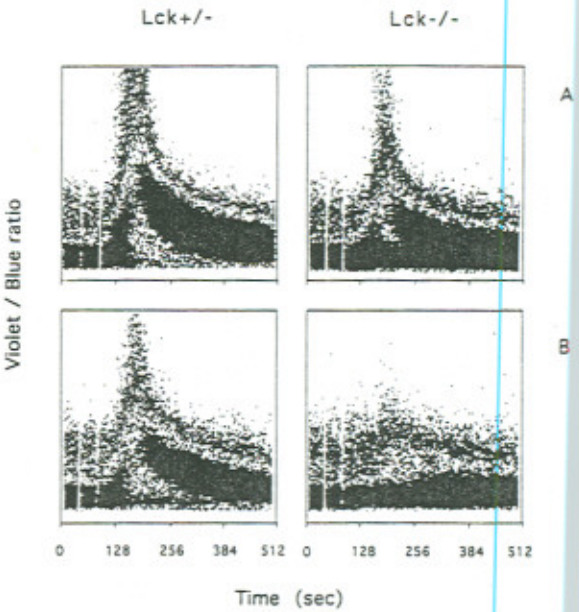

Figure 6. Impaired Ca^{2+} Mobilization in *lck*-Deficient Thymocytes

Thymocytes were isolated from heterozygous littermates (left) or *lck*-deficient (right) mice at day 8 after birth, stained for CD4 and CD8, and loaded with indo-1. Analysis was performed on a FACStar Plus flow cytometer. CD4$^+$CD8$^+$ DP cells were 83% and 42% for *lck*$^{+/-}$ and *lck*$^{-/-}$ mice, respectively. Density plots represent the course of the violet/blue ratio, which is linearly related to the molarity of intracellular Ca^{2+} over time. Ca^{2+} mobilization is detected in *lck*-deficient mice when thymocytes are stimulated at 40 s with 3 μg/ml anti-CD3ε MAb, followed at 80 s by 30 μg/ml anti-hamster IgG (A). When 0.3 μg/ml anti-CD3 was used (B), the response of *lck*-deficient thymocytes was abolished

SP thymocytes. Surprisingly, CD3ζ-deficient mice hav up to normal numbers of peripheral T cells. In this respec *lck*-deficient mice differ from CD3ζ-deficient mice, as the possess only 5%–10% of the normal number of peripher T cells (Molina et al., 1992). The initial aim of the prese study was to use these mice to test our hypothesis th cross-linking of CD3ε on DN thymocytes induces their d ferentiation to the DP stage by a process involving sign transduction (Levelt et al., 1993a, 1993b). In addition, th elicitation of synchronous responses of thymocytes anti-CD3ε MAb allowed us to delineate the different roles of CD3ζ and *lck* during early thymocyte differenti tion in more detail than has been previously possible phenotypic analysis alone.

We first investigated whether CD3ζ was involved in s nal transduction through the immature TCRβ–CD3 co plex, and if it had a specific or only an amplifying ro Recent studies have shown that not only CD3ζ, but al CD3ε can mediate TCR signaling (Letourneur and Klausn 1992; Wegener et al., 1992). It was suggested that diff ent PTKs are involved in signal transduction through t CD3ζζ module and the CD3γδε module (Letourneur a Klausner, 1992). Recently, evidence accumulates that t same PTKs mediate signaling through both CD3ε a CD3ζ (Weiss and Littman, 1994; Wange et al., 19 Duplay et al., 1994), though with differential affinities. S nal transduction through CD3ζ may be more efficient th

through CD3ε because its cytoplasmic tail contains three ARAMs, signaling motifs containing two tyrosines that can be phosphorylated by specific PTKs, as compared with a single motif in the cytoplasmic tail of CD3ε (Weiss and Littman, 1994). A second reason why CD3ζ may be important in regulating the strength of the signal through TCR–CD3 is that, in the absence CD3ζ, the assembly and surface expression of TCR–CD3 is extremely inefficient (Weissman et al., 1989).

To address these questions, we made use of our previous finding that in FTOC of mice deficient in TCRβ gene rearrangement, cross-linking of CD3 with anti-CD3ε MAb fully restored early thymocyte differentiation. By injection of newborn mice with anti-CD3ε MAb (Jacobs et al., 1994; Shinkai and Alt, 1994), we found that *RAG2*-deficient and *RAG2*/CD3ζ double-deficient mice were equally competent in the generation of DP thymocytes upon anti-CD3ε MAb treatment. Induced DP thymocyte numbers were similar to that in normal untreated newborn mice of the same age. We conclude that signaling through CD3γδε is sufficient for the full induction of this maturation step.

Subsequently, we addressed the question whether signal transduction through the immature CD3γδε module involved primarily PTKs other than *lck*, used by the CD3ζζ module. In that case, induction of thymocyte development in *lck*/*RAG1* (or *RAG–2*) double-mutant mice by anti-CD3ε MAb treatment should be successful. By injecting anti-CD3ε MAb into *lck*/*RAG1* double-deficient mice, we observed production of DP cells, but the absolute number did not exceed 15% of that obtained in anti-CD3ε MAb-treated *RAG1*-deficient mice. Moreover, down-regulation of IL-2Rα on DN cells was incomplete. These results suggest that *lck* is the main PTK in signal transduction through both the CD3ζζ and CD3γδε modules at this stage of thymocyte development.

The developmental block in *lck*-deficient mice was not restricted to the proliferation of DP thymocytes, but also affected the proliferation and differentiation of DN thymocytes. We found that during early thymocyte selection, the early activation marker CD69 was transiently expressed, similar to its expression upon late positive selection of DP thymocytes (Bendelac et al., 1992; Yamashita et al., 1993; Swat et al., 1993). In *lck*/*RAG1* double-mutant mice, expression of CD69 after 1 day of anti-CD3ε MAb treatment occurred on fewer thymocytes than in *RAG1* mutant mice. Furthermore, down-regulation of IL-2Rα, proliferation of DN thymocytes, and reduction in cell size all were less effective in the absence of *lck*. Whether this represents a complete block in the development of a proportion of the cells, or a general slowdown for all cells, cannot be decided from our results. In either case, the data suggest that *lck* is important in signal transduction through the pre-TCR already before the acquisition of the DP phenotype. In this context, it is interesting that allelic exclusion of the TCRβ locus in TCRβ transgenic *lck*-deficient mice is almost complete (Wallace et al., 1995). It is possible that different responses to pre-TCR signaling are associated with different biochemical pathways or are restricted by different quantitative thresholds.

In *lck*- or CD3ζ-deficient mice, DP thymocytes differ phenotypically from normal DP thymocytes. It has been described before (Crompton et al., 1994) that in CD3ζ-deficient animals, IL-2Rα expression is detected on many DP thymocytes. This was also observed on *lck*-deficient DP thymocytes (C. N. L., unpublished data). In addition, we observed functional differences between DP thymocytes from wild-type mice and those from *lck*- or CD3ζ-deficient mice. *lck*- or CD3ζ-deficient DP thymocytes were highly resistant to deletion by in vivo treatment with anti-CD3ε MAb. This could partially be due to inefficient signal transduction through the TCR–CD3 complex in the absence of either CD3ζ or *lck*. Indeed, Ca^{2+} mobilization was absent in DP thymocytes of CD3ζ-deficient mice, and reduced in *lck*-deficient DP thymocytes. However, impaired signaling may not be the only explanation for the resistance to deletion, as reduced sensitivity to dexamethazone-induced apoptosis was also observed. It is therefore likely that, in addition, maturation of DP thymocytes is incomplete in CD3ζ- or *lck*-deficient mice and does not proceed efficiently to an apoptosis-sensitive stage. In line with this idea is our finding that in CD3ζ-deficient mice, DP thymocytes remained sensitive to anti-CD3ε-induced proliferation, a response typical of immature thymocytes. The lack of this immature response in *lck*-deficient DP thymocytes may highlight the importance of *lck* in the proliferative response of immature thymocytes, as also observed in anti-CD3ε-treated *RAG1*/*lck* double-deficient animals. Our observation that *lck*-deficient DP cells can mobilize Ca^{2+} does not argue against this: the signal inducing early thymocyte proliferation is not likely to depend on Ca^{2+} mobilization, as suggested by previous results (Levelt et al., 1993b), and by the proliferation induced in CD3ζ-deficient DP thymocytes.

Is the cross-linking of CD3 complexes on $TCR\beta^-$ thymocytes indeed comparable to signaling through the pre-TCR? In this context, it is interesting that the cytoplasmic tail of the pre-TCRα chain contains phosphorylation sites consistent with an involvement in signal transduction (Saint-Ruf et al., 1994). While CD3 complexes have been detected on $TCR\beta^-$ thymocytes with biochemical means (Wiest et al., 1994), it is not known whether the pre-TCRα chain can be expressed on the cell surface without TCRβ as part of these incomplete CD3 complexes. Alternatively, CD3 cross-linking by MAb may generate a strong signal that overrides the requirement for pre-TCRα, or this molecule may not be involved in signal transduction.

Taken together, the results presented in this report show that maturation of DN thymocytes into the DP stage, including proliferation of immature DP thymocytes, depends on signaling through the CD3 complex. For the signals dictating pre-TCR-dependent thymocyte maturation, a cooperation between *lck* and CD3γδε seems essential. CD3ζ does not play a specific role in this process, but may be of importance in the augmentation of signaling through the pre-TCR–CD3 complex.

Experimental Procedures

Mice

Newborn *RAG1*$^{-/-}$, *RAG2*$^{-/-}$, CD3ζ/η$^{-/-}$, and *lck*$^{-/-}$ mice or crosses of these strains, were obtained from the specific pathogen-free breeding

facility at the Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts, and at the Max Planck Institut für Immunbiologie, Freiburg, Federal Republic of Germany. *RAG1*-deficient and *lck*-deficient mice were typed by PCR analysis of tail DNA. The following primers were used: *RAG1* sense, 5′-TACCCTGAGCTTCAGTTC-3′; *RAG1* antisense, 5′-CAACATCTGCCTTCACGTC-3′; Neomycin sense, 5′-TATCAGGACATAGCGTTGGCTACCC-3′ (Molina et al., 1992); *lck* antisense, 5′-CTTAGACTCACGTGCTCCACAGGTA-3′ (Molina et al., 1992); *lck* sense, 5′-AGACCTGACAACTGTCCGGAAGAGC-3′.

RAG2-deficient mice were typed by staining thymocytes for intracellular TCRβ chain expression and FACS analysis. If results were not evident because of the anti-CD3ε MAb treatment, the mice were tested for *RAG2* expression in the thymus by RT–PCR, using the following primers: *RAG2* sense, 5′-CACATCCACAAGCAGGAAGTACAC-3′ and *RAG2* antisense, 5′-GGTTCAGGGACATCTCCTACTAA-3′.

CD3ζ-deficient mice were typed by intracellular staining of thymocytes for CD3ζ.

MAbs

Anti-CD3ε antibody 500A2 (Havran et al., 1987) was isolated from culture supernatants by affinity chromatography over protein A columns (Pharmacia, Freiburg, Federal Republic of Germany). Flow cytometry employed labeled anti-Lyt-2 (53.6-7), anti-L3T4 (RM-4-5), anti-TCRβ (H57-597), anti-CD3ε (500A2), anti-CD69 (H1.2F3), anti-IL-2Rα (7D4) (all purchased from PharMingen), and biotin-labeled anti-CD3ζ antibody H146-968 (Punt et al., 1991), using a FACScan flow cytometer (Becton Dickinson, San Jose, California).

Flow Cytometry

Two- and three-color stainings were performed using fluorescein isothiocyanate–, phycoerythrin-, and biotin-labeled antibodies. As a third color, Red 670-conjugated streptavidin (GIBCO, Gaithersburg, Maryland) was used. Intracellular stainings were performed as described (Levelt et al., 1993a, 1993c). Events were collected using a FACScan flow cytometer (Becton Dickinson, San Jose, California).

Antibody Treatment of Newborn Mice

Newborn mice were injected intraperitoneally with 10 μg/g bodyweight anti-CD3ε MAb. To avoid variations in thymocyte numbers by differences in the age of the newborn mice, analyses were always performed at day 8 after birth. Injections were given at varying days before analysis, as indicated with each experiment. Because no effects of intraperitoneal injections of normal hamster IgG were detected, some control newborn mice were not treated.

Mobilization of Intracellular Free Calcium

Calcium mobilization studies were performed as described (Rabinovitch et al., 1986). Cells were isolated from thymi derived from 8-day-old newborn mice. Thymocytes (5 × 10^6/ml) were loaded with indo-1 by incubation with its acetoxy–methyl ester (Molecular Probes, Eugene, Oregon) (5.3 M) and 0.027% pluronic acid (Molecular Probes) for 45 min at 37°C. Cells were washed and resuspended in Iscove's modified Dulbecco medium supplemented with 1% fetal calf serum and 1% glutamine. Analysis followed immediately and was performed on a FACstar flow cytometer (Becton Dickinson), in combination with staining for CD4 and CD8.

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